

A Microrobotic Tissue Factory for Guided Assembly of Cell Clusters and Drug Screening in Microtumors

Philipp Richard Harder

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1. Prof. Dr. Berna Özkale Edelmann
2. Prof. Dr. Josep Puigmartí-Luis

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To the Scientific Method and Engineering Principles

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Abstract

Soft microrobots are rapidly emerging as transformative tools in biomedical research, offering precise control over cellular microenvironments for applications in diagnostics, therapy, and tissue engineering. These systems aim to bridge the gap between traditional microfabricated tools and dynamic biological environments by enabling untethered operations and seamless integration at the cellular scale. This dissertation presents the development of a hydrogel-based microrobot platform that combines photothermal actuation, fluorescence-based thermal sensing, and mechanical cell stimulation to investigate cell behavior in complex two-dimensional (2D) and three-dimensional (3D) environments.

The fundamental design of these microrobots is grounded in the integration of functional materials into a biocompatible hydrogel matrix. A microfluidic fabrication strategy was employed to create monodisperse, cell-sized soft microrobots capable of responding to optical stimuli. Gold nanorods enable photothermal heating, while embedded fluorescent dyes such as Rhodamine B serve as a temperature sensor. The use of ionically cross-linked alginate provides a tunable and biocompatible scaffold, whose mechanical properties can be precisely adjusted for biological interfacing. Additionally, stimuli-responsive polymers like poly(N-isopropylacrylamide) (PNIPAM) were incorporated to achieve reversible shape change for mechanical actuation.

The first results section introduces thermally activated cell-signal imaging (TACSI) microrobots, which use light-induced heating to stimulate cells with high spatial and thermal precision. These microrobots exhibit dynamic thermophoretic locomotion and allow fine temperature control up to 60 °C. By stimulating individual cells or small cell clusters and measuring intracellular calcium signaling responses, the TACSI system enables single-cell resolution studies of temperature-dependent cellular pathways. The second section focuses on the mechanical characterization of alginate microgels, providing essential insight into the structural stability and tunability of the microrobot matrix. Nanoindentation experiments revealed that gel stiffness and viscoelasticity are highly sensitive to the ionic composition of the surrounding medium, particularly calcium and sodium concentrations. These findings establish key design guidelines for ensuring mechanical reliability under physiological conditions. The final section explores advanced applications in spatially organized tissue modeling and localized therapy. Microrobots were surface-functionalized to enable single-cell pickup

and guided self-assembly into 3D spheroids, offering a new level of control compared to stochastic spheroid formation. Furthermore, light-responsive PNIPAM-based microrobots were developed for reversible mechanical stimulation in both 2D cultures and spheroid systems, allowing researchers to probe mechanotransduction under dynamic physical constraints. As a demonstration of combined functionality, microrobots were used to modulate invasive cancer behavior *in vitro* by integrating photothermal stimulation with chemotherapeutic treatment.

Together, this dissertation establishes a multifunctional soft microrobot platform for light-driven, programmable single-cell manipulation and environmental sensing in three-dimensional biological systems. The TACSI microrobot system and its PNIPAM-actuated counterparts offer new options for studying cellular signaling, mechanobiology, and disease modeling.

Zusammenfassung

Weiche Mikroroboter entwickeln sich rasch zu transformativen Werkzeugen in der biomedizinischen Forschung, die eine präzise Kontrolle über zelluläre Mikroumgebungen für Anwendungen in der Diagnostik, Therapie und Gewebezüchtung ermöglichen. Diese Systeme sollen die Lücke zwischen traditionellen mikrofabrizierten Methoden und dynamischen biologischen Umgebungen schließen, indem sie programmierbare, ungebundene Operationen im zellulären Maßstab ermöglichen. In dieser Dissertation wird die Entwicklung einer Hydrogel-basierten Mikroroboter-Plattform vorgestellt, die photothermische Aktivierung, fluoreszenzbasierte Wärmesensorik und reversible mechanische Manipulation kombiniert, um das Zellverhalten in komplexen 2D- und 3D-Umgebungen zu untersuchen.

Das grundlegende Design dieser Mikroroboter basiert auf der Integration funktioneller Materialien in eine biokompatible Hydrogelmatrix. Eine mikrofluidische Herstellungsstrategie wurde angewandt, um monodisperse, zellgroße weiche Mikroroboter zu fabrizieren, die auf optische Anregung reagieren können. Goldnanostäbchen ermöglichen die photothermische Erwärmung, während eingebettete Fluoreszenzfarbstoffe wie Rhodamin B als Temperatursensoren dienen. Die Verwendung von ionisch vernetztem Alginat bietet ein abstimmbares und biokompatibles Gerüst, dessen mechanische Eigenschaften für biologische Schnittstellen präzise eingestellt werden können. Zusätzlich wurden Polymere wie Poly(N-isopropylacrylamid) (PNIPAM) eingearbeitet, um eine reversible Formveränderung der Mikroroboter für mechanisches Anregen von Zellen zu erreichen. Im ersten Ergebnisteil werden thermisch aktivierte TACSI-Mikroroboter (TACSI = thermally activated cell-signal imaging) vorgestellt, die mit Hilfe von lichtinduzierter Erwärmung Zellen mit hoher räumlicher Präzision stimulieren. Diese Mikroroboter weisen thermophoretische Fortbewegung auf und ermöglichen eine feine Temperatursteuerung bis zu 60 °C. Durch die Stimulierung einzelner Zellen oder kleiner Zellcluster und die Messung intrazellulärer Kalzium-Signalreaktionen ermöglicht das TACSI-System Untersuchungen von temperaturabhängigen zellulären Signalwegen in Einzelzellauflösung. Der zweite Abschnitt befasst sich mit der mechanischen Charakterisierung von Alginat-Mikrogelen, die einen wesentlichen Einblick in die strukturelle Stabilität der Mikroroboter-Matrix bietet. Nanoindentationsexperimente zeigten, dass die Steifigkeit und Viskoelastizität des Gels sehr empfindlich auf die ionische Zusammensetzung des umgebenden Mediums, insbesondere auf die

Kalzium- und Natriumkonzentration, reagieren. Diese Ergebnisse stellen wichtige Konstruktionsrichtlinien für die Gewährleistung der mechanischen Zuverlässigkeit unter physiologischen Bedingungen dar. Der letzte Abschnitt befasst sich mit fortgeschrittenen Anwendungen in der räumlich organisierten Gewebemodellierung und lokalisierten Therapie. Mikroroboter wurden oberflächenfunktionalisiert, um die Aufnahme einzelner Zellen und den geführten Selbstaufbau zu 3D-Sphäroiden zu ermöglichen, was im Vergleich zur stochastischen Sphäroidbildung ein neues Maß an Kontrolle bietet. Darüber hinaus wurden lichtempfindliche PNIPAM-basierte Mikroroboter für die reversible mechanische Stimulation sowohl in 2D-Kulturen als auch in Sphäroid-Systemen entwickelt, die es den Forschern ermöglichen, die Mechanotransduktion unter dynamischen physikalischen Bedingungen zu untersuchen. Weiterhin wurden die Mikroroboter zur Modulation des invasiven Krebsverhaltens *in vitro* eingesetzt, indem die photothermische Stimulation mit einer chemotherapeutischen Behandlung kombiniert wurde.

Zusammengefasst stellt diese Dissertation eine multifunktionale, weiche Mikroroboter-Plattform für die lichtgesteuerte Manipulation von Einzelzellen und die Erfassung der Umgebung in dreidimensionalen biologischen Systemen dar. Das TACSI-Mikrorobotersystem und seine PNIPAM-gesteuerten Gegenstücke bieten neue Optionen für die Untersuchung zellulärer Signalübertragung, Mechanobiologie und Krankheitsmodellierung.

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1 Introduction

The conceptual and technical foundations of microrobotics emerged during the 1980s with the development of microelectromechanical systems, which enabled the batch fabrication of miniature mechanical elements integrated with electronic components [1]. The emergence of nanomanipulation technologies, such as atomic force microscopy (AFM), optical tweezers, and magnetic tweezers, that operate under atmospheric and physiological conditions marked a pivotal advancement in the field of biomedical engineering. These tools enabled precise interactions with single molecules and cells under externally controlled conditions, significantly broadening their adoption in biomedical and materials research. AFM, for example, became a cornerstone for single-molecule force spectroscopy, allowing researchers to probe molecular adhesion under ambient conditions [2]. Optical tweezers facilitated pioneering studies on DNA elasticity by stretching individual molecules in aqueous environments [3], while magnetic tweezers have been used to investigate mechanotransduction in live cells, revealing how mechanical forces propagate across the cell membrane and the cytoskeleton [4]. These tools collectively demonstrated the power of microscale force application and measurement in biological contexts, but they remained limited by their reliance on complex, stationary setups. Building on the principles of targeted actuation and microscale control established by these techniques, systems that could operate in an untethered manner within biological environments were developed. This transition was fueled by advances in stimuli-responsive materials, high-resolution imaging, and wireless actuation strategies employing magnetic, optical, or acoustic fields, ultimately giving rise to microrobots [5, 6].

This new class of wireless machines unlocked new possibilities for operation within biological systems, combined with a strong motivation to reach and repair disease sites in the human body that are otherwise inaccessible. Early biomedical applications envisioned microrobots as *in vivo* agents for targeted drug delivery, local biopsy, and minimally invasive microsurgery under remote control [7]. Their compact size would allow them to access anatomically constrained regions, bypass conventional surgical routes, and deliver therapeutic payloads with spatial specificity while minimizing systemic side effects. Research conducted over the last 20 years demonstrated the ability of microrobots to navigate through confined spaces *in vivo* and perform precise actions such as drilling

through occlusions, releasing therapeutic cargo, and enabling local removal of diseased tissues under the guidance of magnetic, optical, or acoustic fields [8, 9, 10, 11, 12, 13].

While these technologies primarily target acute conditions such as vascular blockages or localized tumors, there is a growing recognition that chronic and systemic diseases, including neurodegenerative disorders, metabolic syndromes, and cancer, often originate from dysregulated behavior at the single-cell level [14, 15, 16]. This realization has sparked a paradigm shift in the biomedical community toward preventative and personalized approaches, where single-cell analysis plays a central role. In response, researchers are now developing microrobotic tools capable of manipulating and interrogating individual cells with high precision. Single-cell omics have revealed the critical influence of individual cells within larger populations, uncovering rare subtypes and dynamic states that shape development, disease, and tissue function [17, 18]. However, this cellular heterogeneity is often obscured in three-dimensional (3D) systems, where traditional large-scale assays fail to resolve differences between individual cells within densely packed and structurally complex tissues. As this biological resolution has increased, so too has the demand for tools capable of interacting with single cells. Microrobots have begun to address this need, owing to their ability to manipulate, stimulate, and transport single cells with high spatiotemporal precision, positioning them as ideal functional complements to rapid bioscreening technologies. This capability is especially valuable in biomedical engineering, where integrating single-cell information into multicellular models remains a central challenge. Simultaneously, organoids and 3D tissue cultures have emerged as powerful, physiologically relevant *in vitro* platforms, offering insight into tissue-level organization and function. To further enhance the spatial and mechanical precision of these models, microrobots present a promising avenue for delivering localized stimuli and manipulating individual cells within complex tissue architectures. However, achieving seamless integration between microrobots and soft biological systems remains an open challenge, particularly because most microrobots to date have been fabricated from rigid materials [11, 19]. Recent advances in soft, hydrogel-based microrobots offer a step toward mechanical compatibility, yet the full realization of biointegrated, sensor-equipped microrobots for functional tissue engineering is still in its early stages. Their ability for scalability, from cellular targeting to tissue-level actuation, is one of the most distinguishing features of biomedical microrobots. These microrobots are capable of positioning individual cells with high precision, locally delivering mechanical or biochemical signals, and assembling multicellular constructs [20, 21]. At the same time, their size, which can be similar to a single mammalian cell, permits embedding within tissues or engineered systems without disrupting function, enabling real-time sensing or actuation within physiologically relevant environments. With the help of biocompatible and responsive materials, including hydrogels, shape-memory polymers, and soft composites, microrobots are in-

creasingly designed for functional integration with biological tissues, offering promising biocompatibility and improved interaction with cellular environments [22]. Contemporary microrobotic platforms incorporate temperature and pH sensing, force feedback, and semi-autonomous navigation [12, 23]. As biomedical science continues to develop personalized and dynamic interventions, microrobots are positioned to become central tools in both diagnostic and therapeutic applications.

1.1 Scope and Structure of the Thesis

The objective of this thesis is to develop a versatile soft microrobot capable of single-cell transport, localized physical cell stimulation, and integration into 3D tissue models. The Thermally Activated Cell-Signal Imaging (TACSI) is designed to be photothermally active, light-guided, and mechanically tunable, enabling it to not only manipulate individual cells but also to sense and deliver thermal and mechanical signals within 2D and 3D cell culture. Built from biocompatible hydrogels and optically responsive nano- materials, this system bridges microscale actuation with functional cell interfacing, offering new avenues for thermal biology, tissue construction, mechanobiology, and drug screening *in vitro*. Chapter 2 outlines the fundamental principles and technical background necessary to understand and contextualize the development and function of soft microrobots. The discussion is organized from the broader landscape of *in vitro* models to the specific challenges and innovations in soft microrobotic systems, including actuation strategies, material choices, and applications in cellular mechanobiology and thermal modulation. Chapter 3 establishes the foundation by detailing the design principles, the fabrication methodology, and the initial biological demonstration of TACSI microrobots. This section includes selecting and engineering biocompatible materials, namely alginate hydrogels and gold nanorods for photothermal actuation, characterizing thermal responsiveness of the resulting microrobots, and demonstrating controlled locomotion capabilities. The chapter explores how these microrobots interface with cells under 2D culture conditions, highlighting precise, thermally induced calcium signaling within cellular environments. Chapter 4 revolves around the characterization of the mechanical properties of TACSI microrobots, crucial to ensure biomechanical compatibility. We employ nanoindentation techniques to assess the impact of ionic crosslinking densities, storage conditions, and culture media on microrobot stiffness, critically linking mechanical properties to functional microrobot performance in biological settings. Chapter 5 transitions to practical applications of TACSI microrobots, demonstrating their capabilities for high-throughput single-cell transport, spheroid assembly, mechanical stimulation in 2D and 3D, as well as integrated drug screening within 3D cell constructs. The chapter highlights advanced functionalities, including surface functionalization for rapid cell adhesion during cell pickup, assembly of microrobot-integrated spheroids, and dynamic drug modulation experiments using thermal and mechanical actuation. We

also explore the interaction dynamics in complex cellular environments, emphasizing viability, signaling responses, and therapeutic efficacy. Finally, the concluding chapter summarizes the key contributions of the thesis and outlines future directions, including future development for 3D cell studies and additional sensing modalities.

2 Fundamentals and Technical Background

2.1 Current *In Vitro* Models and the Need for Dynamic Tools

In natural tissue, cells reside within a dynamic and multifaceted microenvironment that guides their behavior through a series of mechanical, biochemical, and structural cues [24]. The native cell microenvironment includes a three-dimensional extracellular matrix (ECM) composed of fibrous proteins like collagen, laminin, and fibronectin, which provide structural support and present biomolecular ligands for cell adhesion [25]. The mechanical properties of the ECM—such as stiffness, viscoelasticity, and topography—vary widely between tissues and are further modulated during development, injury, or disease [26]. Cells encounter gradients of oxygen, nutrients, and signaling molecules, and interact with neighboring cells through direct contact and paracrine signaling [27]. In specialized tissues, additional stimuli such as electrical cues (e.g., action potentials in neural or cardiac tissue) or compressive forces (e.g., in cartilage) further refine cellular responses [28, 29, 30]. Importantly, the microenvironment is not static: cells constantly remodel their surroundings through matrix deposition and degradation, leading to an evolving, reciprocal relationship that governs function and fate [31]. Understanding and replicating this complexity *in vitro* is crucial for advancing mechanobiology, regenerative medicine, and tissue engineering.

Early *in vitro* models sought to recreate elements of the native microenvironment in simplified systems. Traditional two-dimensional (2D) cultures on plastic or glass substrates have provided valuable insights into cellular behavior; however, they failed to recapitulate the complex three-dimensional (3D) architecture and mechanical properties of native tissue environments [32]. The need for recreating the natural conditions of cells led to the development of 3D culture systems, which better emulate the spatial and mechanical context of native tissues. Among these systems, hydrogels emerged as foundational materials. Their high water content, tunable mechanics, and permeability allow for cell encapsulation, nutrient diffusion, and mechanical support, making them ideal ECM substitutes [33]. Natural hydrogels such as collagen, fibrin, and Matrigel offer intrinsic bioactivity, while synthetic variants like polyethylene glycol (PEG) allow for precise control over stiffness, degradability, and ligand density [34]. Hydrogels also support the formation of biochemical gradients and permit cell–matrix remodeling, facilitating studies of dynamic cellular processes [35]. However, despite

these advances, hydrogel systems are largely passive in nature. While they provide a tunable and supportive matrix, they do not actively deliver or modulate stimuli in response to biological signals.

To capture more physiologically driven organization, models based solely on cell–cell interactions have been developed, most notably spheroids and organoids. Spheroids, typically ranging from 100–500 μm in diameter, form via spontaneous aggregation and serve as models for studying tumor growth, embryogenesis, and tissue morphogenesis [36]. Organoids, often exceeding 1 mm in size, are derived from pluripotent stem cells and undergo self-organization to produce miniature organ-like structures exhibiting region-specific differentiation and functional compartmentalization [37, 38, 39]. These systems represent critical features of native tissues, including cell–cell and cell–matrix interactions, mechanical feedback, and nutrient diffusion gradients. Despite their biological relevance, 3D constructs remain limited by practical and technical constraints: Their reliance on intrinsic self-assembly processes restricts spatial control, often resulting in considerable heterogeneity in size, morphology, and cellular composition [39]. Furthermore, the closed, three-dimensional geometries of *in vitro* spheroids and organoids hinder real-time interrogation and targeted stimulation. Delivering precise inputs—such as mechanical forces, biochemical gradients, or thermal cues—to specific regions within these dense structures remains technically challenging. As a result, stimulation is often applied globally, affecting the entire model rather than discrete cellular populations. This limits the ability to dissect spatially resolved responses and to link localized microenvironmental conditions to single-cell behavior.

To overcome these challenges and enable precise stimulation and manipulation at the single-cell level within 3D environments, various microscale technologies have been developed. Early tools such as micropipette aspiration and glass microneedles allowed for the isolation, positioning, and manipulation of individual cells through direct physical interaction, including aspiration, microinjection, and microsurgery [40, 41]. These techniques are highly spatially precise due to their fine-tip geometry, enabling targeting of single cells with micrometer accuracy. However, they are inherently low-throughput, require direct contact, and depend heavily on skilled manual operation. Microfluidic systems offer an alternative by enabling the parallelized control of fluid flow, chemical gradients, and cell encapsulation through precisely engineered microchannel networks [42]. These platforms are widely adopted for high-throughput applications such as cell sorting and modeling physiological flow conditions, and have also been employed for controlled spheroid formation [43, 44, 45], but their ability to deliver localized stimulation and manipulate individual cells within a fully 3D structure remains limited. Other advanced approaches, such as optical tweezers and dielectrophoretic traps, enable contactless manipulation using light radiation pressure and spatially varying electric fields, respectively [46, 47, 41]. While these methods offer sub-micron precision and can position cells with

minimal physical contact, they often rely on complex equipment that may expose cells to harmful levels of light and strong electric fields that may jeopardize cell health [48].

Despite the diversity of available technologies for manipulating cells and tissues, current systems share a fundamental limitation: they are externally programmed and typically lack feedback from the biological environment. Actuation signals—whether mechanical, optical, magnetic, or chemical—are applied from the outside and do not adapt in real time to spatial or temporal changes within complex 3D systems. However, biological tissues are inherently heterogeneous and dynamic. They present spatial gradients in stiffness, pH, and signaling cues, and undergo continuous remodeling during processes such as morphogenesis, wound healing, or tumor progression [49, 50]. These microenvironmental changes occur locally and evolve over time, making it difficult for rigid, pre-programmed tools to interact effectively with such complex systems. As a result, many existing platforms struggle to maintain precise control in dense 3D cultures. This limitation has prompted the development of next-generation microrobotic systems that incorporate environmental sensing and adaptive behaviors, enabling more nuanced and responsive interactions with living tissues.

This challenge highlights a critical technological limitation: the need for responsive, multifunctional systems capable of real-time interaction with single cells inside multicellular assemblies, in a way that mimics the dynamic interplay seen between cells and their ECM *in vivo*. Addressing this need requires more than just passive materials—it demands active, cell-scale tools that can sense, respond, and adapt within living systems. In response to this need, microrobots have emerged as a transformative class of miniaturized systems for precision manipulation in 3D biological environments. These untethered, mobile devices—operating at length scales ranging from tens to hundreds of micrometers—can directly interface with individual cells and tissue microenvironments [23, 21]. Depending on their design, they can be actuated wirelessly by magnetic fields [51], focused light [23], ultrasound [52], or local chemical gradients [53]. When constructed from soft, biocompatible, and stimuli-responsive materials—such as hydrogels, shape-memory polymers, or liquid crystal elastomers—microrobots are able to navigate tissue-like matrices, adapt to local mechanical conditions, and integrate into 3D cell cultures without compromising the *in vitro* environment [20, 54].

Notably, many of these microrobots are made from hydrogel-based materials, bringing the well-established role of hydrogels as passive ECM mimics into an entirely new functional context. While traditional hydrogels have long been used to recapitulate ECM stiffness, porosity, and ligand presentation in tissue models, they generally lack active behavior [25]. Microrobots composed of responsive hydrogel networks, however, can deform, contract, and move in response to external signals—effectively turning passive ECM analogs into programmable agents [22]. This combination of mechanical compliance, biocompatibility, and actuation represents a significant leap in functional

complexity over both conventional hydrogel scaffolds and rigid microtools. Furthermore, these soft microrobots are uniquely suited to the challenges posed by 3D *in vitro* models such as spheroids and organoids. These self-assembled constructs have proven valuable for replicating tissue-level organization, but their closed geometry and structural heterogeneity limit the delivery of localized cues and real-time readouts. Microrobots overcome these barriers by infiltrating dense tissues and enabling spatially resolved operations [55]. For example, cell-sized microrobots have been introduced into spheroids to locally deliver signaling molecules, mechanically stimulate subpopulations, or monitor environmental parameters such as pH and oxygen tension [56, 12].

In addition to probing existing tissues, microrobots have been used to construct them. Magnetically and optically guided devices can transport single cells or aggregates to defined positions, facilitating the bottom-up assembly of multicellular structures—a process that mimics key aspects of embryonic morphogenesis, but in a controllable, programmable fashion [57, 58]. The cell-level microrobotic construction offers superior spatiotemporal precision compared to conventional 3D culture approaches, which rely on uncontrolled self-assembly and often result in variability in size, composition, and architecture. Soft microrobots also serve as mechanical actuators and biosensors: Light-responsive devices made from liquid crystal elastomers have been demonstrated to walk, bend, or grip in response to optical stimuli, enabling local mechanical stimulation of cells—an important trigger of stem cell fate and morphogenesis [59]. Similarly, magnetically controlled microrobots have been shown to move robustly in confined, gel-like environments, such as hydrogel matrices and microfluidic channels, demonstrating compatibility with common 3D culture systems [60, 61].

Altogether, these advances suggest a powerful solution: a new generation of soft microrobots that combine the biocompatibility and ECM-mimicking properties of hydrogels with the actuation, sensing, and manipulation capabilities of microrobotics. Rather than treating the matrix as a static scaffold, this approach embeds functional intelligence into the materials developed to culture the cells. When integrated into organoid or spheroid systems, these microrobots can function as autonomous agents—sensing microenvironmental changes, delivering cues on demand, and enabling adaptive experimentation. Such a paradigm shift could unlock new opportunities in tissue engineering, disease modeling, and cell-based regenerative therapies.

2.2 Classification and Use Cases of Microrobots

Microrobots used in biomedical research can be systematically classified along several key dimensions: operational environment, material composition, actuation modality, and functional role (Figure 2.1). This framework reflects both engineering constraints and biomedical objectives, offer-

ing a basis for understanding microrobot capabilities and informing design decisions. While early efforts focused mainly on *in vivo* interventions, recent advances—particularly in cell and tissue engineering—have expanded the field toward *in vitro* applications. The following classification outlines both categories, with a technical emphasis on *in vitro* microrobots relevant to this thesis.

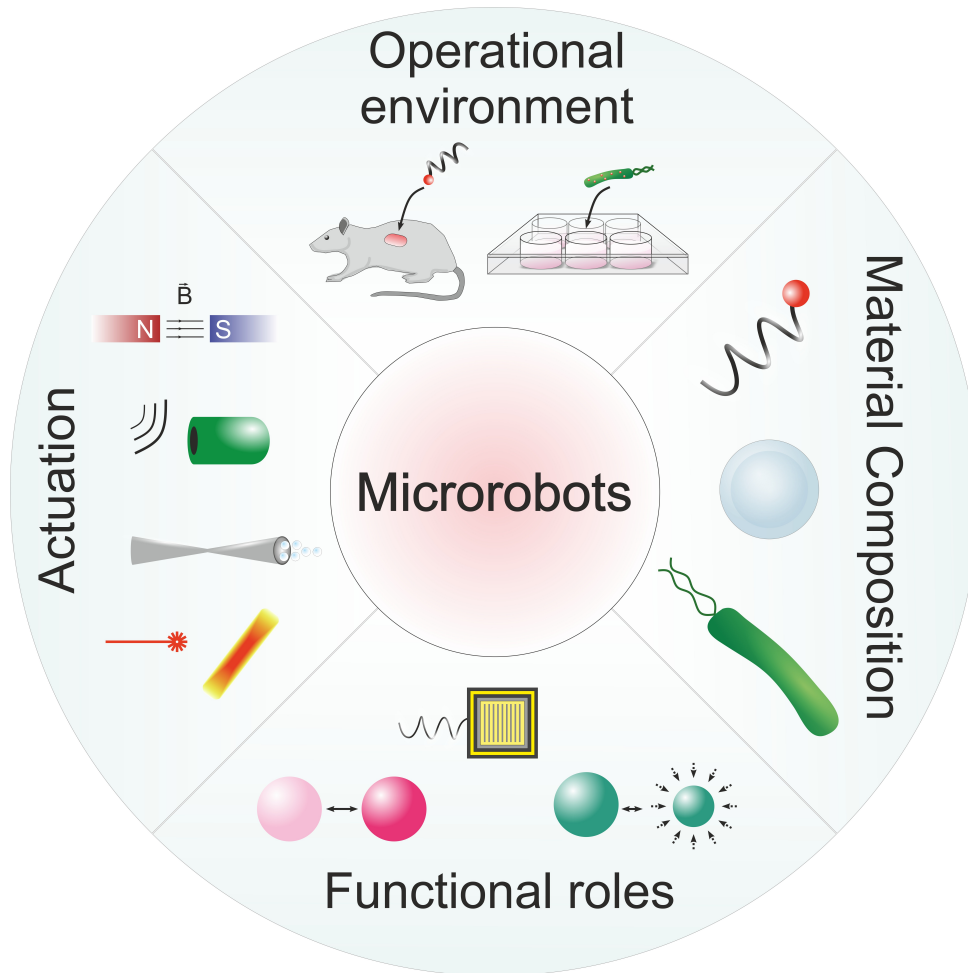


Figure 2.1 Schematic of different microrobotic classifications ranging from operational environment, material composition, actuation modality, and functional role.

Operational Environment: *In vivo* microrobots are designed to function within living organisms and must meet stringent requirements for biocompatibility, biodegradability, and remote actuation. They have been explored for targeted drug delivery, site-specific diagnostics, and minimally invasive surgery. Representative systems include magnetically driven helical swimmers for intravascular navigation [62], thermoresponsive microgrippers for gastrointestinal biopsy [63], and cell-based microrobots for tumor ablation via photodynamic therapy [64, 65]. Despite their potential, clinical translation is limited by challenges including immune system clearance, imaging depth, and reliable localization. While autonomous drug delivery via urease-powered nanobots for cancer therapy in mice has shown great promise [66], the translation to human-scale systems remains a significant challenge due to anatomical and physiological differences. *In vitro* microrobots operate within con-

trolled systems, such as 2D and 3D cultures, organoids, and lab-on-a-chip platforms, where they are used for precise manipulation, stimulation, and sensing at the single-cell level [55, 12]. These platforms typically span spatial dimensions from hundreds of micrometers to a few millimeters, closely matching the scale of individual cells (10–30 μm) or small tissue assemblies, and often feature well-defined boundaries, low fluidic complexity, and optically accessible environments [7]. This controlled microenvironment allows microrobots to perform tasks such as targeted drug delivery and localized sensing with high precision and minimal interference [67, 68].

Material Composition: Microrobots can be broadly grouped into rigid, soft, and biohybrid systems. Rigid microrobots, typically fabricated from metals or ceramics, offer high structural precision and mechanical stability, and are suited for magnetic and chemical control [69, 70]. Soft microrobots, often composed of hydrogels such as alginate or Gelatin Methacrylate (GelMA), are mechanically compatible with soft tissues and can be used to deliver spatiotemporally controlled biomechanical and biochemical cues that guide cell behavior. These microrobots enable applications such as mechanical stimulation, localized heating, and 3D tissue assembly owing to the stimuli-responsive nature of the hydrogels used to construct them [58, 67, 71]. Biohybrid microrobots combine living cells and microorganisms with synthetic materials, exploiting natural motility and biochemical compatibility. Examples include sperm-driven microrobots for cell transport [72], Spirulina-based microrobots for neuromodulation [73], and pollen-derived vehicles for targeted therapeutic delivery [74].

Actuation Mechanisms: Microrobots are typically actuated using magnetic, optical, acoustic fields, or chemical gradients. Magnetic control remains the most prevalent due to its deep penetration, high precision, and compatibility with biological tissues [75, 76]. Optical actuation, especially via near-infrared lasers, enables precise spatiotemporal control in transparent and shallow tissues, and it is commonly enabled via the use of photothermally active materials [23, 73]. Acoustic and electric fields have been explored for coordinated swarm behavior and distributed control, though their use in 3D biological matrices remains comparatively limited. Actuating microrobots via chemical gradients offers unique advantages in terms of autonomy and localized control, particularly in *in vitro* settings, where the local concentration of ions, fuels, and signaling molecules can be tightly regulated to guide microrobot movement via chemotaxis or self-diffusiophoresis [77, 78].

Functional Roles: Building on the distinction between microrobotic components and their applications, microrobots can be classified into functional categories such as micromotors, microswimmers, microactuators, and sensing microrobots, each tailored to specific biomedical tasks. These categories reflect the dominant functional role of the microrobot based on its configuration of actuators, sensors, and control systems. Micromotors are primarily designed for locomotion, often powered by magnetic fields, catalytic reactions, acoustic forces, or light, particularly through photoresponsive

materials and photocatalytic propulsion mechanisms that enable wireless and highly controllable actuation in fluidic environments [23]. Microswimmers, inspired by natural organisms such as bacteria or spermatozoa, exhibit biomimetic propulsion strategies—such as helical or flagellar motion—that are particularly suited for navigating complex microenvironments [79]. These systems often serve in diagnostic applications, performing tasks like cell capture or microscale biopsies by incorporating functionalized surfaces for selective adhesion or molecular recognition [63, 80]. Microactuators focus on the mechanical output of microrobots, using light, magnetic, or electric stimuli to generate forces or induce localized heating [81]. These are particularly valuable in mechanobiological studies, where physical perturbations are used to modulate cellular behavior or influence tissue morphogenesis [71]. By applying strain, compression, or other mechanical cues, microactuators enable researchers to probe the mechanosensitivity of individual cells or multicellular assemblies. Sensing microrobots are equipped with components that detect changes in temperature, pH, strain, or molecular composition within the surrounding microenvironment [20]. These microrobots are used in theranostic platforms—integrating therapy with diagnostics—or to collect physiological data from difficult-to-access regions *in vitro* or *in vivo* [82, 67]. Their feedback capabilities also enable closed-loop control in responsive microrobotic systems.

2.3 Locomotion in Low Reynolds Number Regimes

As robotic systems are miniaturized to micrometer dimensions, the physical principles governing their motion deviate significantly from those at the macroscale. In large-scale systems, inertia dominates motion; however, at the microscale, viscous forces overwhelm inertial effects [83]. This fundamental shift is governed by scaling laws: inertial forces scale with the cube of length, while viscous forces scale linearly. As a result, microscale objects cannot glide—once external actuation ceases, motion stops immediately. Gravitational forces also become negligible, while surface effects such as drag, adhesion, and interfacial tension become increasingly prominent [84]. This transition in fluid dynamics is quantitatively described by the Reynolds number, a dimensionless parameter that expresses the ratio of inertial to viscous forces in a fluid system:

$$\text{Re} = \frac{\rho V L}{\eta}, \quad (2.1)$$

where ρ is the fluid density, V is the characteristic velocity, L is the characteristic length, and η is the dynamic viscosity. For devices operating at the submillimeter scale, the Reynolds number is typically in the range of 10^{-4} to 10^{-6} , firmly placing them in the low Reynolds number regime [85].

In this regime, fluid flow becomes linear and time-reversible, and locomotion is dominated by viscous drag, defined by Stokes' law:

$$F_{\text{drag}} = 6\pi\eta rv, \quad (2.2)$$

where F_{drag} represents the drag force on a spherical object moving at velocity v , r is the object's radius, and η is the viscosity of the surrounding fluid. Unlike macroscopic systems, where drag scales quadratically with velocity, this linear relationship implies that a continuous force must be applied to maintain motion. Small-scale robots, particularly those in the nanometer scale, simultaneously experience Brownian motion—random displacements caused by thermal fluctuations of fluid molecules, which impose further constraints on precise positioning and control [86]. Despite these challenges, microorganisms have evolved robust locomotion strategies tailored to low Reynolds regimes. Bacteria such as *Escherichia coli* achieve propulsion by rotating bundles of helical flagella, powered by molecular motors [87]. This rotary mechanism breaks time-reversal symmetry, a requirement for generating net displacement in viscously dominated environments [88]. Eukaryotic cells, such as *spermatozoa* and *Chlamydomonas*, rely on flexible flagella or cilia that generate non-reciprocal beating patterns to move effectively through fluid [89, 90]. This reliance on asymmetric, time-irreversible motion is essential at low Reynolds numbers, where viscous forces dominate. In fact, Purcell's scallop theorem arises from these observations: reciprocal motions—those symmetric under time reversal—fail to produce propulsion in such environments [85]. These biological strategies have inspired the development of synthetic microrobots capable of locomotion in similar conditions. Rigid helical microrobots actuated by rotating magnetic fields replicate the corkscrew-like propulsion of bacterial flagella [62, 75]. Soft microrobots, often composed of stimuli-responsive hydrogels, undergo bending, swelling, and peristaltic deformation in response to external cues such as light or heat, mimicking muscular motion [23, 58]. In biohybrid systems, living microorganisms such as sperm and algae are integrated into synthetic frameworks, exploiting their natural motility for navigation within biological fluids [91, 64]. All of these strategies share a common principle: to generate propulsion under low Reynolds number constraints, the actuation must be non-reciprocal in time and asymmetric in space, while achieving enough net propulsion force that overcomes drag.

2.4 Actuation Mechanisms in Microrobotics

2.4.1 Magnetic Systems

Magnetic actuation is among the most widely adopted methods for guiding and manipulating microrobots due to its compatibility with soft matter systems, wireless controllability, and ability to penetrate biological tissues without significant attenuation in field strength. Unlike optical and acoustic

stimuli, magnetic fields do not require line-of-sight access or specific fluidic properties, making them well-suited for both *in vivo* and *in vitro* environments [92, 93]. At the microscale, magnetic forces and torques are commonly used to actuate microrobots by integrating magnetically responsive materials into their structure. Rigid microrobots often incorporate ferromagnetic components to enable direct torque transfer, while soft microrobots typically embed superparamagnetic nanoparticles within polymeric matrices to allow for flexible, shape-adaptive actuation [94, 91]. The rotational torque $\vec{\tau}$ generated on a microrobot with magnetic moment $\vec{m}$ in an external magnetic field $\vec{B}$ is expressed as:

$$\vec{\tau} = \vec{m} \times \vec{B} \quad (2.3)$$

This torque facilitates controlled reorientation or continuous rotation [93]. Translational motion arises from magnetic field gradients and is given by the force:

$$\vec{F} = \nabla(\vec{m} \cdot \vec{B}) \quad (2.4)$$

In uniform fields ($\nabla \vec{B} = 0$), torque-based locomotion dominates, while spatially varying fields enable directed translation. Magnetic actuation platforms often rely on programmable electromagnetic coil systems. Helmholtz coils generate uniform rotating fields for torque-driven propulsion, while Maxwell coils and gradient coil configurations provide translational control [95, 96]. Advanced platforms combine multiple coils to achieve full 3D orientation, rotation, and positioning of single and collective microrobots with high spatiotemporal resolution. Magnetic microrobots use diverse locomotion strategies depending on their geometry and material properties:

i. Helical propulsion: Rigid, helical microrobots rotate in uniform magnetic fields to generate corkscrew-like forward motion, mimicking bacterial flagella. These devices are commonly used for navigation in vascular and gastrointestinal systems [62, 75]. *ii. Flexible tail propulsion:* Microrobots with soft tails or filaments bend periodically under alternating magnetic fields, creating traveling wave motions akin to spermatozoa and cilia [23]. This feature enables navigation in confined and viscous environments, such as extracellular matrices and cerebrospinal fluid [97]. *iii. Surface rolling and walking:* Flat or asymmetric microrobots can roll and crawl along boundaries using whole-body rotation or frictional anchoring. These modes are advantageous for transporting cargo along mucus-covered surfaces [98].

Magnetically actuated microrobots have been demonstrated in a variety of biomedical contexts. *In vivo*, they have been used for targeted drug delivery, biopsy collection, embolism removal, and localized hyperthermia [63, 65]. Applications include navigating through vasculature, crossing tissue barriers, and actuating on specific tissue sites under imaging guidance. *In vitro*, magnetic systems

can be integrated into tumor models, 3D cultures, and microfluidic devices for dynamic manipulation, including mechanical stimulation, cell positioning, and cargo delivery [55, 71, 99, 61, 100]. Soft hydrogel-based microrobots embedded with magnetic particles have also been developed as fully biodegradable carriers for targeted stem cell delivery. These systems can encapsulate human stem cells, navigate using external magnetic fields, and enzymatically degrade to release viable cells that subsequently proliferate and differentiate at the target site, demonstrating potential for regenerative therapies [58].

A particularly powerful feature of magnetic actuation in these contexts is its ability to enable swarm control. Rather than being a locomotion mode, swarm behavior represents a higher-order control strategy that can be applied to rolling, helical, or flexible microrobots [101]. Through external magnetic fields, large numbers of microrobots can be organized into programmable collectives—such as chains, clusters, or dispersed swarms—that respond collectively to dynamic field patterns. These swarms can aggregate, reconfigure, or generate flow fields to enhance functionalities like targeted delivery, tissue remodeling, and clot disruption [76]. This scalable, programmable coordination is especially advantageous in complex *in vivo* environments where individual microrobot navigation may be limited.

Overall, magnetic actuation provides a flexible and minimally invasive strategy for precise microrobot control in biological systems. Its adaptability across different materials, geometries, and environments makes it foundational to both experimental and translational microrobotics. However, because magnetic actuation relies on global fields, it is difficult to selectively control individual microrobots when many are present in a single experiment, limiting spatial resolution at small scales. At the same time, this global nature can be advantageous for synchronizing and actuating large numbers of microrobots simultaneously, enabling collective behaviors and swarm-based functionality.

2.4.2 Acoustic Systems

Acoustic actuation offers an advantageous approach for driving microrobots, especially in biological environments where non-invasive, deep-tissue manipulation is desired. Unlike optical actuation, which is limited by scattering and absorption in tissue, ultrasound propagates efficiently through opaque media and can penetrate complex 3D constructs. These characteristics make acoustic fields attractive for *in vitro* systems such as spheroids and organoids, and even for targeted *in vivo* delivery under ultrasound imaging guidance [68, 102].

At the microscale, acoustic fields interact with suspended objects primarily through two mechanisms: *acoustic radiation* pressure and *acoustic streaming*. In a standing wave field, compressional and rarefaction phases produce pressure gradients that act on particles, leading to a net time-

averaged force known as the *acoustic radiation force*. This force can trap and displace particles toward nodes or antinodes of the pressure field depending on their physical contrast with the surrounding medium [103, 104]. The force is derived from the gradient of the acoustic potential energy U_{ac} , expressed as:

$$\vec{F}_{rad} = -\nabla U_{ac} \quad (2.5)$$

The acoustic potential energy itself depends on particle size, density, and compressibility:

$$U_{ac} = \frac{4\pi a^3}{3} \left(\frac{1}{2} f_1 \left(\frac{\rho_p - \rho_m}{\rho_p + 2\rho_m} \right)^2 + f_2 \left(\frac{c_p - c_m}{c_p + 2c_m} \right)^2 \right) \langle p^2 \rangle \quad (2.6)$$

Here, a is the particle radius, ρ_p and ρ_m are the densities of the particle and medium, and c_p and c_m represent the speeds of sound in the particle and medium, respectively. The term $\langle p^2 \rangle$ denotes the time-averaged square of the acoustic pressure. The dimensionless coefficients f_1 and f_2 quantify the monopole and dipole contrast between the particle and the fluid, determining whether the particle is attracted to pressure nodes and antinodes [103, 104].

A second form of propulsion arises from *acoustic streaming*, a nonlinear effect in which oscillating boundaries and internal asymmetries generate steady microflows within the fluid. This flow can be harnessed to propel microrobots that contain structural asymmetries, such as tilted fins, cavities, or embedded bubbles, among which bubble-based designs have been widely studied. When subjected to ultrasound, a trapped air bubble oscillates, producing localized flow fields that generate net propulsion around the microrobot [105]. These systems can achieve rapid movement and complex trajectories without requiring magnetic materials and direct physical contact. For example, acoustic streaming has also been utilized to assemble living cells into predefined patterns within hydrogels by leveraging holographically shaped ultrasound fields. In such approaches, phase-engineered acoustic landscapes produce localized convection flows that guide suspended cells into complex geometries, which are then fixed by gelation. These methods preserve cell viability and structure, offering a noncontact strategy for biofabrication in tissue engineering and mechanobiology [106]. Acoustically actuated microrobots have been explored for diverse biomedical applications, leveraging their wireless control, biocompatibility, and scalability. *In vivo*, they have been used for targeted drug delivery, including bubble-based cargo release and site-specific deposition via acoustic resonance [105]. However, bubble-based systems often suffer from limited stability, as bubbles can collapse or dissolve under prolonged acoustic exposure or in variable physiological conditions. Systems employing armored microbubbles or porous spiral structures have demonstrated drug release in response to ultrasound in confined spaces [107], though their fabrication complexity and potential

for clogging in dense tissues may limit scalability. Hybrid acoustic-magnetic microrobots have been developed for drug delivery and show great potential as biohybrid biocompatible tools for *in vivo* therapy [91], but their integration of multiple actuation modalities increases system complexity and may pose challenges for precise control.

2.4.3 Chemical and Catalytic Systems

Chemical and catalytic microrobots represent a distinct class of autonomous systems that convert chemical energy directly into motion. These systems are particularly attractive for their ability to navigate complex microenvironments, exploit local chemical gradients, and perform tasks such as targeted drug delivery, environmental sensing, or microscale remediation. Their self-propelling nature also enables collective behaviors and swarm dynamics that can enhance functionality at scale. However, their broader application in biological contexts remains limited due to challenges such as fuel toxicity, limited directional control, and sensitivity to environmental conditions [108].

The propulsion mechanisms for these microrobots rely on local physicochemical gradients generated by surface-bound reactions. Several distinct modes have been identified, including bubble propulsion, self-diffusiophoresis, and self-electrophoresis, each governed by different interfacial phenomena [109, 110]. The most well-studied and visually striking among these is bubble propulsion, which uses catalytic decomposition of hydrogen peroxide (H_2O_2) to generate oxygen gas:



In microrobots featuring confined geometries—such as conical tubes or hemispherical cavities—this reaction leads to asymmetric bubble nucleation and expulsion, producing a net thrust [53]. The directional recoil from gas ejection drives forward propulsion. This mechanism is enhanced by surfactants like sodium dodecyl sulfate (SDS), which lower surface tension and promote bubble detachment. The approximate propulsion velocity v can be related to the bubble generation frequency f_b and the average bubble radius R_b as:

$$v \propto f_b R_b^2 \quad (2.8)$$

This relation implies the sensitivity of propulsion efficiency to reaction kinetics, fuel concentration, and fluidic properties [111]. Although bubble-based systems offer high thrust, they are typically limited to environments where bubble formation and release are unobstructed, which may exclude dense or high-viscosity biological media. Moreover, in *in vivo* applications, the uncontrolled generation of gas bubbles poses a significant risk of embolism, particularly in microvascular networks, and may trigger immune responses or local inflammation [112]. The use of chemical fuels such as

hydrogen peroxide also introduces cytotoxic effects, making this propulsion method unsuitable for applications involving delicate tissues, live cell cultures, or immune-reactive environments.

An alternative strategy is self-diffusiophoresis, wherein catalytic reactions on asymmetric surfaces generate local concentration gradients that induce slip flow along the particle surface. Janus particles, with catalytic coatings on one hemisphere, are widely used in this context. The asymmetric reaction results in solute accumulation on one side of the microrobot, driving interfacial flow and particle motion in the opposite direction [110]. A closely related mechanism is self-electrophoresis, where electrochemical reactions across bimetallic surfaces create ion gradients and electric fields. For example, platinum–gold nanorods in H_2O_2 produce directional ion flux and electroosmotic flow, resulting in axial propulsion from the platinum to the gold end [113, 78]. Beyond metal-catalyzed propulsion, enzyme-powered microrobots have emerged as biohybrid alternatives that operate using biologically compatible fuels such as glucose or urea. These systems harness natural enzymatic reactions—e.g., catalase, urease, and glucose oxidase—immobilized on the microrobot surface to produce motion-driving gradients [77, 114, 115]. Enzyme-driven propulsion offers improved biocompatibility and functional integration with living systems, though it also introduces sensitivity to pH, temperature, and enzyme inhibitors. *In vitro*, catalytic and chemically powered microrobots have demonstrated utility in microfluidic environments and synthetic tissue models. They have been used for cargo transport, mixing, targeted delivery, and environmental sensing. However, their applicability in biological systems remains constrained by the toxicity of common fuels like hydrogen peroxide, hydrazine, and sodium borohydride [116]. Alternative fuels, including magnesium, glucose, and even biological metabolites, offer safer profiles but at the cost of reduced propulsion force and duration. Additionally, chemical propulsion lacks intrinsic directionality unless supported by geometrical asymmetry and auxiliary steering mechanisms such as magnetic alignment.

Environmental factors such as salinity, pH, and viscosity, typical of *in vitro* gels and cell media, also affect catalytic efficiency and gradient formation. These limitations necessitate careful material design and consideration of application context. Still, the ability of chemically powered microrobots to operate without external systems makes them attractive candidates for distributed and scalable microrobotic platforms.

2.4.4 Optical Systems

Light offers a powerful tool for wireless microrobot control, delivering energy with exceptional spatial and temporal resolution. In soft microrobotic systems, optical inputs allow precise manipulation and deformation without direct contact, making them particularly suitable for *in vitro* applications and engineered tissue environments. This section first focuses on locomotion strategies enabled by light-

induced thermal and interfacial phenomena, followed by a discussion of the actuation mechanisms and materials responsible for light-to-motion conversion.

Optical Locomotion Mechanisms: Locomotion in soft microrobots can be achieved through fluid-mediated forces triggered by localized heating. One prominent example is thermophoresis, or the Soret effect, where particles migrate along temperature gradients. By irradiating absorptive materials—such as gold nanoparticles or metallic coatings—with focused light, localized heat is generated, creating spatial temperature gradients in the surrounding medium. In addition to gold, other photothermal materials such as carbon-based nanostructures (e.g., carbon nanotubes and graphene oxide), conducting polymers, and semiconductor nanomaterials can be employed to achieve similar heating effects [117]. This phenomenon drives motion without mechanical contact, especially effective in low Reynolds number environments. The resulting thermophoretic velocity, $\vec{v}_{th}$, is described by:

$$\vec{v}_{th} = -D_T \nabla T \quad (2.9)$$

where D_T is the thermal diffusion coefficient and ∇T is the temperature gradient [118]. Beyond thermophoresis, Marangoni propulsion enables motion at fluid interfaces through surface tension gradients. These gradients emerge when localized heating alters the interfacial tension, leading to fluid flow along the interface and propulsion of nearby microrobots [119, 120]. At higher thermal loads, buoyancy-driven convection may occur, inducing vertical and circulatory flow patterns around the heat source [121]. These flow fields are well-suited for microscale mixing, particle transport, or facilitating penetration into dense tissue matrices.

Several light-powered microrobot designs have demonstrated successful implementation of such locomotion strategies. Composite microrobots based on Poly(N-isopropylacrylamide) (PNIPAM) and gold nanorods (GNRs) exhibit rolling and inchworm-like crawling behaviors under modulated NIR light. Controlled thermal gradients along the structure induce directional movement over centimeter-scale distances despite the small device footprint [21]. In another approach, liquid crystalline elastomer (LCE) walkers functionalized with azobenzene undergo cyclic bending and relaxation in response to alternating ultraviolet (UV) and visible light. This periodic deformation enables crawling along substrates by harnessing photomechanical contraction during the isomerization cycle [122].

Photothermal Actuation and Stimuli-Responsive Materials: The locomotion effects described above rely on underlying actuation mechanisms that convert light energy into mechanical motion. In soft microrobots, this conversion primarily occurs via three physical mechanisms: photothermal heating, photoisomerization, and optical gradient forces [23]. Among them, photothermal processes are the most extensively employed due to their reliability and broad material compatibility.

A key component of photothermal actuation is the use of light-absorbing transducers that efficiently convert optical energy into heat. Gold nanorods are particularly effective for this purpose, owing to their tunable localized surface plasmon resonance (LSPR) in the near-infrared range. When illuminated, GNRs rapidly generate localized heat, establishing thermal gradients with high spatial resolution (Figure 2.2) [21]. This heating can be precisely controlled by adjusting nanorod size, surface functionalization, and dispersion within the microrobot matrix.

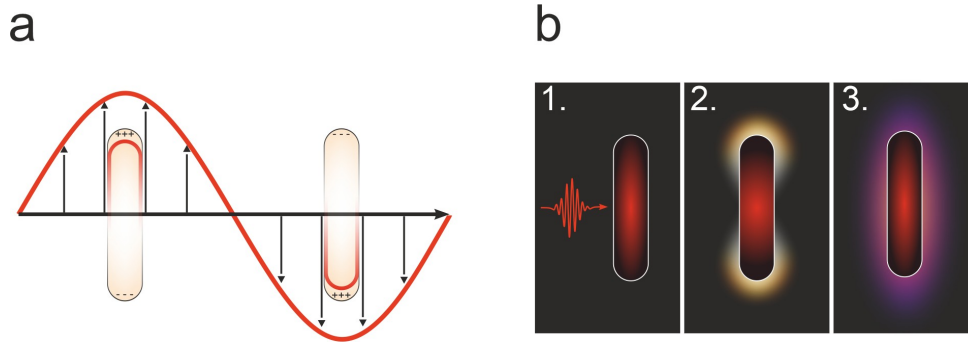


Figure 2.2 Plasmonic actuation and heating of gold nanorods. a) Interaction between an incident electromagnetic wave and a gold nanorod aligned along its longitudinal axis. Plasmonic polarization enables efficient energy absorption. b) Photothermal conversion mechanism. (1) A photon is absorbed by the nanorod. (2) Plasmonic oscillation induces polarization at the nanorod ends. (3) Absorbed energy is dissipated as localized heat, which drives actuation in thermoresponsive host materials.

When embedded within thermoresponsive polymers such as poly(*N*-isopropylacrylamide) (PNIPAM), this localized heating drives rapid and reversible deformation. PNIPAM exhibits a volume phase transition around 32 °C: it swells at lower temperatures and collapses when heated beyond the threshold [123]. By leveraging this thermal response, focused light can be used to trigger controlled shape changes such as bending, contraction, or folding. The ability to confine these transformations to submicron domains using tightly focused beams makes PNIPAM–AuNR systems highly effective for applications in cell manipulation and targeted actuation [122]. Other materials utilize alternative activation modes. For instance, azobenzene-doped LCEs undergo photoisomerization when exposed to UV and visible light. This reversible conformational change enables soft actuators to cyclically bend, elongate, or contract without thermal input [59]. Such mechanisms underpin many microrobotic walkers and grippers, particularly where direct heating is less desirable. Light-driven actuation also supports advanced biofunctional operations. Microgrippers, designed from thermoresponsive or light-sensitive bilayers, have been used for the capture, release, and manipulation of biological specimens. By exploiting controlled heating, the gripper structure can fold around individual cells and return to its original shape when the stimulus is removed. This reversible behavior offers a minimally invasive method for interacting with delicate biological samples [63, 124].

In summary, light actuation offers a uniquely powerful and multifunctional approach to microrobot control, combining wireless energy delivery with submicron spatial precision and diverse response

modes. Through photothermal conversion—most notably using gold nanorods in conjunction with thermoresponsive materials such as PNIPAM—a single light input can simultaneously enable structural reconfiguration, induce fluid-mediated locomotion (including thermophoretic, Marangoni, and convective motion), and facilitate complex tasks such as cargo transport, targeted cell manipulation, and environmental sensing.

2.5 Material Choices and Fabrication Techniques for Soft Microrobots

Soft microrobots represent a specialized class of untethered, mobile microdevices designed to operate in highly constrained and dynamic environments, particularly within biological systems [54, 64]. Their defining feature is mechanical compliance—constructed from flexible, deformable materials, these microrobots can adapt to irregular surfaces, navigate confined tissue spaces, and interact safely with cells and tissue structures [20]. Compared to their rigid counterparts, soft microrobots offer enhanced biocompatibility and reduced invasiveness. Importantly, they can also interface more effectively with cells and tissues at a biochemical level, as their constituent materials—often borrowed from bioengineering and tissue engineering—are inherently designed to mimic or integrate with biological environments. These qualities are especially advantageous in *in vitro* systems such as organoids, spheroids, and engineered tissue models, where maintaining structural integrity and physiological relevance is critical [125]. Soft microrobots enable minimally disruptive cell manipulation, localized stimulation, and dynamic microenvironment reconfiguration, making them ideal for probing biological processes with high spatiotemporal control. At the same time, their potential for *in vivo* deployment remains strong, particularly in scenarios requiring safe navigation through soft tissues or vascular systems [54]. As such, material selection is foundational to their design, influencing not only mechanical compliance but also actuation, sensing, and degradation behaviors. Table 2.1 summarizes representative materials commonly employed in soft microrobots, highlighting their functional properties and relevance across various biomedical applications.

Table 2.1 Categories of soft microrobots and example materials with corresponding functions.

Category	Example Materials	Functions / Features
Artificial Soft Microrobots	Hydrogels: alginate, PEG, PNIPAM; Elastomers: PDMS, Ecoflex; Stimuli-responsive polymers: PAA, A6ACA, azobenzene, LCEs; Biodegradable polymers: PLGA, PCL	Hydrogels mimic ECM, offer biocompatibility and environmental responsiveness; Elastomers provide flexibility and moldability for locomotion; Stimuli-responsive materials enable thermal, pH, and light actuation; Biodegradable polymers allow safe <i>in vivo</i> deployment and degradation
Biohybrid Soft Microrobots	Synthetic scaffolds combined with muscle cells or motile microorganisms	Merge engineered structures with biological control or propulsion; enable tasks like guided navigation and adaptive response
Biological Soft Microrobots	Living entities: bacteria, algae, sperm cells	Use natural motility and sensing; inherently biocompatible; respond to biochemical cues
Functional Additives (cross-category)	Gold nanorods, magnetic nanoparticles (iron oxide, Nd-FeB), graphene oxide, carbon nanotubes	Enable photothermal actuation, magnetic control, electrical conductivity, and biosensing

Soft microrobots can be broadly categorized into artificial, biological, and biohybrid systems, each employing distinct material strategies [21]. While biological and biohybrid microrobots harness living components such as bacteria or muscle cells to achieve motility and biochemical responsiveness [126, 127, 128], the majority of current developments—and the focus of this work—lie in artificial soft microrobots composed entirely of synthetic materials. These systems are typically built from hydrogels or polymers tailored to exhibit specific mechanical and chemical characteristics. Hydrogels such as alginate, poly(N-isopropylacrylamide) (PNIPAM), and polyethylene glycol are central to this category [129, 130, 131]. These hydrated polymer networks mimic key features of the ECM, offering not only biocompatibility and permeability but also the ability to support cell culture through the incorporation of adhesion ligands (e.g., RGD peptides), tunable mechanical properties, and adjustable porosity and microstructure. Such properties enable controlled cell attachment, proliferation, and migration, while also allowing dynamic responsiveness to environmental cues such as pH, temperature, or biochemical signals [132, 133]. Alginate, for instance, is a naturally derived hydrogel known for its pH and ion sensitivity, enabling applications in drug delivery and shape-morphing microrobots [134, 135]. Polyethylene glycol derivatives like Polyethylene Glycol Diacrylate (PEGDA) can be photopolymerized for high-resolution fabrication, while PNIPAM exhibits thermoresponsive behavior with a volume phase transition near body temperature, supporting actuation and cargo release.

In addition to hydrogels, elastomers such as polydimethylsiloxane (PDMS) and Ecoflex are frequently used in soft microrobots for their stretchability and mechanical resilience. PDMS is widely adopted due to its optical clarity and moldability, as demonstrated in multilegged soft robots designed for gastric navigation using snap-through buckling mechanisms [136]. Ecoflex, with even lower stiffness, facilitates biomimetic motion and has been employed in jellyfish-inspired robots with embedded magnetic nanoparticles for rhythmic actuation [137].

To enable responsive behavior, stimuli-sensitive polymers are integral. PNIPAM, as mentioned, changes shape with temperature shifts and has been used in fluorescent microrobots for thermal sensing [138]. pH-sensitive polymers like polyacrylic acid (PAA) and acryloyl 6-aminocaproic acid (A6ACA) allow microrobots to respond to acidic environments, with A6ACA facilitating self-adhesion and phase transitions useful in applications such as aneurysm embolization [139]. Light-responsive materials, including azobenzene-based systems and liquid crystal elastomers (LCEs), permit precise remote actuation, as in the case of kirigami-inspired LCE microrobots that twist and roll under laser stimulation [140].

Biodegradable polymers further expand the utility of soft microrobots for *in vivo* applications. Polymers such as poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone (PCL) degrade into biocompatible byproducts, allowing for safe bodily clearance post-deployment. PLGA-based microrobots embedded with magnetic nanoparticles have been used for magnetically guided drug delivery and shown to degrade completely under physiological conditions [141]. PCL, responsive to enzymatic degradation, has enabled tumor-targeted drug release via lipase-sensitive microrobots [142]. Hybrid designs such as PNIPAM–PCL microgrippers exemplify the integration of responsive actuation with controlled degradation [143].

To add advanced functionalities, conductive and magnetic nanocomposites are incorporated into soft matrices. Gold nanorods embedded in hydrogels like gelatin and PEG permit localized photothermal actuation when irradiated with near-infrared light, enabling controlled drug release and microrobot deformation [144]. Magnetic nanoparticles—such as iron oxide and neodymium-iron-boron—are integrated into materials like chitosan, PLGA, or Ecoflex to enable magnetic guidance and locomotion [141, 137, 145].

The evolving material landscape of soft microrobotics reflects a convergence of softness with multifunctionality. Innovations in dynamic polymers, nanocomposites, and hybrid architectures continue to unlock new possibilities, empowering microrobots to undertake increasingly complex biomedical and environmental tasks with precision and adaptability.

2.5.1 Fabrication Techniques for Soft Microrobots

The fabrication of soft microrobots demands a precise alignment between material properties and the requirements of their intended biomedical and environmental functions. As these systems rely on soft, often stimuli-responsive materials, their fabrication processes must support high-resolution patterning, 3D shaping, and compatibility with embedded functional components. Over the past decade, a variety of microfabrication techniques have emerged to meet these needs, each offering distinct advantages in scalability, resolution, and functional integration (Table 2.2). These methods can be broadly grouped into photopolymerization-based approaches (e.g., stereolithography and two-photon polymerization for high-resolution 3D structuring), template- and mold-assisted techniques (such as soft lithography or replica molding), microfluidic fabrication (including droplet-based microgel generation), fiber-forming strategies (notably electrospinning for producing nanofibers or 3D printing), and biointegration-driven methods (which incorporate living cells or ECM components during fabrication) [21].

Table 2.2 Comparison of fabrication methods for soft microrobots.

Method Category	Typical Materials	Resolution	Scalability	Geometry / Examples
Photopolymerization	PEGDA, PNIPAM, GelMA	Submicron– μm	Low–High	2D/3D complex; UV-cured, two-photon polymerization
Template / Mold-Based	Alginate, Chitosan, Elastomers	μm –100s μm	Medium–High	Regular, 2.5D shapes; micromolding, layer-by-layer, electroplating
Microfluidic	PEGDA, Alginate, GelMA	μm –100s μm	High	Spherical, capsule-like; microgels, emulsions
Fiber / Printing-Based	PNIPAM, PCL, composite hydrogels	100 nm– μm	Medium	Filaments, 3D folding; 4D-printed grippers
Biohybrid Integration	Live cells + hydrogels / nanogels	Cell-scale	Low	Natural forms; spermbots, RBC–bacteria hybrids

Photopolymerization-based techniques are among the most widely employed strategies, with ultraviolet polymerization offering a straightforward means to fabricate microrobots from photosensitive polymers such as PEGDA and PNIPAM [130, 146]. This approach utilizes photomasks to control crosslinking patterns, enabling the parallel production of microstructures with tailored mechanical properties. For example, spatially tunable hydrogel microclips have been created by modulating UV exposure to define crosslinking gradients [147]. Multilayered microrobots combining thermoresponsive PNIPAM and magnetically responsive Fe-PEGDA have been produced through sequential UV curing steps [148]. In another example, self-folding microrobots were formed by layering PEGDA onto a PNIPAM base, where differential swelling upon NIR exposure induced complex 3D bending

[130]. While UV photopolymerization offers rapid throughput and ease of use, its ability to fabricate fully 3D structures is limited without the inclusion of folding mechanisms and advanced light projection techniques.

To overcome these dimensional limitations, two-photon polymerization (2PP) enables true three-dimensional microfabrication with submicron precision [129, 149]. This method exploits the nonlinear absorption of femtosecond laser pulses to locally polymerize photosensitive materials like PEGDA or GelMA. Through 2PP, researchers have developed microrobots with embedded magnetoelectric nanoparticles for targeted neural cell transport and stimulation [150], and capillary-compatible hydrogel microrollers with precisely controlled geometries [151]. Additionally, programmable microrobots have been constructed for dynamic environmental sensing with submicron resolution [152]. Despite its unmatched spatial precision, the slow serial nature of 2PP limits its scalability for high-throughput applications.

Template- and mold-assisted techniques represent another robust class of fabrication strategies. Micromolding is commonly used to produce soft microrobots by casting polymeric precursors into predefined molds, then curing and gelling the material. This method has been applied to generate cylindrical magnetic microrobots using PDMS molds filled with elastomeric composites [153], and to form gelatin–platinum micromotors within polycarbonate molds, later released via solvent dissolution for hydrogen peroxide-driven propulsion [154]. Template-assisted layer-by-layer (LbL) assembly allows for the deposition of multilayered structures through electrostatic and molecular interactions. Bovine serum albumin/poly-L-lysine (BSA/PLL) multilayered microrockets have been created within porous templates, incorporating gelatin and gold nanoparticles for photothermal drug release [144], while spiral microrobots for drug delivery have been fabricated by depositing alternating chitosan and alginate layers onto calcium-alginate microfibers [155]. Although LbL offers intricate chemical functionality, it is time-intensive due to repeated washing and deposition steps.

Electrochemical deposition offers a straightforward and accessible method for fabricating microscale structures by layering electrically conductive or electroactive materials within patterned templates [156]. Using electric fields to drive localized deposition, researchers have created cylindrical microrobots composed of polymer–metal composites such as polypyrrole (PPy) or poly-3,4-ethylenedioxythiophene (PEDOT) with embedded magnetic materials, enabling magnetically steerable devices with tunable geometry and composition [157]. While this technique provides precise vertical control and good reproducibility, it is generally limited to materials that can undergo electrodeposition and may require careful environmental control for reproducibility, but does not necessitate complex instrumentation—typically relying only on a potentiostat. Compared to high-resolution

methods like two-photon polymerization, electrodeposition remains a low-cost and scalable alternative for specific microrobot geometries.

Microfluidic and flow-based fabrication techniques are indispensable for producing large numbers of soft microrobots with defined size and structure [158]. Droplet microfluidics is commonly used to create monodisperse hydrogel spheres by emulsifying polymer solutions in immiscible carrier fluids, with droplet solidification triggered by ionic crosslinking or photopolymerization. This technique has enabled the production of alginate-based microgels for cell encapsulation and controlled release, often incorporating additional components such as magnetic particles, cells, and proteins to enhance functionality [159, 160, 161]. Stop-flow and continuous flow lithography extend microfluidic capabilities by using UV light to define complex, anisotropic shapes. In these systems, flow is paused while a UV mask projects onto the photosensitive prepolymer, producing defined microstructures [162]. For instance, PEGDA-based microrobots with embedded temperature sensors have been fabricated using this approach [163]. Microfluidic fabrication offers excellent throughput, fine control over microrobot dimensions, and compatibility with live-cell encapsulation [164]. However, it is constrained by challenges in producing high-aspect-ratio or fully 3D structures, and material compatibility is limited by the fluidic and interfacial properties of the system.

Fiber-based fabrication strategies, particularly electrospinning, allow the production of soft microrobots in the form of flexible, nanoscale fibers. In this high-voltage technique, polymer solutions are drawn into fine filaments that can be embedded with functional agents. Double-tailed sperm-inspired microrobots and magnetically steerable swimmers have been constructed by embedding magnetic nanoparticles within electrospun fibers [165, 166]. Hygroscopically responsive actuators capable of bending with humidity changes have also been developed using multilayered electrospun composites [167]. While electrospinning supports high throughput and anisotropic morphology, its use is primarily limited to fibrous geometries and structures with lower mechanical robustness.

Emerging fabrication approaches integrate smart materials to enable shape transformation in response to external stimuli—a concept known as 4D printing. By embedding responsive material gradients, microrobots can be programmed to undergo reversible shape changes, such as uniaxial or biaxial contraction in hydrogels triggered by environmental stimuli [168]. Other implementations include pH-responsive microcapsules capable of reversible expansion and collapse for therapeutic trapping and release [169]. These techniques provide unprecedented control over dynamic shape modulation, yet face ongoing challenges in scaling down complex actuation systems to the microscale.

Finally, biointegration and hybridization methods uniquely incorporate biological components into soft microrobotic systems. These biohybrid approaches exploit the motility, sensing, and metabolic

functions of living organisms such as bacteria, sperm, or red blood cells. In one strategy, red blood cells have been chemically linked to magnetically steerable *E. coli* to create autonomous drug carriers [64], while hydrogel microrobots functionalized with neutrophils and *E. coli* membranes have demonstrated chemotactic targeting of brain tumors [170]. Physical integration methods have also been used, where motile cells are embedded or tethered to synthetic scaffolds. For example, sperm cells have been guided using synthetic magnetic anchors for targeted drug delivery under flow conditions [171], and asymmetric binding of bacteria to polymer beads has produced directional propulsion systems [172, 173]. These biohybrid systems offer self-powered mobility and natural biointerface compatibility, though their fabrication remains complex and difficult to scale due to variability in biological components.

Altogether, the field of soft microrobot fabrication continues to evolve toward methods that combine high structural precision, functional responsiveness, and biological integration. Each fabrication approach offers unique strengths and trade-offs, and future progress will likely depend on hybridizing techniques to combine their respective advantages for increasingly sophisticated and application-ready microrobots.

2.6 Microrobotic Approaches for Mechanobiology and Thermal Biology

Microrobots have demonstrated considerable potential in biomedical sciences, ranging from navigation through biological fluids to targeted cargo delivery and localized actuation. Beyond these translational goals, there is a growing interest in employing microrobots as investigative tools for fundamental cell biology. To fully realize this potential, microrobotic systems must interact with cells in ways that are biologically informed and mechanistically relevant. This requires a deep understanding of the molecular machinery that governs how cells sense physical cues—such as force, substrate stiffness, and temperature—and translate them into biochemical responses [174, 175, 176, 177]. These mechanosensitive and thermosensitive pathways define the thresholds and kinetics that microrobots must engage with to elicit controlled and interpretable cellular outcomes. This section outlines the key components of cellular force and temperature sensing, including the ECM–integrin–cytoskeleton axis, nuclear mechanotransduction, mechanosensitive ion channels such as Piezo1, and thermally activated transient receptor potential (TRP) channels [178, 179, 180]. Together, these form the foundational map for designing microrobotic systems capable of not just interfacing with cells, but also uncovering the rules that govern their dynamic responses to the physical microenvironment.

Cellular mechanotransduction begins at the interface between cells and the ECM, where matrix stiffness and ligand composition vary widely across tissues—from less than 1 kPa in brain tissue to more than 30 kPa in bone [181, 182, 183]. Cells probe this environment through integrin-based adhesion complexes that physically link the ECM to the actin cytoskeleton (Figure 2.3). When integrins cluster upon ECM binding, they recruit talin and vinculin to form focal adhesions (FAs), which act as hubs for signal transduction and force transmission. According to the “molecular clutch” model, mechanical load on integrins promotes talin unfolding, exposing binding sites for vinculin and reinforcing the adhesion [184, 185]. This process exhibits a threshold-like response: only when substrate stiffness exceeds approximately 3–5 kPa does significant talin unfolding and FA maturation occur [175, 186].

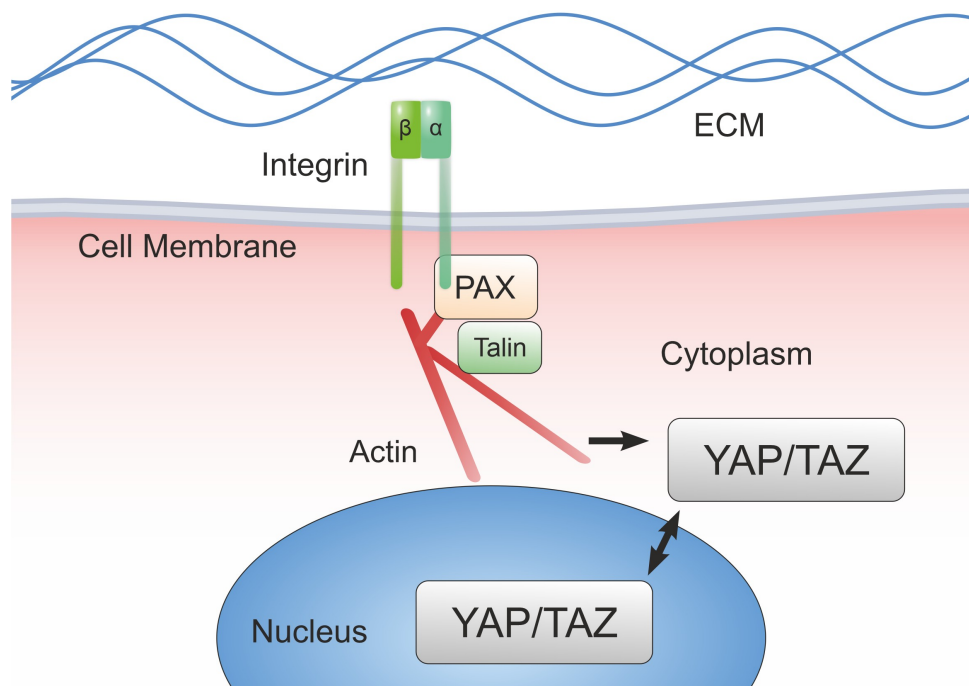


Figure 2.3 Schematic of mechanotransduction through the ECM–integrin axis. Integrins bind ECM components and cluster at focal adhesions, where intracellular adaptor proteins such as talin and paxillin link integrins to the actin cytoskeleton. Mechanical signals transmitted through this complex regulate cytoskeletal tension and cellular architecture. These mechanical cues influence the nuclear localization of transcriptional regulators YAP and TAZ, which translocate into the nucleus under high tension to promote gene expression, or remain cytoplasmic under low mechanical input.

These signals are relayed to the nucleus via actin stress fibers connected through the LINC (linker of nucleoskeleton and cytoskeleton) complex [176, 177]. Nuclear deformation induced by cytoskeletal tension affects chromatin organization and gene regulation. One of the most studied mechanotransduction pathways involves Yes-associated protein and Transcriptional coactivator with PDZ-binding motif (YAP/TAZ), which accumulate in the nucleus in response to high mechanical input [187, 188, 189]. Experiments using tunable-stiffness polyacrylamide gels have shown that YAP nuclear localization increases sharply beyond an ECM stiffness of 18 kPa, saturating around 60

kPa [190, 175]. Notably, pathways such as those mediated by Piezo1 channels, which respond to membrane tension and pressure, further highlight the diversity of cellular force sensors beyond focal adhesions [191, 178].

Thermal cues are similarly integrated through ion channels of the Transient Receptor Potential Vanilloid (TRPV) family (Figure 2.4). TRPV4, for instance, activates around 27–34 °C and responds to both temperature and mechanical stress, placing it at the intersection of thermo- and mechanosensation. At higher temperatures, channels like TRPV1 (activation >42 °C) and TRPV2 (>52 °C) are triggered, resulting in calcium influx and downstream activation of cytoskeletal and transcriptional pathways [180, 192]. Calcium signaling via these channels often activates mitogen-activated protein kinases (MAPKs), including ERK1/2 and p38, further integrating mechanical and thermal signals with nuclear responses such as cell proliferation or migration [193, 194]. Mechanosensory and thermosensory pathways often intersect at shared signaling nodes such as calcium influx and MAPK activation, underscoring the relevance of integrated approaches in microrobotic stimulation [194].

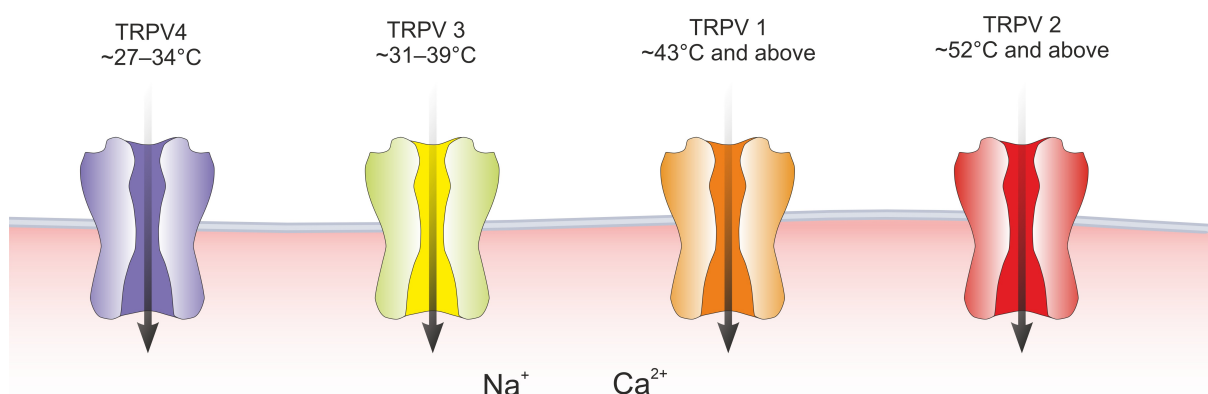


Figure 2.4 Heat-sensitive TRPV channels and their temperature thresholds. Schematic representation of the four thermally activated TRPV channels—TRPV4, TRPV3, TRPV1, and TRPV2—arranged by increasing activation temperature. TRPV4 responds to mild warmth (27–34 °C), TRPV3 to moderate heat (31 – 39 °C), TRPV1 to noxious heat (>42 °C), and TRPV2 to extreme temperatures (>52 °C). Upon activation, these channels open to allow influx of Ca²⁺ and Na⁺, triggering downstream cellular responses such as cytoskeletal remodeling, changes in cell volume, and activation of signaling pathways.

Despite the wealth of biological knowledge about how cells sense and respond to mechanical and thermal cues, through mechanisms such as ECM-mediated mechanotransduction or TRP channel activation, there remains a clear lack of tools capable of engaging these pathways at the single-cell level with both spatial and temporal precision. As discussed earlier in this chapter, there is a growing demand for technologies that can apply controlled stimuli to individual cells in dynamic and physiologically relevant environments. Conventional techniques, including atomic force microscopy, bulk heating, or deformable substrates, offer valuable insights but often lack the resolution and flex-

ibility needed to target specific cells or operate in real time. Microrobots offer a promising solution to this challenge. Their small size, wireless operation, and ability to deliver localized mechanical or thermal inputs position them as uniquely suited for probing and manipulating sensory pathways at the cellular scale. A number of studies have begun to demonstrate this potential—magnetic microrobots have been used to measure tissue stiffness and examine YAP/TAZ pathway activation in spheroids [67], while magnetically actuated microstructures have applied forces to single cells to investigate cytoskeletal dynamics [195]. In our own work, the TACSI microrobot was developed to apply localized heating and activate TRP channels, resulting in measurable intracellular calcium responses [120]. These examples highlight the emerging role of microrobots as tools for investigating mechanosensory and thermosensory signaling. Further development in this direction could enable new strategies for real-time cellular interrogation and precise control in both *in vitro* models and future *in vivo* applications.

3 Design, Fabrication, and Biointegration of Thermally Activated Cell-Signal Imaging Microrobots

This chapter presents the design, fabrication, and characterization of a laser-driven microrobot for spatiotemporally controlled thermal stimulation of single cells. Emphasis is placed on the integration of photothermal and thermosensitive elements, microfluidic encapsulation strategies, and real-time temperature sensing capabilities. Experimental results demonstrate targeted stimulation of human embryonic kidney cells and effective locomotion in three-dimensional (3D) workspaces using thermophoretic convection. This work lays the foundation for the microrobot-assisted tissue building system developed in the following chapters; as a first step, it was essential to engineer a soft, bio-compatible microrobot capable of precise actuation and cellular interaction. The work presented in this chapter was published under: Philipp Harder, Nergishan Iyisan, Chen Wang, Fabian Kohler, Irina Neb, Harald Lahm, Martina Dreßen, Markus Krane, Hendrik Dietz, Berna Özkale. A Laser-Driven Microrobot for Thermal Stimulation of Single Cells. Advanced Healthcare Materials. 2023, 12, (26)

3.1 Introduction

Thermal cell stimulation has emerged as an effective therapeutic strategy for applications such as tumor ablation and bacterial eradication [196, 197, 198]. By using light-activated nanoheaters to convert light into heat, this approach enables non-contact delivery of localized noxious doses—often exceeding 70 °C—to induce cell death [199, 200, 201]. Beyond ablation, photothermal materials facilitate the remote actuation of diverse biological processes, including protein activation and controlled cellular responses. Interestingly, lowering the dose of thermal stimulation to mild temperatures of 45 °C enhanced the wound healing process in a mouse skin model [202]. More recently, thermal neuromodulation has shown promise as a tool to manipulate brain activity and treat disorders such as depression and arrhythmias [203]. While the influence of noxious heat on mammalian cells is well studied, it remains unclear how mild heat doses affect cell behavior. Understanding this re-

lationship could enable new therapeutic strategies, yet delivering carefully regulated heat doses to single mammalian cells in physiologically relevant microenvironments remains technically challenging. Early methods to study thermal biology relied on resistive heaters and perfusion chambers that targeted large cell populations [204, 205]. While being informative, these techniques are typically constrained by poor spatial resolution, thermal hysteresis, and slow response times. Advances in microtechnologies and nanomaterials have led to the development of localized and rapid small-scale heaters. Among these, plasmonic gold nanomaterials have emerged as highly efficient photothermal converters. When exposed to near-infrared (NIR) light, gold nanoparticles generate heat via localized surface plasmon resonance, enabling fine control over magnitude, location, and timing of generated heat loads [206, 207]. Gold nanoparticles have been used to investigate temperature-sensitive processes such as ion channel gating, integrin signaling, and apoptosis at the single-cell level [208, 209, 210, 211].

However, a persistent limitation of existing plasmonic tools is their inability to interact dynamically with cells in three-dimensional (3D) environments. Static nanoheaters embedded in hydrogels or tethered to substrates lack the mobility and adaptability needed to target specific cells within heterogeneous populations [212, 213, 214]. In contrast, soft microrobots offer an attractive alternative. Previous microrobotic systems have demonstrated capabilities in guiding cell migration, triggering signaling pathways, and probing mechanical properties [213, 215, 216]. Yet, few have achieved the combination of real-time thermal feedback, controlled heating, and light-driven mobility within a biocompatible framework.

In this study, we introduce the *Thermally Activated Cell-Signal Imaging* (TACSI) microrobot, a novel soft microrobotic platform designed for localized thermal stimulation and real-time temperature sensing at the single-cell level (Figure 3.1). The TACSI microrobot is composed of gold nanorods (GNRs) embedded within an alginate-based hydrogel matrix and functionalized with the thermosensitive fluorescent dye Rhodamine B (RhB), enabling photothermal heating and *in situ* thermal readout under near-infrared light actuation. Fabricated via high-throughput microfluidic encapsulation, the microrobots exhibit uniform nanocomposite distribution, tunable thermal and mechanical properties, and compatibility with standard cell culture systems. Under optical stimulation, TACSI microrobots achieve temperature increases of up to 40.9 °C above ambient and support multiple light-induced locomotion modes—including rolling, vertical propulsion, and Marangoni motion—with speeds ranging from 5 to 65 $\mu\text{m/s}$. These locomotion capabilities allow precise 3D targeting of individual cells, while RhB fluorescence offers real-time thermal feedback. To demonstrate biological functionality, we employed human embryonic kidney (HEK293) cells, a widely used immortalized cell line known for its reliability, ease of transfection, and suitability for studying intracellular signaling

pathways [217]. Upon localized heating by TACSI microrobots, we observed temperature-dependent intracellular calcium responses, with significant elevations in calcium levels at temperatures exceeding 52 °C. This response is consistent with the activation of thermosensitive ion channels such as TRPV1 and TRPV2, highlighting the utility of TACSI microrobots for probing heat-mediated cell signaling.

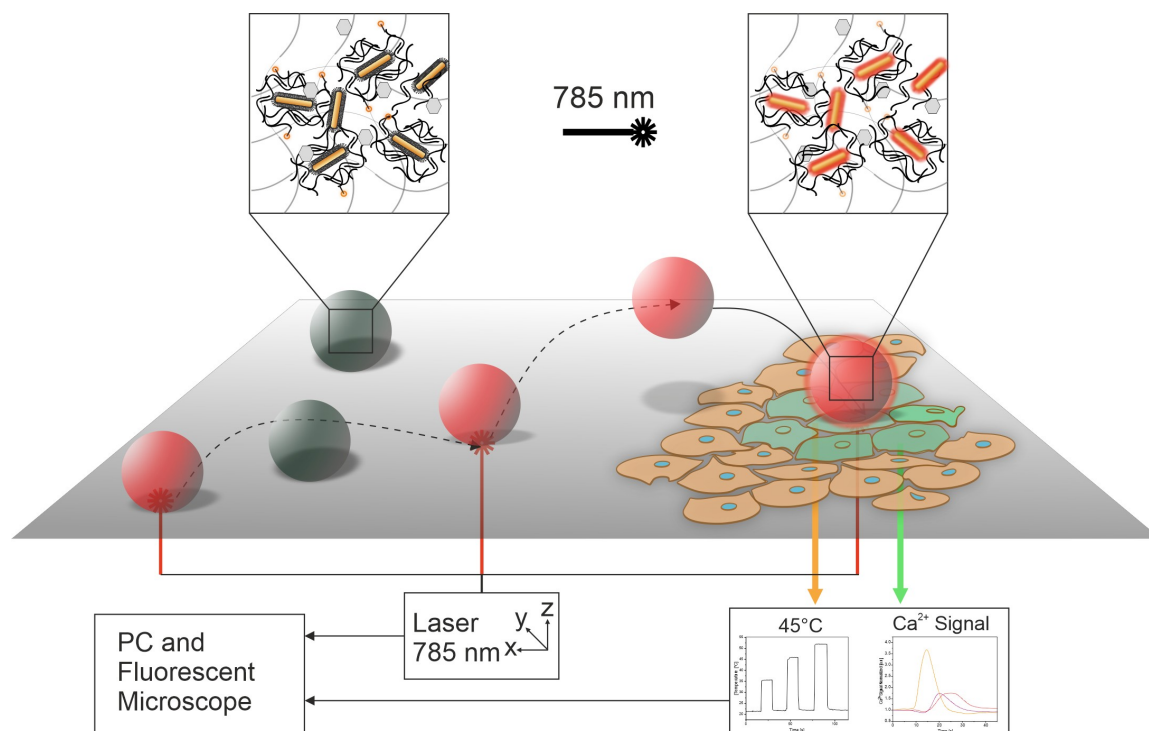


Figure 3.1 Functional capabilities of the TACSI microrobot. Schematic illustration of TACSI operation highlighting its integrated functions: gold nanorods enable precise local heating upon NIR laser illumination for 3D navigation via thermally induced convection. Concurrently, embedded Rhodamine B provides real-time temperature readout through fluorescence changes. The localized heating further enables targeted thermal stimulation of adjacent cells, eliciting measurable changes in intracellular Ca²⁺ signal.

The TACSI microrobot platform integrates photothermal actuation, thermometry, and 3D locomotion within a soft, cell-compatible design. This multifunctionality provides a unique capability to stimulate and monitor cells *in situ*, offering a new pathway to investigate thermal biology at the single-cell level. Importantly, these capabilities also lay the groundwork for downstream applications in tissue construction, where precise cell-by-cell manipulation and stimulation are critical. The following sections present a comprehensive characterization of the system, beginning with material selection and microrobot fabrication (Section 3.2.1), thermal performance and locomotion analysis (Section 3.2.4), and finally, biological demonstration of thermal cell stimulation and calcium imaging (Section 3.2.6).

3.2 Results and Discussion

3.2.1 Design and Fabrication of TACSI Microrobots

The functionality of the TACSI microrobot is fundamentally rooted in the rational design of its hydrogel matrix. To fulfill the requirements for localized single-cell stimulation and monitoring, the material had to meet several key criteria: it needed to be biocompatible to support cell viability and downstream culture, mechanically tunable to match soft tissue environments, and compatible with embedded nanoscale components—specifically nanoheaters and nanosensors—without compromising their function. Selecting a hydrogel that preserved the performance of these integrated elements was therefore essential.

Sodium alginate was chosen as the base material because it satisfies all these requirements and supports the formation of nanocomposites with minimal interference. As a naturally derived polysaccharide, alginate is highly biocompatible and widely used as a synthetic extracellular matrix (ECM) mimic [218, 219]. Its stiffness can be tuned across a broad physiological range (100–100,000 Pa) by modulating the concentration of crosslinking ions, making it adaptable to diverse cellular microenvironments. Moreover, alginate features abundant carboxyl groups, which provide ease of chemical functionalization through carbodiimide-mediated coupling reactions—enabling stable integration of functional molecules without altering the gel's mechanical properties.

To create a multifunctional nanocomposite, we embedded two key functional nanomaterials into the alginate matrix. For thermal sensing, we covalently conjugated RhB, a temperature-sensitive fluorescent dye, to the alginate backbone using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) coupling chemistry. This reaction forms stable amide bonds between alginate's carboxyl groups and an amine-modified RhB derivative [220], ensuring uniform dye distribution and preventing leaching—thereby enabling reliable, long-term temperature monitoring in biological settings.

For photothermal actuation, we embedded gold nanorods into the hydrogel as light-responsive transducers. GNRs are well suited for biological applications due to their high absorption cross-section and strong longitudinal plasmon resonance in the near-infrared region [221]. Upon irradiation with NIR light, they convert absorbed photon energy into heat via non-radiative decay, enabling rapid and localized temperature increases with minimal thermal hysteresis [209, 222]. Previous studies have demonstrated that alginate matrices preserve the photothermal responsiveness of embedded GNRs in macroscale systems [223], supporting their effective use in our microscale microrobot.

In summary, the engineered alginate-based nanocomposite integrates soft mechanical properties, NIR-responsive heating, and *in situ* thermal sensing into a single, cell-compatible microrobot. We

conjectured that these properties would enable fast and reversible thermal actuation of the final microrobot.

3.2.2 Synthesis of plasmonic nanoheaters

Gold nanorods (GNRs) with tailored optical properties were synthesized using a seed-mediated growth protocol in a cetyltrimethylammonium bromide (CTAB)- and silver ion (Ag^+)-rich environment. This approach promotes anisotropic growth through selective surface adsorption, producing nanorods with controlled aspect ratios and narrow size distributions (Figure 3.2 a) [224]. Transmission electron microscopy (TEM) confirmed rod-shaped particles with consistent morphology and minimal byproduct formation (Figure 3.2 b), while ultraviolet–visible (UV–Vis) spectroscopy revealed a dominant plasmon resonance peak at 785 nm (Figure 3.2 c), suitable for near-infrared-triggered actuation.

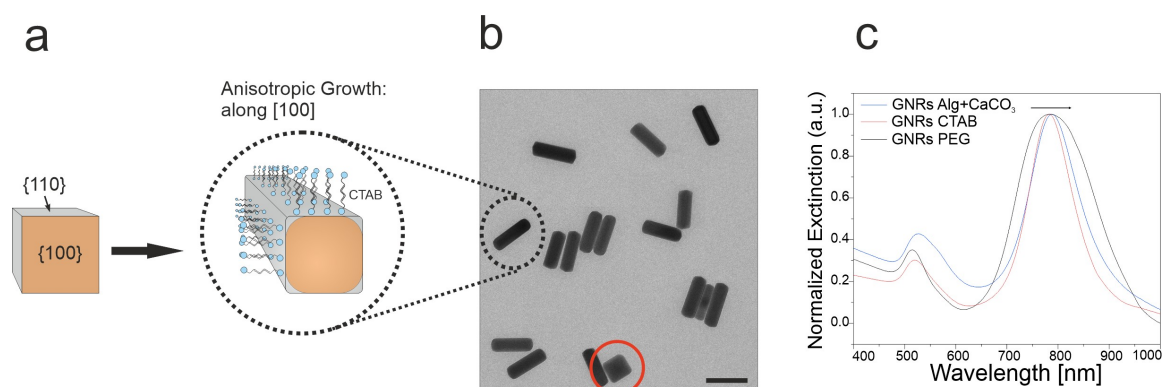


Figure 3.2 Synthesis and optical characterization of gold nanorods for TACSI microrobots. a) Schematic of seed-mediated growth of GNRs in a CTAB and Ag^+ -rich environment. b) TEM image of synthesized nanorods, showing aspect ratio control and minor byproduct formation (red outline). Scale bar: 100 nm. c) UV-Vis absorption spectra showing strong longitudinal plasmon resonance near 785 nm GNRs without a coating and with PEG- and alginate-coatings.

To ensure compatibility with both biological environments and the alginate matrix, gold nanorods (GNRs) were surface-modified via electrostatic complexation with alginate. This modification not only replaces the cytotoxic CTAB bilayer but also stabilizes the nanorods in aqueous media. Zeta potential measurements confirmed successful functionalization, with a charge inversion from $+45.1 \pm 1.9$ mV to -48.5 ± 1.1 mV (Table 3.1). For comparison, we also tested functionalization using polyethylene glycol (PEG), a common biocompatible coating [225]. However, the PEG-modified GNRs exhibited only modest surface charge (-11.7 ± 0.1 mV), resulting in weak electrostatic interactions and poor retention within the alginate matrix. In contrast, alginate-coated GNRs exhibited uniform dispersion in ultrapure water or cell culture media, forming a reliable foundation for encapsulation within the microrobot structure.

Table 3.1 Hydrodynamic size and zeta potential of different surface-functionalized gold nanorods.

Type of Nanorod	Dimensions [nm]	Zeta Potential [mV]
GNRs@CTAB	$23.6 \pm 3.4 \times 95.6 \pm 15.1$	$+45.1 \pm 1.9$
GNRs@PEG	$24.6 \pm 2.2 \times 87.0 \pm 11.4$	-11.7 ± 0.1
GNRs@Alginate	384.3 ± 154.4	-48.5 ± 1.1
GNRs@Alg.+CaCO ₃	362.1 ± 122.5	-45.7 ± 1.6

3.2.3 Microfluidic Encapsulation of Alginate–GNR Precursor Droplets

Achieving uniform gold nanorod (GNR) dispersion at the microscale proved more challenging than at the macroscale, particularly during microfluidic encapsulation, where nanoparticle aggregation led to uneven loading and occasional channel clogging. To address this, tip sonication was introduced as a critical dispersion step during precursor preparation. Applying 15 s of sonication at 60% amplitude immediately before mixing disrupted residual aggregates and ensured homogeneous nanoparticle integration into the Rhodamine B-conjugated alginate matrix (Figure 3.3). Transmission electron microscopy and ultraviolet–visible spectroscopy confirmed that GNR morphology and optical properties were preserved post-sonication, while dynamic light scattering (DLS) measurements demonstrated thermal stability up to 70 °C, verifying particle robustness under operational conditions (Figure A.2).

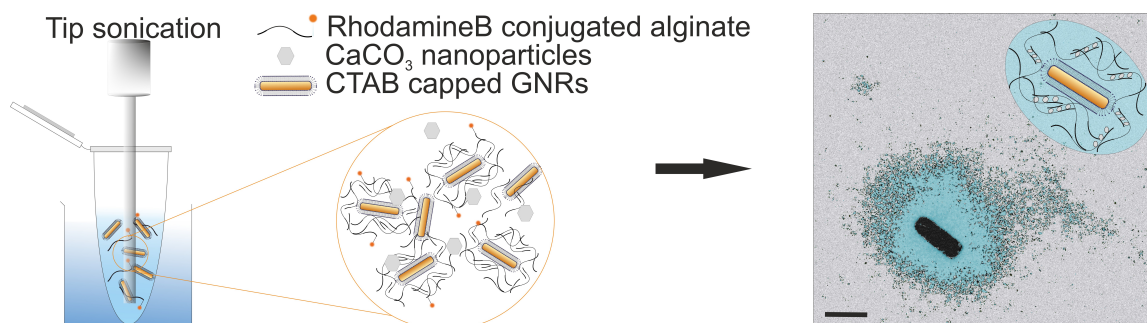


Figure 3.3 Preparation of Rhodamine B-conjugated alginate solution with embedded gold nanorods and CaCO₃ nanoparticles. Tip sonication ensures uniform dispersion of CTAB-stabilized GNRs within the alginate matrix. TEM image shows GNR encapsulation with an alginate shell (false-colored blue). Scale bar: 100 nm.

To fabricate monodisperse hydrogel microrobots, the sonicated pre-polymer solution—containing Rhodamine B-conjugated alginate, alginate-coated GNRs, and CaCO₃ nanoparticles—was used as the dispersed aqueous phase in a two-inlet microfluidic chip (Figure 3.4). The chip, produced via standard soft lithography using a previously established protocol in our lab, featured one inlet for the aqueous precursor solution and another for the continuous oil phase comprising hydrofluoroether (HFE) oil and acetic acid. Droplets were formed at the cross-junction and collected at the outlet.

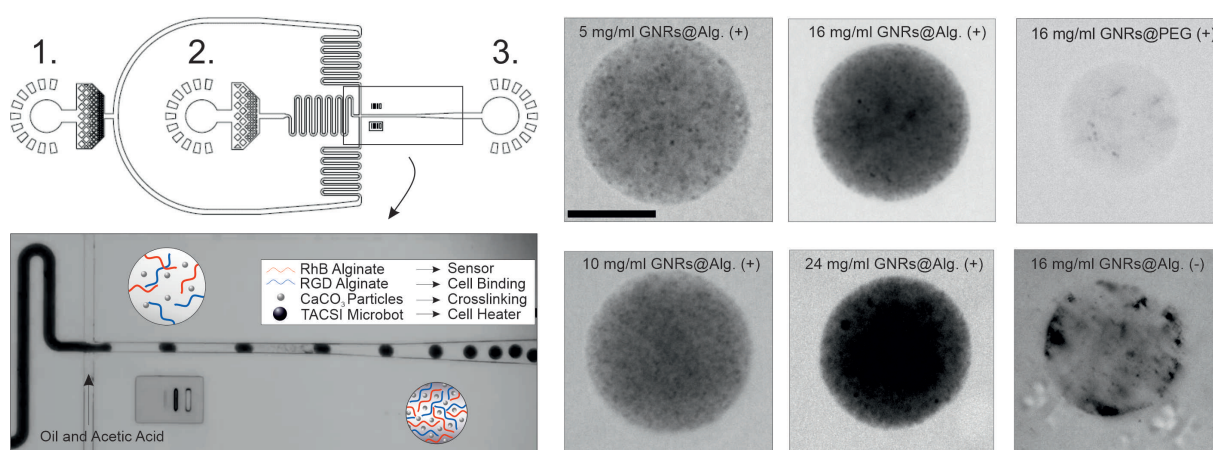


Figure 3.4 Microfluidic encapsulation of RhB-alginate, CaCO_3 , and gold nanorods into monodisperse hydrogel microrobots. CAD schematic of the microfluidic chip showing the oil inlet (1), the aqueous inlet (2), and the outlet (3). (Inset) Microscopy image showing the black aqueous phase—containing RhB-alginate, CaCO_3 , and GNRs—being pinched off by the continuous oil phase. Brightfield images of microgels produced with GNR concentrations of 5, 10, 16, and 25 mg/mL. Samples marked with (+) were tip-sonicated, resulting in homogeneous GNR distribution. Control samples include PEG-coated GNRs and non-sonicated CTAB-GNRs, which show aggregation. Scale bar: 15 μm .

To initiate droplet generation, the aqueous phase was introduced at 2 $\mu\text{L}/\text{min}$ via a syringe pump. At the junction, alginate rapidly crosslinked due to acetic acid-mediated dissolution of CaCO_3 , which released Ca^{2+} ions and triggered immediate ionic gelation. This process produced spherical hydrogel microrobots with average diameters ranging from $(30.6 \pm 1.2 \mu\text{m})$ at 0 mg/mL GNRs to $(28.2 \pm 1.8 \mu\text{m})$ at 24 mg/mL GNRs, confirming size uniformity across formulations (Figure A.1). The method yielded approximately 1,000,000 microrobots per hour, as quantified by hemocytometry.

Post-processing involved initial oil removal and addition of bead buffer (BB), followed by treatment with 33% perfluorooctanol in hydrofluoroether oil. This mixture was gently combined with the microgels and left for 5 minutes to break the emulsion. After phase separation, all oil and perfluorooctanol were removed, and the microgels were centrifuged and washed with BB. For applications involving cell culture, an additional sterilization step was performed by washing the microgels with 70% ethanol, after which they were stored in sterile cell culture media.

With optimized dispersion established, formulation parameters and surface chemistry were next refined to maximize encapsulation performance. Alginate-coated GNRs readily integrated into the alginate matrix due to favorable electrostatic interactions (Table 3.1), while polyethylene glycol (PEG)-coated GNRs, though similar in size and optical properties, exhibited poor retention and weak signal intensity, even at higher concentrations. Achieving a homogeneous nanocomposite, long recognized as a key challenge in materials science due to issues like aggregation and poor nanoscale dispersion, was addressed by sonication [226, 227]. Microrobots produced from sonicated mixtures showed homogeneously distributed GNRs and consistent optical uniformity. In contrast, skipping

the sonication step, particularly with CTAB-stabilized GNRs, led to aggregation, diminished optical contrast, and inconsistent photothermal behavior. These findings highlight the combined importance of surface charge compatibility and physical dispersion strategies. Notably, the TACSI platform enabled reliable GNR encapsulation across a broad concentration range (5–24 mg/mL) using a single, scalable microfluidic workflow.

Beyond photothermal performance, GNR incorporation was carefully balanced to preserve the mechanical and biochemical properties of the hydrogel matrix. Nanoparticles are known to influence gel stiffness and swelling behavior [228], and excessive loading can compromise cell interaction.

3.2.4 Thermal Calibration and Guided Locomotion of TACSI Microrobots

Rhodamine B is a temperature-sensitive fluorescent dye that exhibits a linear decrease in emission intensity over a broad range of 20–90 °C [229, 230, 201]. To enable real-time thermal monitoring during photothermal actuation, each TACSI microrobot is embedded with covalently bound RhB, transforming it into a microscale thermometer capable of reporting local temperature changes across physiological and moderately elevated ranges. A quantitative calibration curve was established by imaging RhB-labeled microrobots on a temperature-controlled microscope stage from 25 to 55 °C (Figure 3.5 a). Fluorescence intensity showed a reliable linear decline with increasing temperature (Figure 3.5 b), with a high correlation ($R^2 > 0.99$) confirming the quantitative accuracy of RhB in this context. Volumetric imaging further confirmed spatially uniform RhB distribution within the hydrogel matrix, supporting consistent signal output and internal temperature homogeneity.

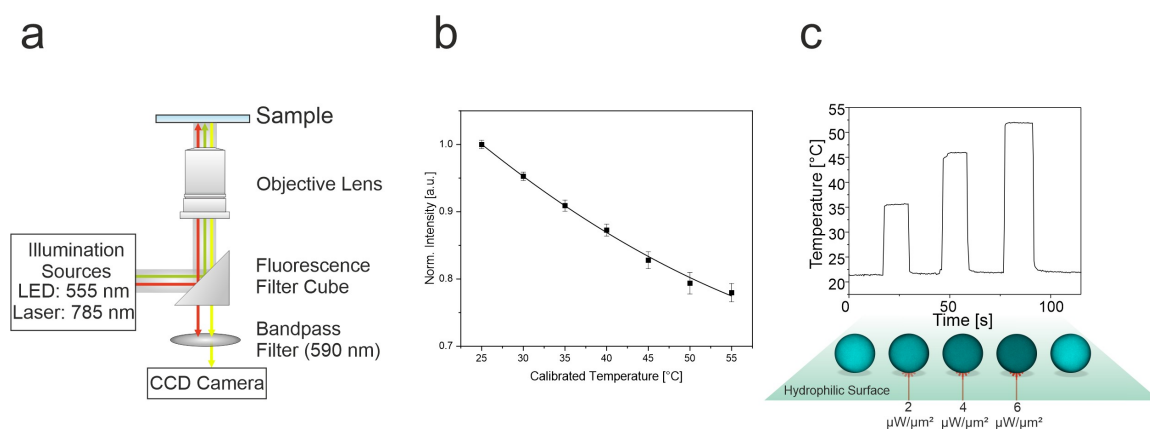


Figure 3.5 Thermal calibration and fluorescence response of Rhodamine B within TACSI microrobots. a) Schematic of the calibration setup using a stage heater. Microrobots were imaged at 25–55 °C under 40× magnification. b) Linear calibration curve of RhB fluorescence versus temperature ($R^2 > 0.99$, $n = 100$ microrobots per point). c) Fluorescence drop of a single microrobot during three laser heating cycles, demonstrating reversibility and photostability.

Under NIR laser stimulation, microrobot fluorescence decreased in a uniform, reversible manner across the microrobot body, indicating isotropic heat generation and stable signal behavior. Step-

wise increases in laser power density ($0 - 6 \mu\text{W}/\mu\text{m}^2$) elicited proportional decreases in fluorescence intensity. This tunable, linear response enabled high-precision thermal control, with minimal hysteresis observed over multiple heating and cooling cycles (Figure 3.5 c). Notably, RhB photostability was retained throughout repeated actuation, supporting its use in dynamic experimental settings. These findings were supported via theoretical calculations.

Finite element modeling showed that thermal energy was dissipated rapidly within the surrounding aqueous medium, as in the experimental case (Figure 3.6 a). More than 50 % of the thermal energy was dissipated within $5 \mu\text{m}$ of the microrobot surface, and the temperature returned to near-equilibrium temperature of 37°C within approximately 40 ms at a distance of $10 \mu\text{m}$ (Figure 3.6 b–c). This spatially and temporally confined heating profile confirmed that TACSI microrobots produce localized, transient thermal fields ideal for single-cell stimulation applications.

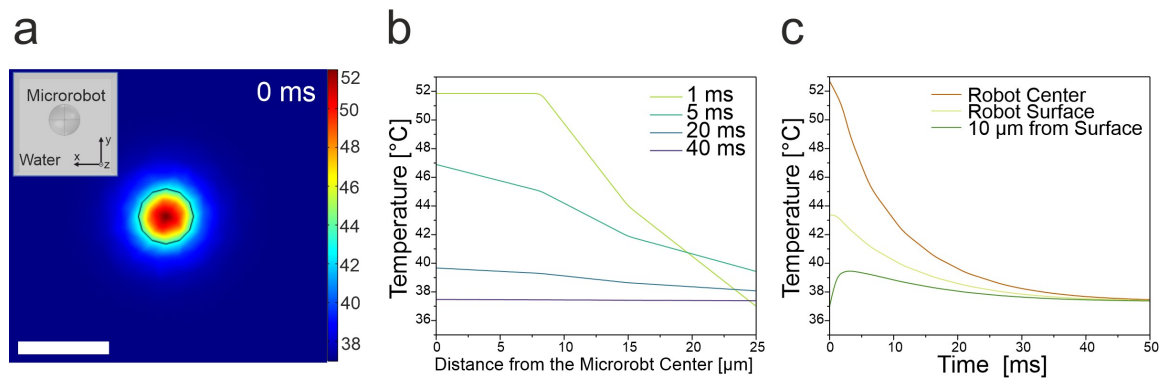


Figure 3.6 Finite element simulation of thermal confinement around a TACSI microrobot. a) Heat map of a $30 \mu\text{m}$ microrobot reaching 52°C within a $200 \times 200 \mu\text{m}$ aqueous domain. b) Radial temperature decay indicating 50 % dissipation within $5 \mu\text{m}$. c) Temporal evolution of heat dissipation at 5 and $10 \mu\text{m}$ from the surface, showing thermal decay to baseline within 40 ms.

To evaluate spatial heating uniformity, three-dimensional fluorescence imaging (z-stacks) was performed before and during laser activation. At baseline (22 °C), RhB fluorescence was uniformly distributed throughout the microrobot volume. Upon heating to 30 °C under near-infrared illumination, fluorescence intensity decreased homogeneously across all optical planes, indicating isotropic temperature increase and the absence of localized hotspots (Figure 3.7 a). This uniform thermal response reflects the even distribution of gold nanorods (GNRs) achieved through optimized encapsulation.

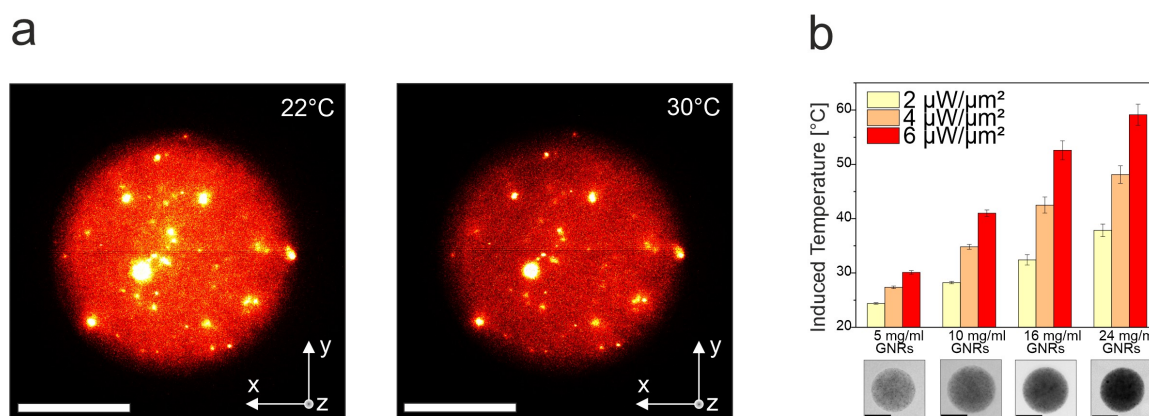


Figure 3.7 Spatial homogeneity of internal heating and thermal output tuning via GNR concentration. a) 3D fluorescence intensity stacks of a single TACSI microrobot imaged before (22 °C) and during (30 °C) laser heating. Rhodamine B fluorescence decreases uniformly throughout the volume upon actuation, confirming spatially homogeneous internal heating. Scale bar: 15 μm. b) Quantification of temperature increase across microrobot populations ($n = 20$ per group) fabricated with varying GNR concentrations (5–24 mg/ml). Higher GNR loading leads to greater thermal output under identical laser conditions, demonstrating the tunability of microrobot heating performance.

To assess the tunability of thermal output, microrobots were fabricated using alginate-coated GNRs at concentrations of 5, 10, 16, and 24 mg/ml under identical microfluidic conditions. Quantitative fluorescence analysis across laser intensities from 2 to 6 μW/μm² revealed a clear concentration-dependent thermal response (Figure 3.7 b). Microrobots with 5 mg/ml GNRs exhibited mild temperature increases (4.3 °C), suitable for gentle stimulation, while those with 24 mg/ml GNRs reached temperature increases up to 40.9 °C, with final actuation temperatures exceeding 60 °C within one second of laser exposure. This range covers both mild hyperthermia levels (e.g., 39 – 42 °C), commonly used for controlled stimulation of cellular responses, and noxious heat conditions (>45 °C).

Notably, the RhB signal remained stable across repeated actuation cycles, and no structural deformation or degradation of the hydrogel matrix was observed, even under high thermal loads. These results confirm the thermal robustness of both the sensing and structural components of the TACSI platform. The observed linear relationship between GNR concentration and temperature increase suggests consistent nanoparticle encapsulation and predictable control over microrobot performance. These findings are consistent with previous studies in nanocomposite PEG hydro-

gels, where increasing GNR concentrations from 0.24 to 9.78 nM led to enhanced photothermal responses up to an optimal range, beyond which heating plateaued due to optical attenuation [201]. This supports our observation that GNR loading is an effective parameter for tuning thermal actuation in hydrogel-based microrobotic systems.

3.2.5 Light-Guided Locomotion and Propulsion Modes

One of the mechanisms for triggering microrobot locomotion in aqueous environments is thermophoretic propulsion [231, 232, 233, 234]. This approach relies on the generation of a localized temperature gradient across the microrobot surface, which induces fluid motion in the surrounding medium and drives directional propulsion. When partially illuminated with a near-infrared laser, a heat differential is established over the microrobot body, creating thermally induced convective flows. These flows are capable of overcoming viscous drag forces at low Reynolds numbers and enable forward propulsion without the need for chemical fuels, relying solely on externally applied optical control. Accordingly, we hypothesized that the heat gradient generated on the surface of the TACSI microrobots would produce sufficient local convection to propel the microrobots directionally through the surrounding fluid.

To validate this hypothesis, we experimentally demonstrated three distinct modes of motion for the TACSI microrobots, each determined by the positioning of the laser and the specific system configuration: (1) substrate-level rolling, (2) vertical propulsion, and (3) surface-level Marangoni motion. Substrate-level rolling was observed at the liquid–substrate interface when the laser power density exceeded $4 \mu\text{W}/\mu\text{m}^2$, resulting in microrobot speeds of up to $5 \mu\text{m}/\text{s}$ (Figure 3.8 a-b). This mode was achieved by asymmetrically heating one side of the microrobot, creating directional convective flow that caused it to roll away from the heated region. Reliable rolling was observed only on hydrophobic surfaces, which reduced adhesion and enabled sustained locomotion. To confirm the presence of flow fields associated with this motion, we tracked $2 \mu\text{m}$ polystyrene beads in the surrounding fluid, which revealed strong convectational flows originating from the microrobot (Figure 3.8 c).

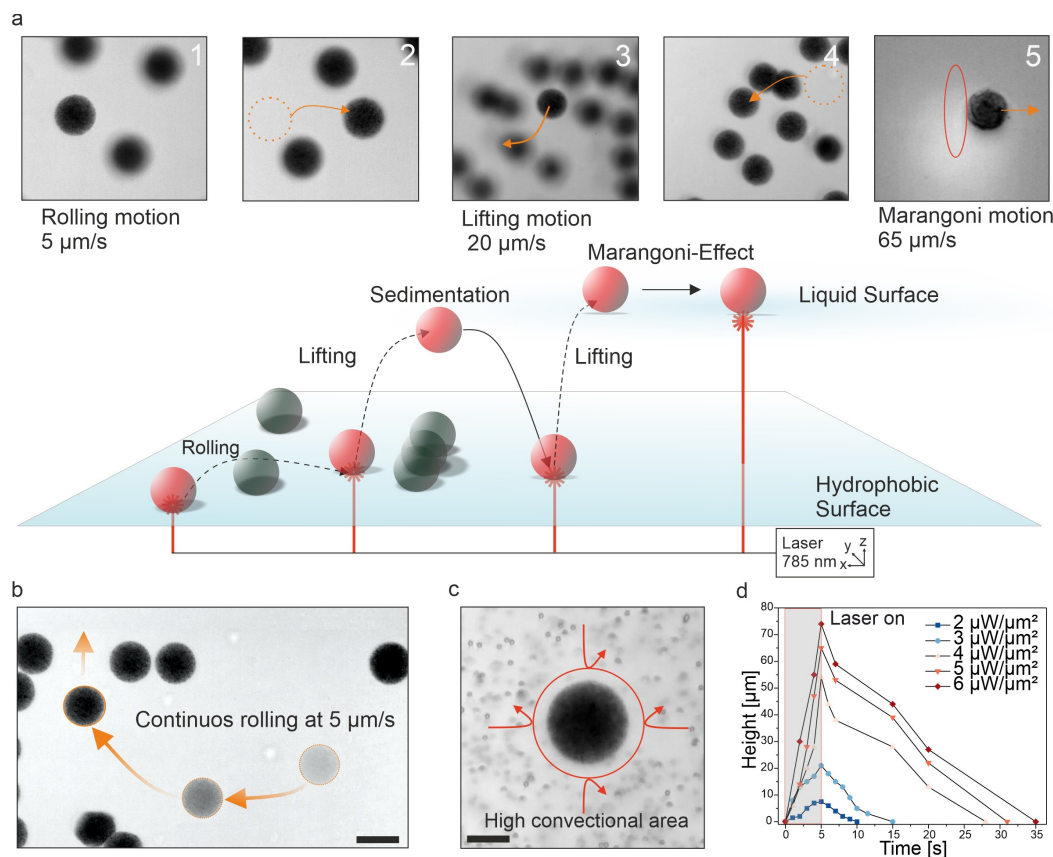


Figure 3.8 Characterization of light-driven microrobot locomotion modalities and speeds. a) Time-lapse images illustrating various movement behaviors: (1) and (2) show planar guidance in the x and y directions, including obstacle avoidance, with a horizontal speed of $5 \mu\text{m/s}$. Image (3) depicts laser-induced lifting of the microrobot over vertical features, reaching a vertical speed of $18 \mu\text{m/s}$, followed by landing in (4). Image (5) shows rapid movement along the air–liquid interface, with recorded speeds up to $65 \mu\text{m/s}$. The inset below schematically illustrates rolling motion driven by asymmetric convective flow. b) Demonstration of directional steering in the x and y axes, with faint outlines indicating previous microrobot positions. Scale bar: $30 \mu\text{m}$. c) Visualization of convectional flow using $2 \mu\text{m}$ polystyrene beads; arrows highlight regions of strong flow around the microrobot. Scale bar: $15 \mu\text{m}$. d) Tracked lifting height (z -axis displacement) of single microrobots with 10 mg/ml GNR loading under varying laser power densities.

Vertical propulsion was induced by uniform illumination of the microrobot from beneath, generating upward thermal gradients and symmetric convection that lifted the microrobot against gravity. Under maximum power, microrobots reached heights of up to $75 \mu\text{m}$ and velocities of up to $20 \mu\text{m/s}$ (Figure 3.8 d), with displacement modulated by adjusting laser power and exposure time. These in-liquid propulsion speeds are notable when compared to other thermally actuated systems. For instance, thermophoretic particle platforms using carbon nanotube absorbers typically report velocities below $2 \mu\text{m/s}$ under similar laser conditions [235], while state-of-the-art light-driven microrobots have reached speeds of up to $20 \mu\text{m/s}$ using structured light fields [236]. TACSI microrobots therefore match these examples, confirming their suitability for precise, fuel-free locomotion in 3D microenvironments.

At the air–liquid interface, microrobots transitioned into a third motion mode driven by the Marangoni effect. To initiate this mode, microrobots were first lifted using vertical propulsion and then asymmet-

rically heated to induce surface tension gradients. This led to rapid planar motion along the interface, with recorded speeds up to 65 $\mu\text{m/s}$ (Figure 3.8, panel 5). This fast and fuel-free transport mode enables long-range movement and repositioning of microrobots across larger distances.

Finally, swarm behavior was observed when multiple microrobots were simultaneously activated under low magnification ($10\times$) (Figure A.3). Sustained laser exposure (up to 60 s) led to the generation of overlapping convection fields, pooling microrobots toward the beam center at collective speeds of approximately 2 $\mu\text{m/s}$. These swarm dynamics offer potential for high-throughput positioning and cooperative tasks in multi-cellular environments.

3.2.6 *In Vitro* Experiments using TACSI Microrobots

An important requirement for implementing TACSI microrobots in live-cell studies was ensuring biocompatibility over time. We tested the cytotoxicity of both encapsulated gold nanorods (GNRs) and complete microrobots by conducting a live/dead assay on human embryonic kidney (HEK 293) cells over 72 hours. Cells were cultured in the presence of three conditions: (1) unencapsulated CTAB-GNRs, (2) alginate-encapsulated GNRs, and (3) TACSI microrobots containing 10 mg/ml GNRs (Figure 3.9 a). A control group without GNRs and microrobots was included.

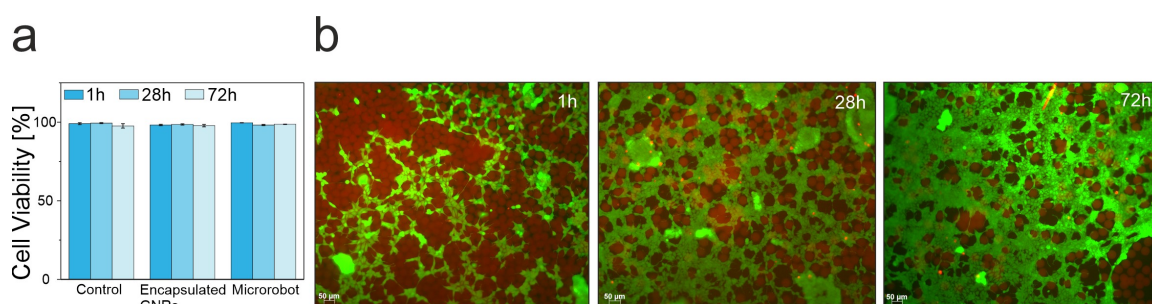


Figure 3.9 Cell viability and fluorescence imaging of HEK 293 cells cultured with TACSI microrobots. a) Quantification of cell viability over a 72-hour period for three experimental conditions: untreated control (no GNRs and no microrobots), alginate-encapsulated gold nanorods (GNRs@Alg) at 10 mg/ml, and TACSI microrobots containing 10 mg/ml GNRs@Alg ($n = 1000$ cells per group). Both GNRs@Alg and TACSI microrobots exhibited high biocompatibility, with viability comparable to control. b) Representative fluorescence microscopy image showing TACSI microrobots (red) distributed within a culture of HEK 293 cells (green). Cells exhibit normal morphology and attachment after 72 hours of co-incubation. Scale bar: 50 μm .

As expected, CTAB-capped GNRs were completely cytotoxic (Figure A.4) [237]. In contrast, encapsulated GNRs and TACSI microrobots exhibited high biocompatibility, maintaining a viability rate of 97% across all time points. Fluorescent live/dead staining confirmed minimal cell death, and brightfield images showed normal cell growth and spreading in direct contact with microrobots over 72 hours (Figure 3.9 b). These findings validate the design choices made for surface chemistry and encapsulation, particularly the coating of cytotoxic surfactants with alginate and the use of ethanol washing to ensure sterility prior to cell exposure.

We finally evaluated the potential of TACSI microrobots for thermal stimulation of single cells. Intracellular calcium signaling was selected as a biological readout due to its central role in thermosensitive ion channel activation and cellular response to environmental stress. To probe this, we used HEK 293 cells loaded with Calbryte™ 520 AM, a live-cell calcium indicator that fluoresces upon Ca^{2+} influx. Upon optical activation of the microrobots at a laser power density of $6 \mu\text{W}/\mu\text{m}^2$, we observed a rapid and robust intracellular calcium signal in adjacent HEK cells, with fluorescence intensities rising sharply during the 1-second laser pulse and returning to baseline within 30 seconds (Figure 3.10 b-c). This response was spatially confined and only occurred when the microrobot was actuated, confirming that the calcium flux was thermally, not mechanically, induced. Quantification across multiple experiments showed a clear dose-dependent effect: stimulation at lower power densities (2 and $4 \mu\text{W}/\mu\text{m}^2$) led to milder or no response, while temperatures exceeding 52°C consistently triggered strong calcium influx, peaking at $2.19\times$ baseline intensity at 57°C (Figure 3.10 c-d).

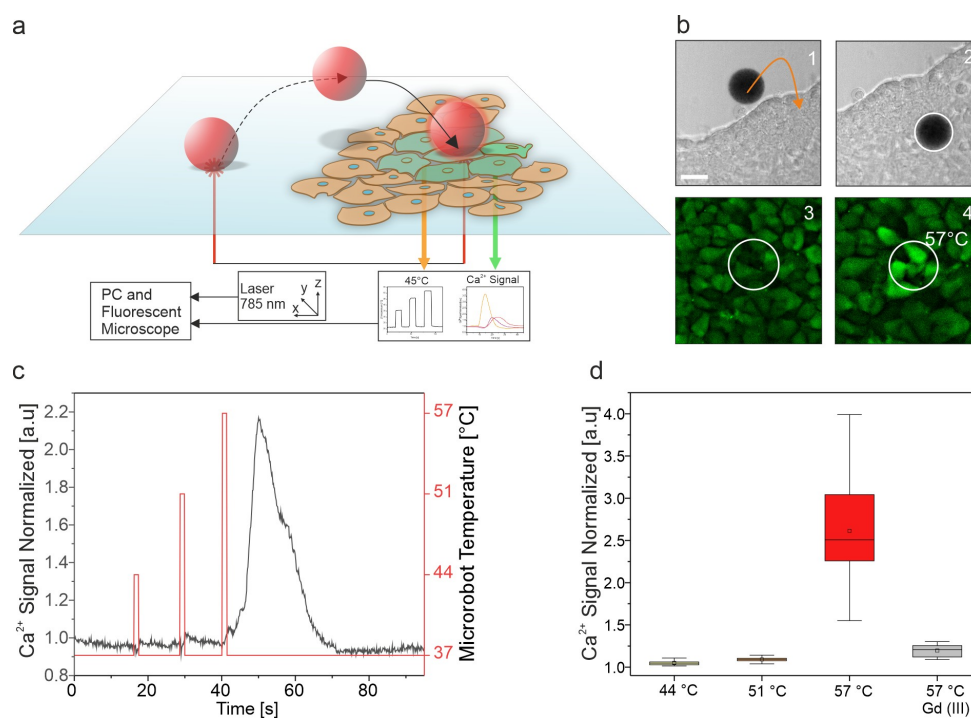


Figure 3.10 Thermally induced calcium signaling in HEK 293 cells triggered by TACSI microrobots. a) Schematic illustration of the experimental sequence: a single microrobot is guided toward the cell monolayer, allowed to settle, and then actuated via NIR laser illumination to generate localized heating. b) Fluorescence microscopy images from a representative experiment. The microrobot is guided onto the cell layer in (1) and (2), settles in close contact in (3), and is activated by laser light, resulting in a strong intracellular calcium response detected in (4). Fluorescence is visualized using Calbryte™ 520 AM as a live-cell calcium indicator. Scale bar: $30 \mu\text{m}$. c) Quantification of normalized intracellular calcium fluorescence intensity from a single HEK 293 cell stimulated by a microrobot at increasing laser power densities (2, 4, and $6 \mu\text{W}/\mu\text{m}^2$). A 1-second laser pulse was used in all cases. d) Summary of calcium signal response across multiple cells ($n = 20$) at different induced microrobot temperatures, with or without Gd^{3+} ion channel blocker. Calcium signaling is suppressed in Gd^{3+} treated cells, confirming a thermally gated, ion channel-mediated mechanism. Laser powers correspond to temperature increases achieved with 2, 4, and $6 \mu\text{W}/\mu\text{m}^2$.

We next investigated the underlying mechanism. Since TRP channels such as TRPV1 and TRPV2 are known to activate at temperatures above 43°C and mediate Ca^{2+} entry into cells [238, 239], we tested whether this pathway was involved. Pre-treatment with 150 μM gadolinium chloride (Gd^{3+}), a channel blocker that has been shown to block temperature-sensitive channels [240], drastically reduced the calcium response under identical thermal conditions, strongly suggesting that the observed signaling was mediated by heat-sensitive ion channels (Figure 3.10 d).

These findings demonstrate that TACSI microrobots can deliver targeted, localized heat sufficient to trigger biologically relevant calcium signaling in single cells. Notably, the ability to position microrobots with micrometer precision using optical propulsion enables selective stimulation of individual cells within dense monolayers. Using the vertical propulsion capability, we positioned a single microrobot next to an isolated HEK 293 cell and applied graded thermal stimuli. The resulting fluorescence confirmed a strong response in the targeted cell, with no detectable calcium flux in neighboring, unstimulated cells (Figure 3.11). This spatial precision highlights the potential of TACSI microrobots as non-invasive tools for single-cell thermal modulation and mechanistic studies of heat-sensitive cellular pathways.

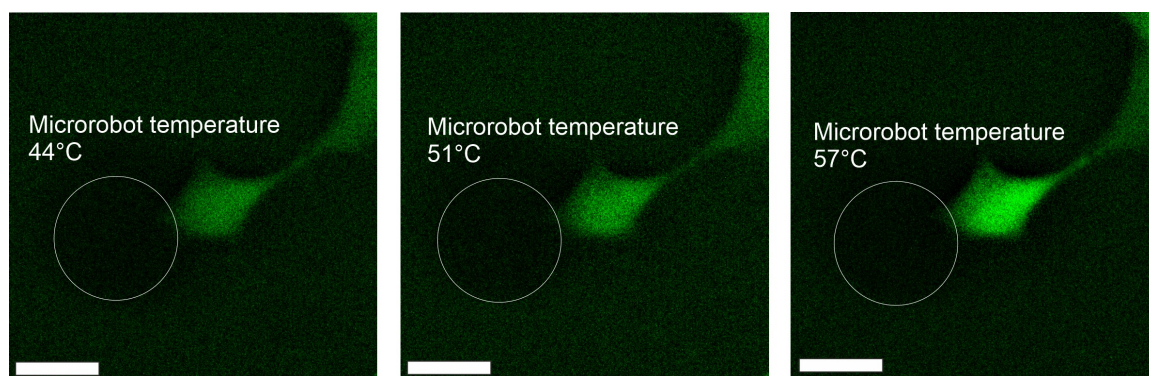


Figure 3.11 Fluorescence images of a single HEK 293 cell during thermal stimulation with three different laser power levels. The position of the TACSI microrobot is outlined in white. Increased intracellular calcium fluorescence is observed with increasing laser intensity. Scale bar: 20 μm .

3.3 Concluding Remarks

This chapter introduced the TACSI microrobot platform — a soft, multifunctional, and biocompatible microsystem capable of delivering localized thermal stimulation with integrated temperature sensing and 3D guidance at the single-cell level. Unlike traditional nanomaterial-based tools that often lack precise control or bio-integration, TACSI microrobots combine photothermal heating, fluorescence-based thermometry, and optically guided locomotion within a scalable hydrogel matrix. This enables direct, reversible interaction with individual cells in a manner that is both controllable and biologically compatible.

We demonstrated that TACSI microrobots can achieve real-time thermal sensing and targeted actuation using embedded gold nanorods and Rhodamine B, achieving thermal resolution and stability comparable to macroscale hydrogel systems [201]. Their light-driven locomotion, through rolling, vertical convection, or Marangoni flow, produced speeds and behavior consistent with previous thermophoretic microrobot studies, validating the propulsion principles in a soft, cell-compatible material [236]. Functional assays confirmed that TACSI microrobots reliably triggered calcium signaling via TRPV-mediated pathways, and their operation showed excellent biocompatibility, in line with other soft microrobotic systems [54]. Critically, their fine spatial resolution enabled precise stimulation of individual cells, a feature rarely demonstrated in microrobots with integrated actuation and sensing.

Looking ahead, the TACSI concept provides a foundation for next-generation microrobots that incorporate mechanical actuation alongside thermal stimulation. Future developments will explore embedding TACSI microrobots into 3D spheroids as autonomous actuators and sensors, enabling local modulation of the microenvironment in organoid systems. A key enabler for these advanced capabilities is the mechanical design of the microrobot itself. Therefore, the next chapter focuses on characterizing the mechanical properties of the hydrogel matrix, which are critical for understanding their deformability, cell integration, and mechanical stability in different cell culture media.

3.4 Experimental Section

1. Gold Nanorod Synthesis and Characterization

Gold nanorods (GNRs) were synthesized via a seed-mediated solution growth method, following a previously established protocol [216]. The synthesis employed a binary surfactant system composed of cetyltrimethylammonium bromide (CTAB) and sodium oleate. Silver nitrate (4 mM, Sigma-Aldrich) acted as a shape-directing agent, while ascorbic acid (0.064 M, Sigma-Aldrich) reduced gold chloride (1 mM, Sigma-Aldrich). The growth solution was incubated overnight at 31.5°C. The morphology of the nanorods was assessed by transmission electron microscopy (TEM; FEI Tecnai) operated at 120 kV. UV-vis absorption spectra were acquired using a Specord 50 plus spectrometer (Analytik Jena), and hydrodynamic diameter and zeta potential were measured in ultrapure water using dynamic light scattering (DLS; Litesizer 500, Anton Paar). GNR concentrations were quantified by freeze-drying aliquots to determine gold mass, and cross-referenced with the absorbance at 400 nm. For biological use, GNRs were sterile-filtered through 0.22 µm membranes. To prepare alginate-encapsulated GNRs (GNRs@Alg), excess CTAB was removed by repeated washing, and uncrosslinked alginate was added to reach the desired GNR concentration. The mixture was sonicated using a tip sonicator (Fisherbrand Model 120 Sonic Dismembrator) for 15 s at 60% amplitude while cooled in a water bath. Additionally, GNRs coated with polyethylene glycol (GNRs@PEG) were obtained commercially (Nanopartz) and exhibited a peak absorbance at 785 nm.

2. Microfluidic Device Fabrication

Two-channel microfluidic devices were fabricated via standard soft lithography using SU-8 3050 photoresist. A 25 µm thick layer was created via spin coating and soft-baked for 15 min. The photoresist was exposed with 250 µJ/cm², post-exposure baked for 5 min, and developed for another 5 min. Sylgard 184 polydimethylsiloxane (PDMS) was poured over the mold and cured. Inlet and outlet ports were punched, and the PDMS was plasma-bonded to a glass substrate.

3. Alginate Preparation and Microrobot Fabrication

High molecular weight alginate (IG1, KIMICA) was functionalized with Rhodamine B ethylenediamine (Thermo Fisher Scientific) at a degree of substitution (DS) of 2, following established methods. The material was dialyzed, charcoal-treated, filtered, and freeze-dried. For microrobot fabrication, dried alginate was reconstituted to 2% (w/v) in MES (2-(N-morpholino)ethanesulfonic acid) buffer. GNRs were mixed with this alginate solution and tip-sonicated to ensure uniform dispersion. The suspension was injected into a two-channel microfluidic chip driven by a syringe pump (Darwin Mi-

crofluidics) at a flow rate of 2 $\mu\text{L}/\text{min}$. The resulting microrobots were collected, washed to remove residual oil, and stored at 4°C.

4. Microscope Platform

A custom optical setup was built around an inverted fluorescence microscope (Leica DMI8) equipped with a 785 nm laser (Thorlabs, 200 mW). The laser beam was reflected by a dichroic mirror and guided through the microscope's optical path to the specimen. Fluorescence imaging and laser control were operated via Leica LAX software and an external Thorlabs controller. A petri dish heater (Bioscience Tools) was used to perform thermal calibration in 5 °C increments from room temperature up to 55°C. Samples were equilibrated at each temperature for 15 min with illumination turned off to prevent photobleaching. Fluorescence was imaged at 40 $\times$ magnification with an exposure time of 300 ms, using laser power densities between 2–6 $\mu\text{W}/\mu\text{m}^2$. Relative fluorescence intensity was normalized to the baseline at 25°C. Power density was measured using a calibrated optical power meter (Thorlabs). Video acquisition was conducted at frame rates between 24 and 300 frames per second (fps) using the LAX software. Particle movement was tracked in Fiji using the TrackMate plugin, and brightfield microscopy was used to observe microrobot locomotion.

5. Simulations

Thermal simulations were performed in COMSOL Multiphysics 5.5 (COMSOL Inc.) using finite element modeling. A spherical microrobot with a diameter of 30 μm was placed in a 200 $\times$ 200 μm water cube. Thermal parameters for the microrobot were set to a thermal conductivity of 0.57 W/(m·K), a density of 1930 kg/m³, and a specific heat capacity of 4000 J/(kg·K). The surrounding water was maintained at 37°C, while the microrobot core temperature was set at 52°C. Temperature distributions and heat dissipation dynamics were analyzed from cross-sections through the microrobot center.

6. Cell Work

Human embryonic kidney (HEK 293) cells were purchased from ACCT and cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 5 % fetal bovine serum (FBS; Gibco) according to the supplier's instructions. Cells were stained using the LIVE/DEAD Cell Imaging Kit (488/570; Thermo Fisher Scientific), with final concentrations of 2 μM Calcein and 4 μM Ethidium homodimer. Cells were then exposed to GNRs@CTAB, microrobots, and GNRs@Alg + CaCO₃ in a 1:1 volumetric ratio. Prior to use, microrobots and composite constructs were washed with ethanol to ensure sterility. Viability assessments were conducted over three days

under standard cell culture conditions. For photothermal experiments, HEK 293 cells were seeded in 96-well plates and incubated with sterile microrobots. All assays were carried out at 37°C and 5 % CO₂ in a stage-top incubator (Bold Line, Oko Lab). Calcium signaling was assessed using Calbryte™ 520 AM (Biomol, 4.5 µM) following a 40 min incubation in isotonic buffer. Stained cells were washed, and controls were treated with ionomycin, while Gadolinium(III) chloride hexahydrate (Merck, 150 µM) was used to block ion channels.

4 Microscale Mechanical Characterization of Ionically Crosslinked Alginate Microgels Using Nanoindentation

This chapter presents a comprehensive framework for the mechanical characterization of ionically crosslinked alginate microgels, key components of the TACSI microrobots that serve as both functional and structural elements in bioengineering systems, using a cantilever-based nanoindentation technique. Emphasis is placed on the microfluidic fabrication of size- and composition-controlled microgels, optimization of indentation protocols, and systematic analysis of how environmental ionic conditions modulate microscale stiffness and viscoelasticity. The study outlines robust guidelines for consistent mechanical measurements, validated by macroscale rheological comparisons. The importance of aqueous medium composition, particularly calcium and sodium ion concentrations, in determining microgel performance and integrity in bioengineering applications is highlighted. The work presented in this chapter was published under: Philipp Harder, Leonard Funke, Jana Reh, Oliver Lieleg, Berna Özkale. Indenting at the Microscale: Guidelines for Robust Mechanical Characterization of Alginate Microgels. ACS Applied Materials & Interfaces, 2025, 17 (9).

4.1 Introduction

Building on the previous chapter, which introduced the Thermally Active Cell Signal Imaging (TACSI) microrobot platform for localized, reversible stimulation of single cells, this chapter turns to a fundamental aspect of microrobot functionality: its mechanical properties. At the core of the TACSI system lies an ionically crosslinked alginate microgel, embedded with photothermal nanomaterials, temperature sensing components, and cell-binding functionalized alginate. This hydrogel matrix forms the structural and responsive body of the microrobot while acting as the primary interface with biological tissues. As such, its mechanical behavior under physiological conditions plays a pivotal role in determining both the efficacy and reliability of microrobot–cell interactions. Although the field of microrobotics has rapidly advanced in the areas of actuation, sensing, and material integration, less focus has been placed on understanding how soft microrobots behave

mechanically once deployed in biological environments. For systems like TACSI, which depend on robust shape retention, tunable deformability, and compatibility with the mechanical microenvironment of tissues, this understanding is essential. Considering the significant influence of mechanical forces on cell behavior—including migration, proliferation, and differentiation—it is crucial to design tissue-interfacing microrobots with biomechanical compatibility and adaptability in mind. In addition to direct mechanical stresses, microrobots are also subjected to osmotic stresses arising from gradients in ion concentration between the microgel interior and the surrounding biological media. These osmotic imbalances can lead to swelling or shrinking of the microrobot body, affecting both mechanical integrity and functional performance. Therefore, a comprehensive understanding of how ionic and osmotic environments influence microrobot mechanics is vital for their successful integration in dynamic, cell-rich systems.

Alginate—a naturally derived, biocompatible polysaccharide—has emerged as an excellent candidate for microrobot fabrication due to its gentle gelation with divalent cations, tunable viscoelasticity, and excellent cell compatibility [218, 219]. Its gelation process involves the formation of “egg-box” junctions, where calcium ions (Ca^{2+}) bridge guluronic acid residues in the polymer chain [241, 242]. These crosslinking interactions directly dictate the hydrogel’s stiffness, elasticity, and stress relaxation properties [243, 244], all of which are critical for maintaining microrobot functionality in dynamic tissue environments. The ease and reversibility of this ionically driven crosslinking process not only make alginate highly adaptable for soft microrobots but also allow its mechanical properties to be tuned for specific cellular and bioengineering applications.

This well-established crosslinking mechanism has been extensively characterized in macroscale alginate hydrogels[245, 246, 247, 248]. However, translating these insights to the microscale remains nontrivial. Conventional macroscale techniques, such as shear and compression testing, lack the spatial resolution necessary to capture local variations in stiffness, especially under biologically relevant ionic environments and temperature conditions [249, 250]. Similarly, atomic force microscopy (AFM), while highly effective for nanoscale surface characterization and topographical mapping, is primarily designed to probe surface properties and requires complex instrumentation and environmental control. This makes it less straightforward to use for assessing the macroscale or embedded mechanical behavior of hydrogel microstructures, particularly in hydrated, biologically relevant environments. [251, 252, 253].

To overcome these limitations, we employ a cantilever-based nanoindentation approach that enables high-resolution mechanical characterization of individual alginate microgels in aqueous environments. This method allows for the direct measurement of key mechanical parameters—including Young’s modulus and time-dependent viscoelastic behavior—while simultaneously enabling environ-

mental modulation to replicate *in vitro* conditions. Using this platform, we find that microgel stiffness is highly sensitive to the ionic environment: alginate microrobots exhibit a peak Young's modulus of 19.9 ± 3.0 kPa when cross-linked at optimal calcium concentrations (45 mM CaCl_2), but soften significantly to 0.2–0.5 kPa in physiological media containing sodium ions such as Dulbecco's Modified Eagle Medium (DMEM). Conversely, exposure to deionized water reversibly restores stiffness, underscoring the dynamic, environment-dependent nature of ionic cross-linking. Furthermore, we demonstrate that gold nanorod and peptide functionalization slightly modulates stiffness without disrupting microgel integrity, enabling tuning of both bioactivity and mechanical performance. The presence of nanorods slightly reduced the measured Young's modulus: 0.5 ± 0.1 kPa compared to 0.6 ± 0.1 kPa measured in bead buffer. Through precise control of fabrication parameters, ionic composition, and indentation protocol, we establish a robust strategy for the reproducible characterization and mechanical tuning of alginate-based microrobots.

This chapter is structured to provide a comprehensive framework for the microscale mechanical characterization of alginate-based microrobots. Section 4.2.1 describes the fabrication of functionalized alginate microgels using a microfluidic platform, enabling precise control over size, cross-linker concentration, and material composition. In Section 4.2.2, we present the development and calibration of a cantilever-based nanoindentation method tailored for hydrated microgels, detailing parameters such as probe selection, indentation depth, and sample immobilization. Section 4.2.5 examines how microgel stiffness is modulated by calcium cross-linking, revealing an optimal stiffness at 45 mM, and we investigated how external ionic environments, including sodium ions and cell culture media, dynamically influence gel mechanics. Finally, we use these insights to derive formulation and measurement guidelines and discuss the broader implications of micromechanical tuning for biofunctional microrobots.

4.2 Results and Discussion

4.2.1 Fabrication of Alginate Microgels

The main goal was to investigate the mechanical properties of alginate microgels and the influence of gold nanorods once incorporated into TACSI microrobots. Towards this goal, microgels with varying size, crosslinking density, and composition were fabricated using microfluidic encapsulation. Microgel diameter was primarily controlled by microfluidic channel geometry and flow rate, as illustrated in the schematic workflow (Figure 4.1 a). Briefly, the aqueous phase containing uncrosslinked alginate and suspended CaCO_3 nanoparticles is pinched into droplets at the cross-junction, where acetic acid in the surrounding oil phase diffuses into the droplets and dissolves the nanoparticles, releasing Ca^{2+} ions to initiate ionic crosslinking. The efficiency and uniformity of this process enable

precise fabrication of spherical microgels under controlled conditions, building on the microfluidic platform introduced in Chapter 3.

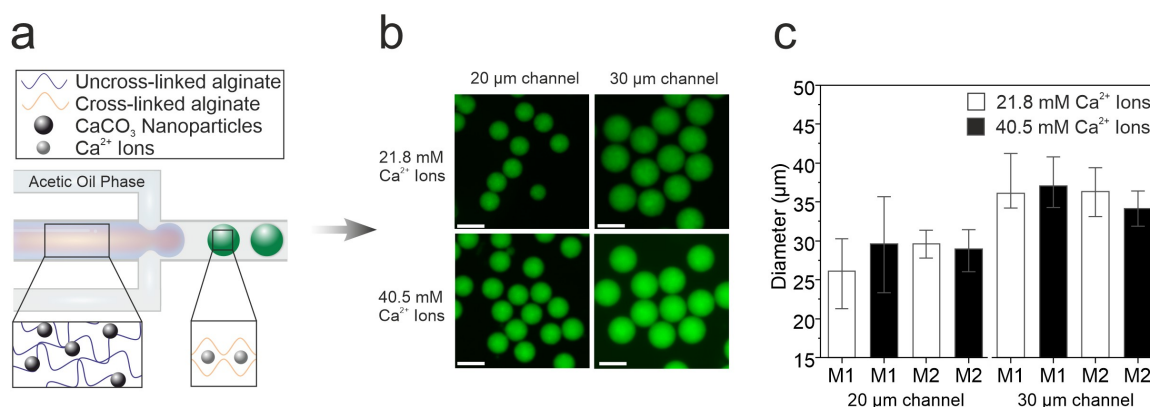


Figure 4.1 Microfluidic fabrication and size analysis of alginate microgels. a) Schematic of the droplet-based microfluidic process for generating monodisperse alginate microgels using an oil phase containing acetic acid. At the cross-junction, the aqueous prepolymer mixture—comprising uncrosslinked alginate and suspended CaCO₃ nanoparticles—is pinched into droplets. Subsequent diffusion of acetic acid into the droplets dissolves the CaCO₃, releasing free Ca²⁺ ions that initiate internal ionic crosslinking of the alginate chains. b) Fluorescence microscopy images of M1-type microgels composed of Rhodamine B-functionalized alginate, categorized by Ca²⁺ crosslinker concentration and microfluidic channel width. Scale bars represent 30 μm. c) Quantitative analysis of microgel diameter for M1 and M2 microgels produced using two different crosslinker concentrations and channel sizes (20 μm and 30 μm). Sample sizes: $n = 400$ for M1 and $n = 100$ for M2.

We began by fabricating M1-type microgels composed of Rhodamine B-labeled alginate to systematically assess the effects of channel width and crosslinker concentration (Figure 4.1 b). At a crosslinker concentration of 21.8 mM Ca²⁺, microgels produced in 20 μm channels had an average diameter of 26.0 ± 2.9 μm, while those fabricated in 30 μm channels reached 36.1 ± 4.1 μm. Increasing the crosslinker concentration to 40.5 mM in the 20 μm device resulted in a slight increase in size to 29.0 ± 2.5 μm. These size variations are attributed to changes in network compaction and swelling behavior driven by the degree of ionic crosslinking.

M2-type microgels—formulated identically to the TACSI microrobots, incorporating RGD-modified alginate and embedded gold nanorods for biofunctionality and photothermal actuation—were fabricated under equivalent conditions to evaluate whether nanomaterials influence droplet formation. These microgels exhibited similar size trends; for example, M2 microgels produced in 30 μm channels at 21.8 mM Ca²⁺ had an average diameter of 36.3 ± 1.3 μm (Figure 4.1 c). This confirmed that microgel size is primarily dictated by channel geometry, while internal composition and nanoparticle content exert negligible influence.

By systematically adjusting flow rate, channel dimensions, and calcium ion concentration, a library of monodisperse microgels with defined crosslinking properties was created. These samples formed the experimental basis for all subsequent nanoindentation and viscoelasticity studies, ensuring that any measured mechanical differences could be directly attributed to known fabrication parameters.

4.2.2 Mechanical Characterization of Alginate Microgels Using Nanoindentation

Quantifying the mechanical properties of alginate microgels at the single-particle level was essential for evaluating their function in *in vitro* applications. To achieve this, we employed a cantilever-based nanoindentation system that enables direct measurement of the elastic response of hydrated microgels in physiologically relevant environments. This technique allows accurate mapping of Young's modulus and viscoelastic parameters while preserving the microgel's geometry and ionic composition during measurement. Nanoindentation experiments were performed using a stage-top instrument, integrated into a fluorescence-compatible inverted microscope (Figure 4.2 a). The system consists of a flexible cantilever mounted on a ferrule, which applies controlled indentation force to individual microgels placed in a multiwell plate. Real-time optical tracking enabled the precise localization of each microgel and the monitoring of the indentation process.

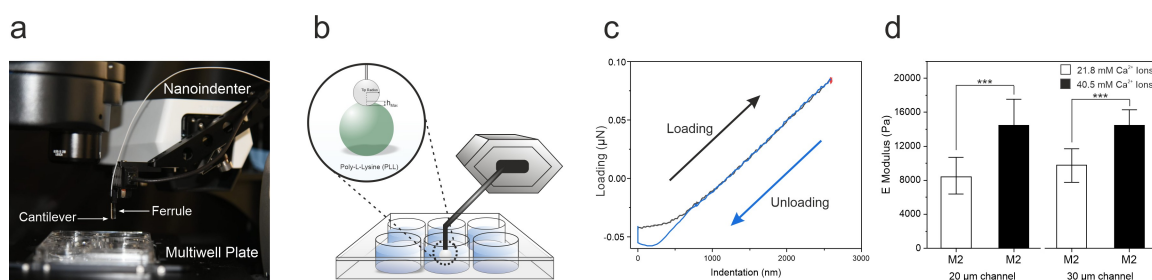


Figure 4.2 Mechanical characterization of alginate microgels using nanoindentation. a) Photograph of the experimental nanoindentation setup, highlighting the cantilever arm and mounted ferrule positioned above a multiwell plate. b) Schematic drawing of the indentation process within a 6-well plate, showing the cantilever (gray), microgel (green), and substrate coating. h_{max} denotes the maximum indentation depth. c) Representative force-indentation curve from a single indentation cycle, showing the loading and unloading response within the elastic regime. d) Young's modulus values for M2-type microgels with different diameters and Ca^{2+} crosslinker concentrations ($n = 30$ per condition). Bars represent mean $\pm$ SD. Statistical analysis via two-way ANOVA; *** $P < 0.001$.

To ensure stability during measurement, microgels were immobilized using a poly-L-lysine (PLL) coating applied to the well surface [254]. PLL has long been used to promote cell adhesion by electrostatically interacting with negatively charged biomaterials, such as cell membranes or modified surfaces [255]. In our system, the alginate microgels carry a net negative charge due to their carboxylated polymer backbone, as confirmed through zeta potential measurements in Chapter 3. Based on this charge profile, we hypothesized that a positively charged PLL layer could serve as a simple and biocompatible means to immobilize microgels on substrate surfaces. Indeed, surface coating with PLL facilitated electrostatic adhesion of the microgels without inducing significant morphological changes, enabling stable sample handling during indentation experiments. As shown schematically in Figure 4.2 b, the cantilever approaches each microgel adhered to a PLL-treated surface, enabling stable indentation without slippage. PLL concentration was found to be critical. At concentrations above 0.5 mg/mL, PLL induced secondary ionic crosslinking within the alginate net-

work, permanently altering microgel size and stiffness (Figure A.5). Similarly, inadequate washing of the PLL layer led to measurable changes in microgel dimensions. Conversely, concentrations below 0.1 mg/mL were insufficient to hold the microgels in place, often resulting in movement during the measurement and incomplete indentation curves. The optimal concentration was determined to be 0.1 mg/mL, combined with thorough drying of the coated surface on a hot plate to prevent undesired crosslinking effects.

All baseline measurements were performed in deionized water to avoid interference from free ions. Indentation was initiated at the apex of each microgel, with the probe mapping the curved surface in 1 μm steps. Consistent stiffness values were observed within the first 5 μm from the pole, averaging 5.4 ± 0.08 kPa, while values decreased at more lateral positions due to partial contact between the spherical probe and the sloped microgel surface (Figure A.6) [256]. Based on these results, all subsequent measurements were restricted to the pole region to ensure contact accuracy. We were also interested in whether cantilever properties—specifically tip radius and stiffness—would influence the mechanical readout. To evaluate this, cantilevers with tip radii between 3 and 11 μm and stiffness values ranging from 0.025 to 0.55 N/m were tested. Across all configurations, Young's modulus values deviated by less than 0.3 kPa (Figure A.7). While softer cantilevers (~ 0.025 N/m) introduced more noise and posed greater challenges during calibration, the stiffer probes yielded more robust and consistent results. A representative force-indentation curve demonstrating a reproducible linear response in the elastic regime is shown in Figure 4.2 c. To satisfy model assumptions, indentation depth was maintained within 5 – 10 % of microgel thickness and below 16 % of the tip radius, in accordance with established guidelines [256].

4.2.3 Method Validation via Macroscale Rheometry

To validate the accuracy of our nanoindentation-based stiffness measurements, we performed rheological analysis on M1-type alginate macrogels prepared using the same Rhodamine B-labeled alginate and CaCO_3 nanoparticle formulation as in microgel fabrication. Two crosslinking strategies were compared: (a) passive gelation via diffusion of 0.037 vol% acetic acid into the prepolymer mixture from the surrounding HFE 7500 oil phase, and (b) active crosslinking initiated by incorporating glucono- δ -lactone (GDL, 200 mg/mL) into the prepolymer solution to facilitate uniform internal acidification (Figure 4.3 a-b).

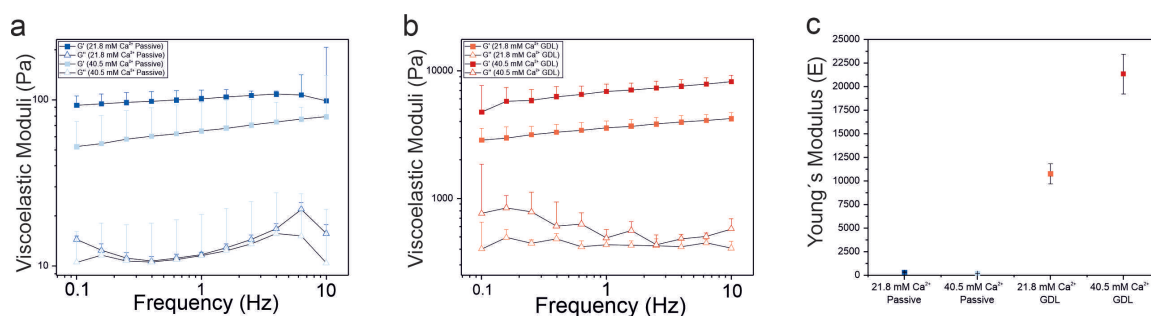


Figure 4.3 Comparison of viscoelastic properties and calculated Young's moduli of M1-type alginate macrogels crosslinked with 21.8 and 40.5 mM Ca^{2+} . a) Frequency-dependent storage and loss moduli of macrogels crosslinked via passive diffusion of Ca^{2+} from CaCO_3 nanoparticles in the presence of 0.037 vol% acetic acid, without GDL in the prepolymer mixture. b) Frequency-dependent viscoelastic moduli of M1-type macrogels crosslinked using GDL to promote active and homogeneous Ca^{2+} release from CaCO_3 nanoparticles. Both formulations were incubated for 12 h to allow full gelation ($n = 3$). c) Calculated Young's moduli based on rheological data from a) and b), demonstrating the effect of crosslinking strategy and calcium concentration. Error bars represent standard deviation.

For both conditions, the final prepolymer solution was prepared by diluting 2 wt % Rhodamine B-labeled alginate to 1 wt % in bead buffer containing suspended CaCO_3 nanoparticles, resulting in final Ca^{2+} concentrations of either 21.8 or 40.5 mM. To compare microscale and macroscale mechanics, the same formulation used for microgel fabrication was cast into cylindrical macrogels and ionically crosslinked. However, unlike microgels formed in microfluidic droplets, where acetic acid diffuses efficiently across the water–oil interface to initiate rapid crosslinking, macrogels lack this favorable surface-area-to-volume ratio. As a result, passive acid diffusion into the gel volume is limited and often leads to incomplete or heterogeneous crosslinking. To address this, glucono- δ -lactone (GDL) was used to trigger controlled internal gelation. GDL hydrolyzes slowly in aqueous solution, lowering the pH over time and releasing calcium ions from insoluble CaCO_3 particles. This method has been widely adopted in previous work to enable uniform ionic crosslinking in alginate macrogels, as it ensures even distribution of calcium throughout the gel volume before network formation begins [257]. As shown in Figure 4.3 a-b, GDL-crosslinked macrogels exhibited significantly higher storage and loss moduli than their passively crosslinked counterparts at both calcium concentra-

tions. Specifically, GDL-mediated gels reached 10.7 ± 1.0 kPa and 21.3 ± 2.1 kPa at 21.8 mM and 40.5 mM Ca^{2+} , respectively (Figure 4.3 c). These values are closely aligned with those obtained via nanoindentation on the corresponding M1-type microgels (11.8 ± 1.4 kPa and 23.3 ± 1.3 kPa), confirming the robustness of the microscale mechanical measurements and validating the reproducibility of gel stiffness across different length scales.

4.2.4 Mechanical Properties of M2-Type Microgels

For all further studies, we focused on M2-type microgels, which represent the functional formulation of TACSI microrobots—alginate microgels embedded with gold nanorods and modified with RGD peptides—due to their suitability for biological integration and photothermal actuation. The Young's modulus of M2 microgels fabricated at 21.8 mM Ca^{2+} was measured to be 8.4 ± 1.0 kPa and 9.8 ± 0.8 kPa for microgel diameters of 29.6 ± 0.9 μm and 36.3 ± 1.3 μm , respectively. Increasing the crosslinker concentration to 40.5 mM resulted in higher stiffness values of 14.4 ± 1.3 kPa and 14.5 ± 1.0 kPa for the same respective sizes (Figure 4.2 d).

Interestingly, microgel size had a negligible influence on stiffness, while calcium concentration clearly modulated mechanical properties. These values are consistent with previous reports on ionically crosslinked alginate gels [258, 259, 260]. To examine the potential mechanical influence of embedded nanomaterials, we compared M2-type microgels with and without gold nanorods. The presence of nanorods slightly reduced the measured Young's modulus: 0.5 ± 0.1 kPa vs. 0.6 ± 0.1 kPa in bead buffer. Although this difference was small, it was statistically significant ($P < 0.001$; Wilcoxon signed-rank test) and suggests that gold nanorods introduce subtle disruption to the ionic crosslinking network. This observation is relevant when designing composite microrobots where embedded nanoparticles serve actuation and sensing functions.

4.2.5 Controlling Hydrogel Mechanics Through Ionic Composition

The ionic composition of the surrounding medium strongly affects the mechanical properties of ionically crosslinked alginate microgels. Calcium chloride (CaCl_2) is commonly used both in the fabrication and storage of alginate hydrogels due to its ability to maintain ionic crosslinks in the alginate matrix [242, 164]. CaCl_2 and NaCl are integral components of cell culture media such as Dulbecco's Modified Eagle Medium (DMEM), where the specific concentrations of divalent and monovalent ions vary across formulations designed for different cell types.

To systematically explore this effect, we investigated the Young's moduli of M2-type alginate microgels under varying concentrations of CaCl_2 . These microgels were chosen for their RGD-mediated cell-adhesion capability and embedded gold nanorods, making them suitable for both mechanobio-

logical modulation and *in vitro* culture applications[216]. Our working hypothesis was that maximal stiffness would be achieved when the molar ratio of Ca^{2+} ions to available carboxyl groups on the alginate was 1:1, corresponding to theoretical full crosslinking. Since alginate consists of alternating mannuronate (M) and guluronate (G) blocks and crosslinking occurs specifically through G blocks [218, 258], and the M/G ratio in our alginate was 0.65 (i.e., 60.6 % G content) [261], we derived the optimal CaCl_2 concentration as:

$$C_{\text{CaCl}_2(\text{opt})} = 0.65 \times C_{\text{Carboxyl}} \quad (4.1)$$

With the total carboxyl group concentration estimated at 0.0124 mmol in 120 μL of 1 wt % alginate, the optimal CaCl_2 concentration was calculated to be 45 mM in the aqueous phase. This value is marked by the red line in Figure 4.4 a, representing normalized peak theoretical crosslinking. Experimental nanoindentation confirmed this prediction. M2-type microgels crosslinked at 21.8 mM Ca^{2+} were tested in measurement media containing 0–100 mM CaCl_2 . In deionized water, the average Young's modulus was 7.3 ± 0.3 kPa. Interestingly, adding just 0.5 mM CaCl_2 reduced stiffness by 39 %, likely due to the dynamic reversal of ionic bridges. Gradual increases in CaCl_2 concentration up to 45 mM led to progressive stiffening, reaching a peak of 19.9 ± 3.0 kPa at 45 mM—corresponding to a 167 % increase in modulus relative to baseline (Figure 4.4 a). Further increases to 75 and 100 mM resulted in a reduction in stiffness (10.7 ± 1.6 and 8.3 ± 1.3 kPa, respectively), likely due to oversaturation and disruption of the optimal egg-box structure [262].

Next, we explored the impact of sodium ions, known to interfere with calcium–alginate interactions by competing for carboxyl binding sites [263, 264]. Introducing 1 mM NaCl led to a pronounced drop in Young's modulus to 4.3 ± 0.9 kPa across both conditions (Figure 4.4 c). At 10 mM NaCl, the microgels began to lose structural integrity and fully dissolved at 20 mM, confirming the chelating behavior of Na^+ ions and their capacity to displace Ca^{2+} from the network (Figure 4.4 d). These findings were consistent with previous reports on macroscale gels, which demonstrated a 63 % reduction in compressive modulus and 84 % drop in shear modulus upon exposure to 150 mM NaCl over 15 hours [265]. Notably, microgels exhibited complete structural breakdown at much lower concentrations and faster timescales, which can be attributed to their larger surface-area-to-volume ratio and faster ionic diffusion rates.

4.2.6 Interplay Between Calcium and Sodium Ions in Alginate Crosslinking

In vitro environments, such as cell culture media, contain dynamic and competing ionic species that can significantly alter the mechanical properties of ionically crosslinked hydrogels. Alginate microgels, commonly stabilized through Ca^{2+} -mediated crosslinking, are particularly sensitive to

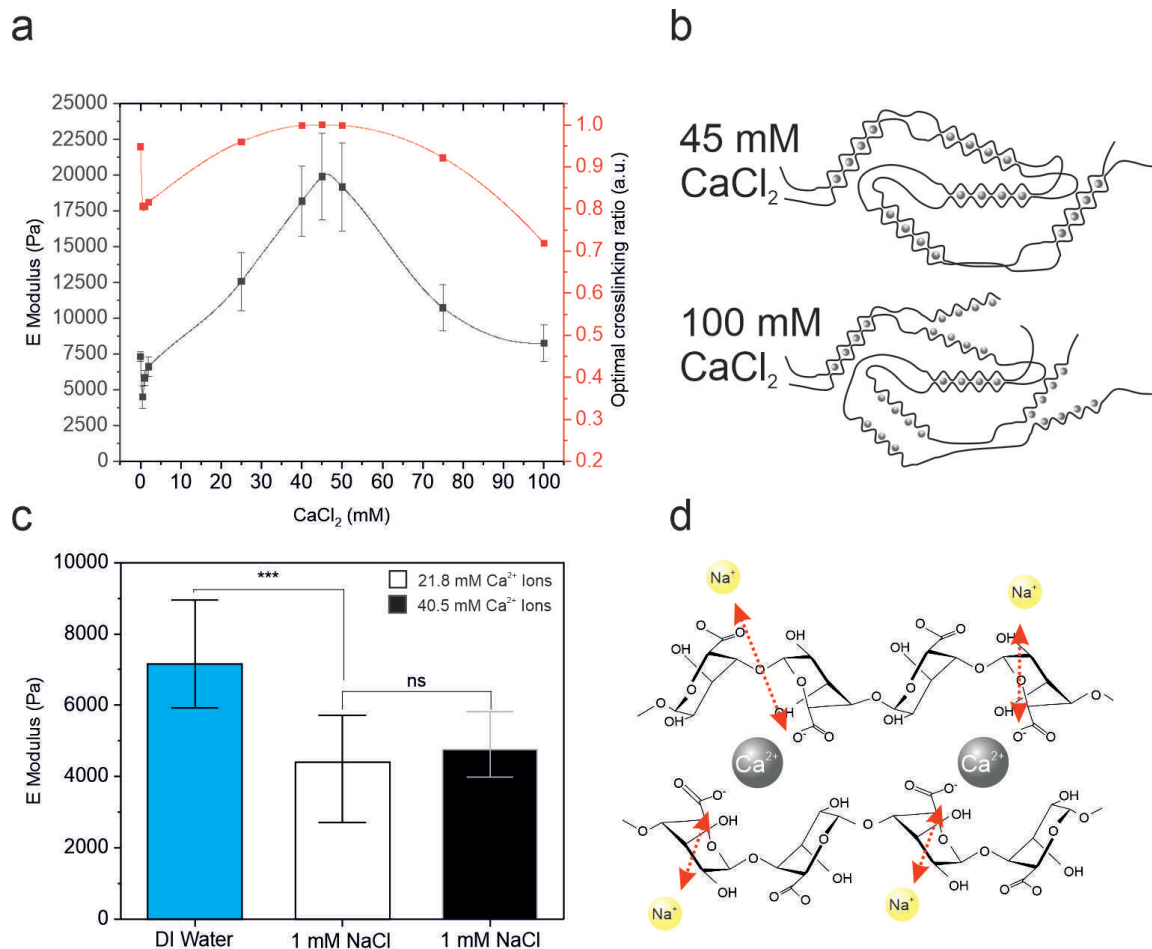


Figure 4.4 Nanoindentation analysis of CaCl_2 and NaCl effects on M2-type alginate microgels. a) Young's moduli of M2-type microgels fabricated at 21.8 mM Ca^{2+} , tested in media with varying CaCl_2 concentrations. The red line marks the theoretical optimal crosslinking point (45 mM CaCl_2). b) Schematic representation of the egg-box model showing optimal Ca^{2+} binding at 50 mM and oversaturation effects beyond this point. c) Microgel stiffness measured in deionized water and 1 mM NaCl for microgels crosslinked at 21.8 and 40.5 mM Ca^{2+} ($n = 15$, $***p < 0.001$, one-way ANOVA). d) Schematic of competitive binding between Na^+ and Ca^{2+} , illustrating ion exchange and network weakening at elevated NaCl concentrations.

fluctuations in extracellular ion composition. Among these, calcium chloride (CaCl_2) and sodium chloride (NaCl) are of primary importance, as they represent the dominant divalent and monovalent ions in formulations like DMEM and Roswell Park Memorial Institute medium (RPMI). Investigating how these salts jointly influence the structure and stiffness of M2-type alginate microgels is essential for predicting their behavior during cell culture, mechanostimulation, and biofunctional assays.

To evaluate this, we systematically varied both CaCl_2 and NaCl concentrations and examined their effects on microgel size and stiffness (Table 4.1, Figure A.8). In the absence of NaCl, increasing CaCl_2 concentration from 2 mM to 50 mM led to a reduction in microgel size from $36.3 \pm 1.3 \mu\text{m}$ to $28.9 \pm 1.0 \mu\text{m}$, consistent with network contraction due to enhanced crosslinking. Introducing NaCl at low concentrations (1–10 mM) into this system reversed the shrinkage effect, increasing the microgel size to approximately 33–34 μm . However, when CaCl_2 was maintained at 100 mM, the

presence of NaCl (1–100 mM) had minimal effect on microgel diameter, indicating that high levels of free Ca^{2+} ions can suppress Na^+ -induced swelling through network reinforcement.

Stiffness measurements corroborated these morphological trends. At 2 mM CaCl_2 , the Young's modulus of M2-type microgels was measured as 6.6 ± 0.7 kPa. The addition of 1 mM NaCl led to a temporary increase in stiffness (8.7 ± 2.3 kPa), potentially reflecting a transient stabilization of the network. However, at higher NaCl concentrations, stiffness declined sharply: 5.5 ± 0.9 kPa at 10 mM, and 0.6 ± 0.3 kPa at 100 mM NaCl. These results reflect a strong disruptive effect of Na^+ ions on ionic crosslinks, likely due to competitive binding and the weakening of Ca^{2+} bridges.

In contrast, microgels immersed in 50 mM CaCl_2 reached their highest stiffness (19.2 ± 4.1 kPa) in the absence of NaCl. Introducing 1 mM NaCl under these conditions decreased stiffness to 14.4 ± 6.6 kPa, and 10 mM NaCl further reduced it to 6.5 ± 0.7 kPa. Interestingly, at 100 mM NaCl, stiffness partially recovered to 11.1 ± 3.8 kPa, suggesting that high concentrations of Na^+ may disrupt overcrowding at crosslinking sites and enable partial reorganization of the network. Microgels exposed to 100 mM CaCl_2 generally showed moderate stiffness (8.3–8.6 kPa) across NaCl concentrations from 0 to 10 mM, with a significant increase to 16.9 ± 3.4 kPa at 100 mM NaCl. This recovery in stiffness at the highest ionic load suggests that in some regimes, Na^+ ions may aid in redistributing and relieving excessive Ca^{2+} saturation, enabling a more stable crosslinked configuration.

Table 4.1 Young's modulus and size of M2-type alginate microgels across varying NaCl and CaCl_2 concentrations. The stiffness trend is noted qualitatively (–, +, ++, +++) to show relative changes.

NaCl / CaCl_2	Young's Modulus (kPa)	Stiffness Trend	n (Stiffness)	Microgel Size (μm)	n (Size)
deionized water	7.3 ± 0.3	baseline	14	36.3 ± 1.3	100
0 mM / 2 mM	6.6 ± 0.7	–	22	32.3 ± 1.2	495
1 mM / 2 mM	8.7 ± 2.3	+	22	33.3 ± 1.0	517
10 mM / 2 mM	5.5 ± 0.9	—	16	34.6 ± 1.8	487
100 mM / 2 mM	0.6 ± 0.3	—	15	36.1 ± 1.0	508
0 mM / 50 mM	19.2 ± 4.1	+++	16	28.9 ± 1.0	395
1 mM / 50 mM	14.4 ± 6.6	++	18	33.3 ± 2.3	429
10 mM / 50 mM	6.5 ± 0.7	–	14	32.9 ± 2.4	479
100 mM / 50 mM	11.1 ± 3.8	+	17	33.9 ± 2.0	342
0 mM / 100 mM	8.3 ± 1.3	+	11	28.9 ± 0.9	258
1 mM / 100 mM	8.4 ± 6.8	+	33	31.2 ± 1.5	849
10 mM / 100 mM	8.6 ± 0.9	+	21	29.0 ± 0.9	620
100 mM / 100 mM	16.9 ± 3.4	+++	37	29.2 ± 0.9	883

To determine whether the changes in stiffness were accompanied by alterations in viscoelastic behavior, we performed dynamic mechanical analysis across a range of NaCl and CaCl_2 combinations. In 2 mM CaCl_2 with 100 mM NaCl, microgels exhibited a phase angle of 38.1° at 2 Hz, indicative of highly viscous behavior. In contrast, samples maintained in 50 mM or 100 mM CaCl_2

displayed lower phase angles (11.6–16.5° at 1–10 Hz), confirming a predominantly elastic response in more robustly crosslinked gels (Figure 4.5).

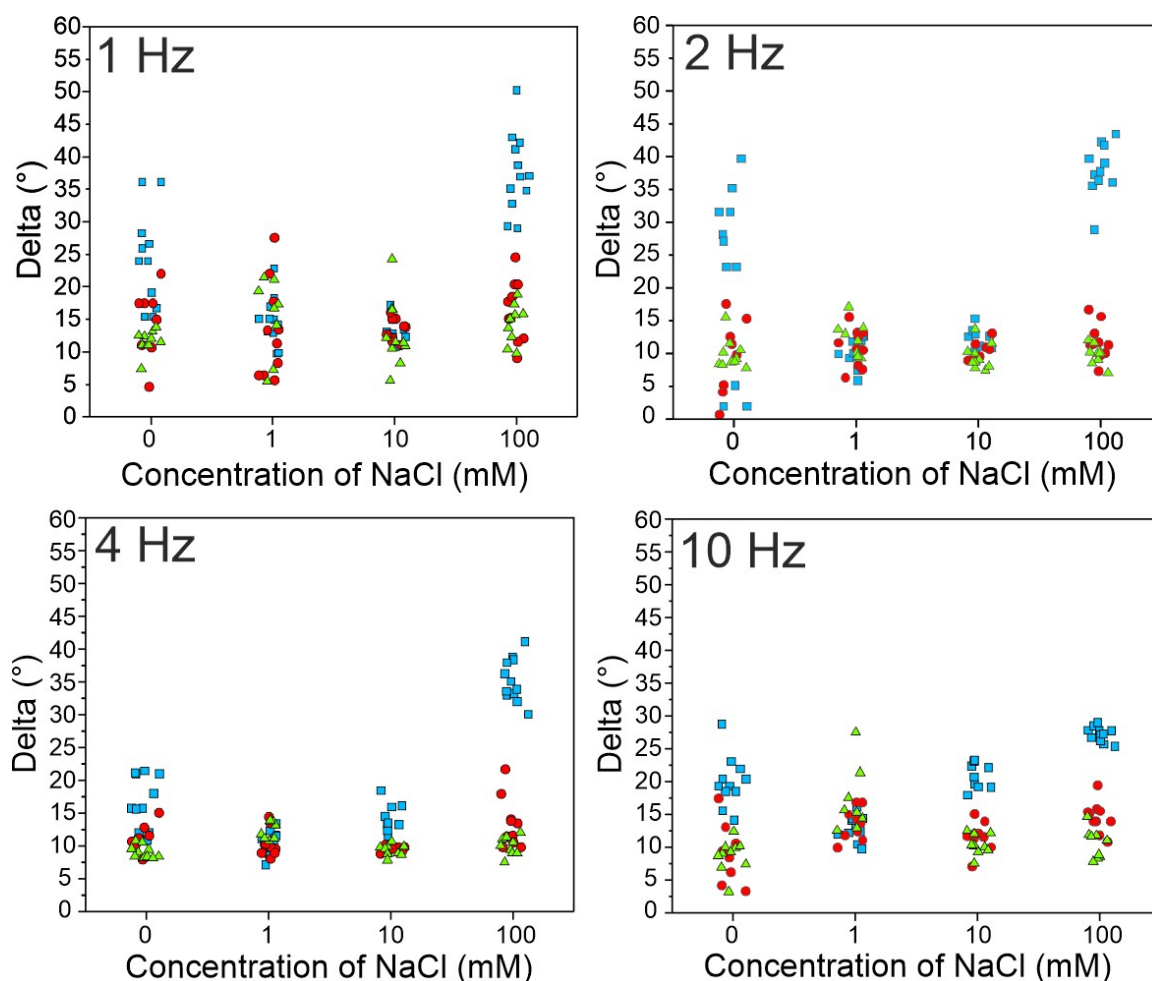


Figure 4.5 Viscoelastic analysis of M2-type alginate microgels. Phase angle measurements for 21.8 mM Ca^{2+} M2-type microgels across varying NaCl concentrations (0, 1, 10, and 100 mM) and CaCl_2 levels (blue squares: 2 mM CaCl_2 ; red circles: 50 mM CaCl_2 ; green triangles: 100 mM CaCl_2) at oscillation frequencies of 1, 2, 4, and 10 Hz.

4.2.7 Impact of Storage Buffers and Cell Culture Media on Microgel Stiffness

To ensure reliable application of alginate microgels in biological systems, it is essential to assess how commonly used storage buffers and culture media influence their mechanical stability. Alginate hydrogels are ionically crosslinked networks, and their stiffness is known to be highly responsive to the ionic composition of their surrounding environment. Given the widespread use of M2-type microgels in cell culture contexts, we systematically investigated how different media affect their mechanical properties.

We first evaluated two standard storage buffers: 25 mM HEPES and bead buffer, the latter consisting of 130 mM NaCl, 2 mM CaCl_2 , and 25 mM HEPES (Table 4.2). While microgels in pure HEPES buffer maintained relatively high stiffness (6.2 ± 1.3 kPa), those in bead buffer exhibited a

drastic reduction in stiffness to 0.7 ± 0.1 kPa. These findings align with previous studies reporting Young's moduli in the range of 1–3 kPa for alginate microgels measured via atomic force microscopy [164, 266]. The observed difference is consistent with the disruptive effect of excess Na^+ on the ionic crosslinking structure and reinforces the need for careful selection of storage conditions.

Table 4.2 Young's modulus, size, and conditions for M2-type microgels in deionized water, storage buffers, and cell culture media.

Media	E (kPa)	Diameter (μm)	Measurable	pH	Reversibility
deionized water	7.2 ± 0.9	36.0 ± 0.7	yes	7	yes
HEPES buffer (25 mM)	6.2 ± 1.3	34.9 ± 1.0	yes	6.5	–
d-glucose (25 mM)	5.5 ± 0.8	33.5 ± 0.8	yes	6.5	–
bead buffer	0.7 ± 0.1	34.7 ± 1.0	yes	6.5	yes
DMEM + FBS (10 %) + P/S (1 %)	0.4 ± 0.1	37.0 ± 1.0	yes	9	–
DMEM	0.2 ± 0.1	38.4 ± 1.4	yes	9	no
DMEM w/o NaHCO_3	0.4 ± 0.1	36.1 ± 0.9	yes	6	–
DMEM + HEPES	0.3 ± 0.1	36.0 ± 1.0	yes	7.5	–
DMEM + HEPES + CaCO_3	0.3 ± 0.1	36.1 ± 0.9	yes	8	–
DMEM/F-12	<0.1	37.3 ± 0.7	no	7	–
RPMI 1640	<0.1	44.9 ± 1.3	no	9	–
mTeSR Plus	<0.1	36.1 ± 1.3	no	8	–

To further evaluate the influence of chemically complex environments, we characterized microgels in various formulations of Dulbecco's Modified Eagle Medium (DMEM), a commonly used culture medium for mammalian cells. In standard DMEM, the average Young's modulus dropped to 0.2 ± 0.1 kPa, indicating substantial softening. This effect is likely due to the high ionic complexity of DMEM, which contains 2 mM CaCl_2 , 1 mM MgSO_4 , 5.4 mM KCl, and 110 mM NaCl, along with bicarbonate buffers, amino acids, and vitamins. The presence of serum proteins (10 % FBS) and antibiotics (1 % Pen/Strep) did not reverse the softening effect, nor did variations such as removing NaHCO_3 or supplementing with HEPES or CaCO_3 . Microgel stiffness was further reduced in other commonly used cell culture formulations, including DMEM/F-12, RPMI 1640, and maintenance medium for Human Pluripotent Stem Cells (mTeSR). In all three cases, stiffness dropped below 0.1 kPa, beneath the sensitivity threshold of the nanoindenter. This mechanical degradation was accompanied by noticeable swelling, particularly in RPMI 1640, where microgels expanded to 44.9 ± 1.3 μm in diameter. The high osmotic pressure and alkaline pH of RPMI have previously been linked to increased water uptake and weakened crosslinking in hydrogels [263, 267, 268].

We then examined whether the stiffness changes induced by different media were reversible. To do so, M2-type microgels, corresponding to the functional formulation of TACSI microrobots, were initially incubated in bead buffer, then transferred to deionized water and measured after a 10-minute equilibration. This process was repeated over five cycles (Figure 4.6 a). With each transfer back to deionized water, the average Young's modulus increased, indicating partial recovery

of stiffness. However, comparing the first and final measurements in deionized water revealed an overall reduction of approximately 55 %, pointing to an irreversible loss of mechanical integrity. In contrast, microgels exposed once to cell culture medium (DMEM) retained their softened state even after being rinsed and measured in deionized water (Figure 4.6 b). These observations suggest that serum proteins and the complex ionic composition of DMEM induce persistent structural changes in the alginate network, consistent with previous findings on the irreversible softening of hydrogels in serum-rich environments.

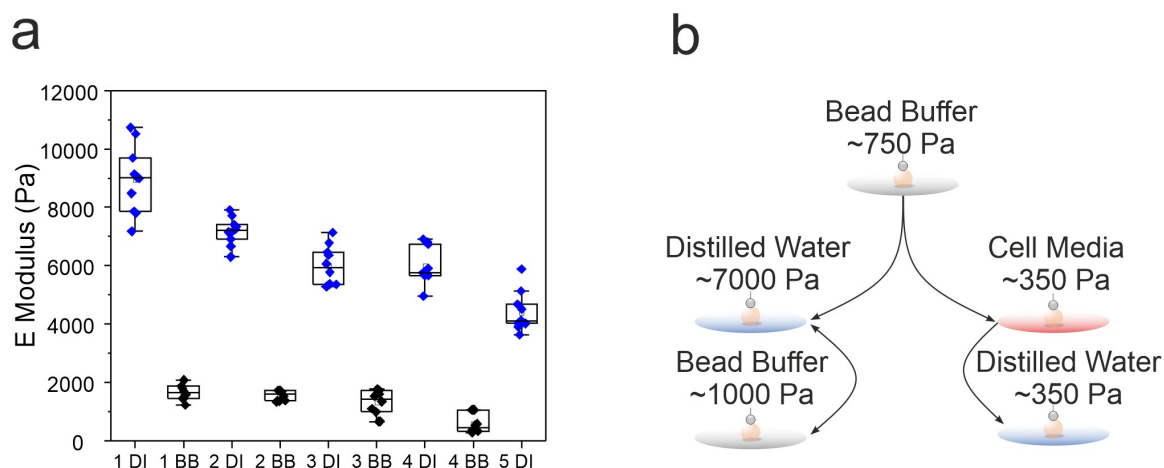


Figure 4.6 Influence of storage and culture media on M2-type microgel mechanics. a) Young's modulus measurements of 21.8 mM Ca^{2+} M2-type microgels in deionized water (DI) and bead buffer (BB), repeated over five cycles. b) Schematic representation of reversible stiffness modulation in microgels for DI water and BB versus irreversible changes induced by cell culture media.

Taken together, these results highlight the critical importance of selecting appropriate liquid media when characterizing and using alginate microgels for *in vitro* context. The dramatic changes in stiffness and size caused by media composition can directly impact cellular behavior, given that Young's modulus influences adhesion, proliferation, and differentiation [269, 270]. Without careful media control, mechanical measurements may become unreliable and poorly reflective of real cellular environments.

4.3 Concluding Remarks

In this chapter, we established a robust mechanical characterization framework for ionically cross-linked alginate microgels, motivated by the need to better understand and optimize the Thermally Activated Cell-Signal Imaging (TACSI) microrobot system for future applications involving direct cell interaction. As these microrobots are designed to function as active scaffolds and dynamic interfaces in biological environments, a precise understanding of their mechanical behavior under physiologically relevant conditions is essential.

To address this, we employed nanoindentation, supported by complementary shear rheology on macrogels, to systematically investigate how formulation parameters, ionic composition, and environmental conditions influence microgel stiffness and viscoelasticity. We found that mechanical properties could be finely tuned by adjusting calcium cross-linker concentrations, with stiffness increasing from approximately 9 kPa at 21.8 mM Ca^{2+} to around 14 kPa at 40.5 mM. The addition of gold nanorods (GNRs) and arginylglycylaspartic acid (RGD)-functionalized alginate further modulated mechanical properties, highlighting the impact of embedded nanomaterials and bioactive ligands on gel stiffness. Interestingly, microgel size did not significantly affect mechanical performance, suggesting that the intrinsic material formulation dominates over geometric scaling in this size regime.

A central insight from this work is the critical influence of the ionic environment on microgel mechanics. We observed that high sodium concentrations, common in biological media, can disrupt calcium-mediated cross-links, resulting in substantial softening of the gel. However, we identified an optimal ionic condition at 45 mM calcium chloride (CaCl_2), which provided maximal stiffness and mechanical stability even in the presence of elevated sodium levels. Viscoelastic measurements revealed a clear shift toward more viscous behavior under suboptimal ionic conditions, whereas higher calcium concentrations helped preserve a more elastic response.

To reflect the intended biological context of TACSI microrobots, we also investigated how commonly used buffers and culture media affect microgel integrity. Simple, low-ionic buffers such as HEPES were found to maintain mechanical stability, but complex media like DMEM, Roswell Park Memorial Institute medium, and mTeSR™ caused marked softening, often leading to irreversible structural changes, particularly in the presence of serum. Notably, even rinsing in deionized water after serum exposure did not fully restore stiffness, emphasizing the lasting impact of media composition on cross-linked alginate networks.

Throughout this work, careful attention was paid to experimental reproducibility and methodological clarity. For consistent sample preparation, substrates were coated with 0.1 mg/mL poly-L-lysine to ensure stable microgel adhesion without unintended secondary cross-linking. Indentation ex-

periments were performed using spherical cantilevers with spring constants around 0.5 N/m, which provided the optimal balance between sensitivity and measurement stability. Just as importantly, mechanical measurements were always performed in the same media conditions as those intended for biological experiments, to ensure physiologically relevant interpretation of results.

Together, these studies offer a comprehensive and reproducible approach for characterizing the mechanical properties of alginate microgels. The insights gained here lay the groundwork for the next chapter, where we will integrate these microrobots into three-dimensional cell constructs and employ them as both mechanical actuators and environmental sensors within tissue-like biological systems.

4.4 Materials and Methods

1. Alginate Preparation

High molecular weight alginate (I-1G, 280 kDa; KIMICA) was used to generate three types of functionalized alginate: RGD-, Rhodamine B-, and biotin-functionalized. Functionalization used EDC/sulfo-NHS carbodiimide chemistry in MES buffer (1 % w/v). For RGD coupling, GGGGRGDSP peptide (Peptide 2.0) was added at a degree of substitution (DS) of 20, aiming for 71.4 mM RGD per g of alginate. This equated to 142.31 mg RGD (MW = 758.75 g/mol, purity = 63.5 %) per g alginate. Assuming a 60 % conjugation efficiency, sulfo-NHS and EDC concentrations were 1.26 mM and 2.53 mM, respectively. Rhodamine B Lissamine (Thermo Fisher Scientific) was used at DS = 2. Biotinylation used EZ-Link Amine-PEG3-Biotin (Thermo Fisher Scientific) at DS = 20. Reactions proceeded for 20 h at room temperature. Functionalized alginates were purified by dialysis against decreasing NaCl concentrations for 3 days, treated with charcoal, filtered (0.22 μ m), and freeze-dried at -50°C for 1 week (FreeZone 4.5L, Labconco). Dried samples were stored at -20°C .

2. Preparation of CaCO_3 Nanoparticles

CaCO_3 nanoparticles (CalEssence, PCC70) were sterilized in an autoclave at 121°C for 20 min (Varioklav). A mass of 14–16 mg was suspended in 600 μL DMEM, sonicated for 15 s at 70 % amplitude (Fisherbrand Model 120 Sonic Dismembrator), diluted in 23 mL DMEM, and centrifuged at 50 rcf for 5 min at 20°C . The top 20 mL was collected and centrifuged again at 1000 rcf. The resulting pellet was resuspended to a final CaCO_3 concentration of 10 mg/mL (99.91 mM) in DMEM.

3. Microgel Fabrication

Two types of microgels were produced: Rhodamine B-loaded (M1) and Au-nanorod-loaded (M2). Alginate was reconstituted at 2 % in MES buffer. M1 used Rhodamine B-functionalized alginate; M2 used 80 % RGD-alginate and 20 % biotin-alginate with 4.875 mg/mL Au-nanorods. The mixture was sonicated (15 s, 60 % amplitude). The aqueous phase included 1 % alginate, 20.8 mM CaCO_3 , and bead buffer (130 mM NaCl, 2 mM CaCl_2 , 25 mM HEPES). The oil phase comprised HFE 7500 (3M), Pico-Surf, and 0.037 % acetic acid. Acetic acid dissolved CaCO_3 post-droplet formation, releasing Ca^{2+} for cross-linking. Final Ca^{2+} concentrations were 3.1 mM (5 μL CaCO_3), 21.8 mM (50 μL), or 40.5 mM (95 μL). Syringes (1 mL aqueous, 3 mL oil) were connected to a microfluidic chip via PTFE tubing and driven by a syringe pump (Darwin Microfluidics) at flow rates of 2–10 $\mu\text{L}/\text{min}$, the same fabrication process as discussed in Chapter 3. Droplets were demulsified using PFO (Sigma) and stored in water or BB at 4°C .

4. Rheological Characterization and Macrogel Fabrication

Rheological measurements were performed on a shear rheometer (MCR302, Anton Paar) with a planar bottom plate (P-PTD200/56) and parallel plate geometry (PP25). The storage modulus G' and loss modulus G'' were recorded at 1 Hz for 10 min, sampled every 30 s. Samples (240 μ L) were loaded with a 400 μ m gap. M1 macrogels were prepared by diluting 2 % RhB-alginate to 1 % with CaCO_3 and BB (final Ca^{2+} = 21.8 mM or 40.5 mM). Passive cross-linking occurred in HFE 7500 oil with 0.037 % acetic acid over 12 h. For GDL-triggered gels, 200 mg/mL GDL was mixed with the prepolymer, and cross-linking proceeded over 12 h. Young's moduli were calculated from the shear modulus using the relation $E = 2G(1 + \nu)$, assuming a Poisson's ratio of $\nu = 0.49$.

5. Nanoindentation and Viscoelastic Properties

A Chiaro stage-top nanoindenter (Optics11 Life) was used for mechanical characterization, employing probes with tip diameters of 3 μ m and 11 μ m and stiffness values of 0.025 N/m and 0.5 N/m, respectively. Indentations were performed to a maximum depth of 1000 nm at a rate of 1250 nm/s. For dynamic mechanical analysis (DMA), a maximum indentation of 10000 nm with superimposed 100 nm oscillations was applied at frequencies of 1, 2, 4, and 10 Hz, with a 2 s rest between each frequency step. Microgels were immobilized in PLL-coated wells (0.05–0.5 mg/mL), which were cured at 35°C for 30 min prior to measurement. Force-indentation data were analyzed using the Hertzian contact model for spherical indenters [271, 272, 256]. Specifically, 90 % of the maximum force range was used for fitting, based on the equation $F = \frac{4}{3}E_{\text{eff}}R^{1/2}h^{3/2}$, where F is the applied force, h is the indentation depth, R is the radius of the indenter tip, and E_{eff} is the effective elastic modulus. To account for the near-incompressibility of alginate hydrogels, the final Young's modulus E was calculated using the relation $E = \frac{E_{\text{eff}}}{1-\nu^2}$, with a Poisson's ratio of $\nu = 0.49$ [273]. Hertzian analysis was selected due to its reliability in the 0-35 kPa range typical of soft biomaterials. The storage modulus E' and loss modulus E'' were further extracted from DMA data to evaluate mechanical suitability for drug delivery and stimuli-responsive applications.

6. Statistical Analysis

All data are shown as mean $\pm$ SD from at least three replicates. Statistical comparisons used a paired Student's *t*-test or Wilcoxon Signed-Rank Test for non-parametric data. One-way ANOVA followed by Tukey's test was used for multiple comparisons. Two-way ANOVA assessed factorial interactions. Significance levels were reported as: ns (not significant), **p* < 0.05, ***p* < 0.01, ****p* < 0.001. All analyses were performed in OriginPro2021b.

5 Spheroid Assembly, Single-Cell Transport, and Microrobot-Enabled Drug Modulation

This chapter introduces the extension of TACSI microrobots toward active biological manipulation in three-dimensional (3D) environments. Building on the thermally actuated design, we integrated two distinct modes of cell adhesion: a fast, electrostatic interaction enabled by poly-L-lysine coating for rapid pick-up and manipulation, and a slower, integrin-mediated adhesion via RGD-modified alginate for stable biological integration. These features allow the microrobots to selectively capture and transport individual cells in 3D workspace. We then demonstrate high-throughput generation of structured spheroids incorporating both microrobots and scaffold microgels, enabling integrated thermal and mechanical stimulation. Emphasis is placed on real-time cellular responses to localized heat delivery, strain-induced compression, and synergistic drug interactions. These results establish TACSI microrobots not only as mobile actuators but also as multifunctional probes for dynamic cellular environments. The work presented in this chapter is currently under review at Advanced Materials.

5.1 Introduction

Despite significant advances in engineered 3D culture systems, most current methods for assembling multicellular tissues still rely on stochastic self-organization, lacking real-time control over cell–cell positioning and environmental cues [39]. While platforms such as organoid culture, spheroid formation, and hydrogel embedding have introduced greater structural and biological complexity, they remain inherently passive, limiting the extent to which researchers can interact with and manipulate individual cells in space and time [274, 275, 276]. In earlier chapters, we introduced TACSI microrobots as a first step toward overcoming these limitations. These hydrogel-based microrobots were engineered with gold nanorods for localized photothermal heating and embedded fluorescent dyes for temperature sensing. We demonstrated robust microfluidic fabrication, thermal calibration, and *in vitro* validation of TACSI microrobots for locomotion and localized thermal stimulation in single-cell environments (Chapter 3). However, while these microrobots were capable of non-contact interactions, they lacked the ability to physically interface with or manipulate cells.

To prepare TACSI microrobots for integration into functional tissue models, we next examined their mechanical behavior under physiologically relevant conditions. While most microrobotic systems have focused on short-term cell transport, only a few have demonstrated the potential for assembling multicellular structures [106, 277]. Unlike these approaches, TACSI microrobots are designed to act not only as mobile actuators but also as mechanically tunable scaffolds that support long-term tissue integration and stimulation. To enable this functionality, we systematically characterized the viscoelastic properties of their ionically crosslinked alginate matrix under varying ionic environments and crosslinking schemes (Chapter 4). This analysis was essential for ensuring that the microrobots could operate as both active tools and passive, biocompatible components within 3D microtissue systems. Building on this foundation, we sought to endow the microrobots with the ability to directly manipulate cells. Specifically, we aimed to enable selective cell adhesion, transport, and release without requiring chemical modification of the cell membrane or complex trapping systems. To achieve fast, reversible adhesion, we coated the otherwise inert alginate surface with poly-L-lysine (PLL), introducing a positive surface charge that facilitates electrostatic interaction with negatively charged cell membranes. This modification produced a new microrobot variant— μ TACSI—with rapid, non-specific adhesion suitable for dynamic cell handling. In contrast, the original TACSI microrobots rely on RGD-functionalized alginate to promote slower, integrin-mediated binding, supporting long-term biological integration. Alongside these functional enhancements, we developed a scalable and efficient method for 3D tissue formation. In a high-throughput, one-pot approach, mammalian cells and RGD-TACSI microrobots were co-incubated in suspension within standard Eppendorf tubes. Under non-adhesive conditions, this setup promoted spontaneous aggregation of cells and microrobots into uniform, self-assembled spheroids. This minimal-handling, scalable process enabled the reproducible fabrication of hundreds of hybrid microtumors containing embedded microrobots—each retaining the capability for localized heating and real-time temperature sensing in a 3D context. In addition to this macroscale fabrication strategy, the unique cell-adhesive and transport capabilities of μ TACSI microrobots offered complementary functionality for manipulating individual cells. While the one-pot method provided simplicity and throughput, the programmable single-cell manipulation enabled by PLL functionalization allowed for precision cell repositioning, spheroid fusion, or localized delivery of stimuli.

Finally, we explored how the previously characterized mechanical properties of the microrobot matrix could be leveraged not only for passive sensing but also for active mechanical stimulation (Figure 5.1). By embedding thermoresponsive polymers such as Poly(N-isopropylacrylamide) (PNIPAM) into the alginate network, we created microrobots and scaffold gels capable of actuation upon heating. This dual-mode functionality—thermal and mechanical—was demonstrated in both 2D monolayers

and 3D spheroids, enabling controlled mechanical cues alongside thermal stimulation. The culmination of these developments is a multifunctional platform for microrobot-assisted tissue engineering and combinatorial stimulation. In the final part of this study, we integrated RGD-TACSI microrobots into tumor spheroids and explored their application in photothermal drug testing. Building on prior literature showing synergistic effects between heat and chemotherapeutics, we demonstrated how embedded microrobots can serve as both actuators and sensors—enabling localized stimulation and real-time feedback in physiologically relevant, multicellular models.

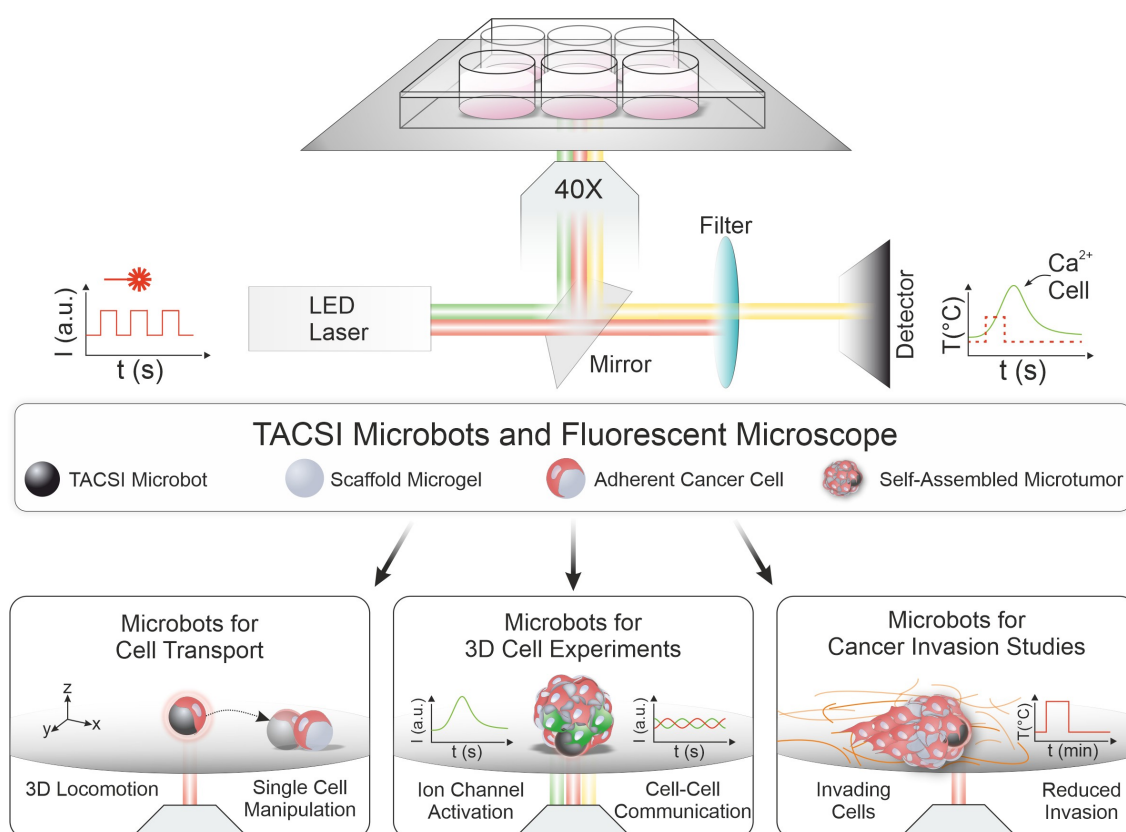


Figure 5.1 Conceptual Overview of μ TACSI Microrobot Integration with 2D and 3D Cell Systems. Schematic representation of the experimental platform for photothermal actuation and cell manipulation. A fluorescence microscope equipped with a 785 nm laser enables localized heating of μ TACSI microrobots and scaffold microgels. These functional microgels facilitate selective single-cell adhesion, transport, and positioning. Upon aggregation, self-assembled microtumors support downstream 3D experiments including thermally gated ion channel activation, cell–cell communication studies, and analysis of cancer cell invasion dynamics.

5.2 Results and Discussion

5.2.1 TACSI Modification for Single Cell Transport

To enable direct microrobot–cell interactions essential for engineered tissue assembly, we sought to extend the functionality of our previously developed TACSI microrobots beyond thermal stimulation and sensing. While the original TACSI design provided precise photothermal control and real-time temperature feedback, it lacked the ability to physically bind and manipulate individual cells. To ad-

dress this, we designed a new generation—termed μ TACSI—by introducing a PLL surface coating, enabling reversible, electrostatic adhesion to mammalian cells without requiring any chemical modification of the cell membrane. This modification was critical for achieving untethered, label-free cell pickup, transport, and release, capabilities that are not only complementary to our previously developed one-pot spheroid formation method but also expand the microrobot's utility in constructing spatially defined multicellular systems. In the following sections, we detail the PLL surface functionalization process, confirm structural and optical integrity post-coating, and demonstrate how μ TACSI microrobots leverage laser-guided locomotion to perform real-time single-cell manipulation in open fluidic environments.

The μ TACSI fabrication process begins with standard TACSI microrobot production via droplet microfluidics, as detailed in Chapter 3. Spherical microgels composed of Rhodamine B and RGD-conjugated alginate and embedded gold nanorods (GNRs) were generated using CaCO_3 -mediated ionic crosslinking within a flow-focusing chip. The resulting photothermally active and temperature-sensitive microrobots were harvested, washed in 70 % ethanol, and transferred to sterile DMEM for downstream functionalization. TACSI microrobots were then suspended in 0.01% (w/v) PLL in DMEM and incubated for 5 minutes under gentle mixing at room temperature (Figure 5.2) for surface modification [266]. The positively charged PLL spontaneously adsorbs onto the negatively charged alginate surface through electrostatic attraction [278]. Following coating, the particles were rinsed three times in DMEM to remove unbound PLL and stabilize the suspension.

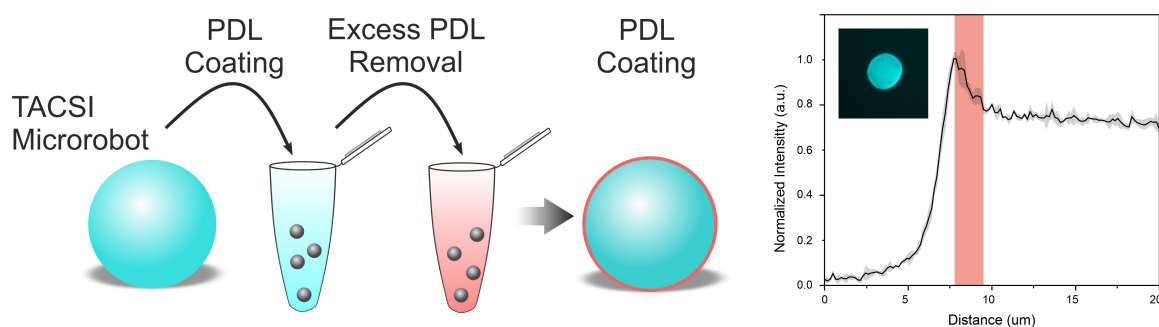


Figure 5.2 Schematic illustration of the PLL coating procedure, in which TACSI microrobots are incubated with PLL and subsequently washed to remove unbound material. Fluorescence image of a μ TACSI microrobot and corresponding quantification of Rhodamine B fluorescence intensity from 10 individual microrobots, confirming surface-localized signal (red highlight) consistent with successful PLL modification. Scale bar: 30 μm .

Fluorescence microscopy revealed increased Rhodamine B intensity around the microrobot perimeter, indicating a denser alginate network near the surface following PLL adsorption. Quantitative image analysis showed an average high-intensity boundary of $2.5 \pm 1.3 \mu\text{m}$ (Figure 5.2), consistent with a conformal but compacted surface layer. Since PLL is not fluorescently labeled, the observed signal arises from Rhodamine B-labeled (RhB) alginate that is more tightly packed at the interface.

The PLL coating introduced a clear change in the microrobots' optical appearance. Compared to the transparent scaffold microgels, both TACSI and μ TACSI microrobots exhibited increased opacity under brightfield microscopy due to GNR incorporation. While visual contrast was highest in GNR-containing samples, μ TACSI microrobots appeared slightly more refractive than their TACSI counterparts, likely due to the added surface layer and altered refractive index at the gel-liquid interface (Figure 5.3 a).

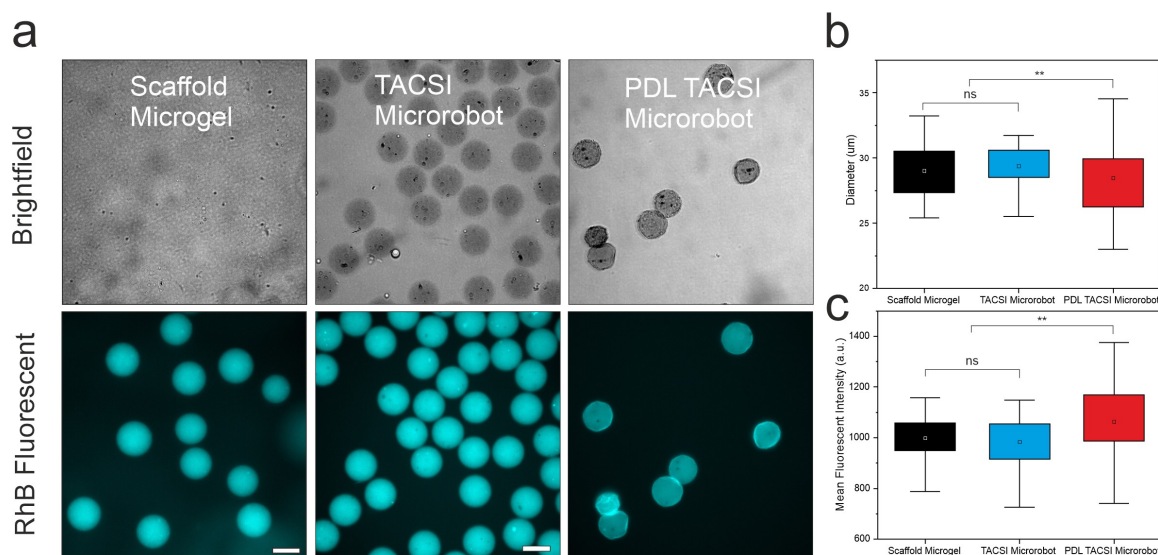


Figure 5.3 Size and Surface Fluorescence Characterization of Scaffold Microgels, TACSI, and μ TACSI Microrobots. a) Representative brightfield and RhB fluorescence images of scaffold microgels, TACSI microrobots, and μ TACSI microrobots. Scale bars: 30 μ m. b) Size distribution analysis of scaffold microgels, TACSI microrobots, and μ TACSI ($n = 200$ per group; one-way ANOVA; ns = not significant, $P > 0.05$; $**P < 0.01$). c) Quantification of RhB fluorescence intensity across the same groups ($n = 200$ per group; one-way ANOVA; ns = not significant, $P > 0.05$; $**P < 0.01$).

Importantly, particle size and fluorescent intensities remained consistent across formulations: scaffold microgels had an average diameter of 28 ± 2 μ m, TACSI microrobots 29 ± 1.5 μ m, and μ TACSI microrobots 29 ± 4 μ m (Figure 5.3 b-c). This confirms that PLL adsorption did not induce bulk swelling and collapse. However, excess PLL exposure (e.g., longer incubation times and higher concentrations) resulted in pronounced microgel shrinkage and aggregation, effects attributed to strong charge neutralization and interparticle bridging. These artifacts were mitigated by optimizing both coating concentration and exposure duration.

To assess the impact of microrobot composition on fluorescence imaging quality, we compared the optical transparency of scaffold microgels, TACSI microrobots, and μ TACSI microrobots placed atop fibrosarcoma HT-1080 cell monolayers (Figure 5.4). HT-1080 cells were chosen for their high motility and sensitivity to microenvironmental stimuli, making them an appropriate model for evaluating microrobot–cell interactions in engineered culture systems [279]. Cells were stained with nuclear and actin markers to monitor signal intensity changes induced by overlying microrobots.

Scaffold microgels exhibited minimal optical interference, allowing fluorescence signals from both nuclei and cytoskeletal filaments to be clearly visualized. In contrast, TACSI and μ TACSI microrobots—containing gold nanorods for photothermal actuation—caused a measurable reduction in fluorescence intensity. Quantification revealed an average decrease of approximately 30% in signal when microrobots were directly positioned over stained cellular regions. These results confirm that scaffold microgels maintain high optical transparency, while TACSI-based systems, despite their added functionality, introduce localized attenuation of fluorescence signals due to their increased optical density.

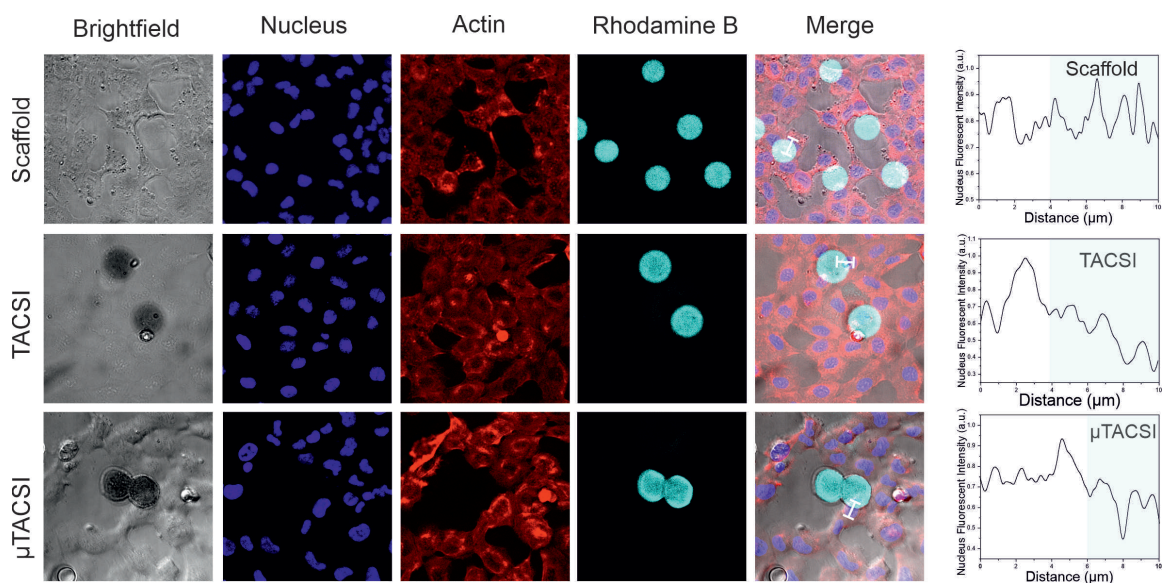


Figure 5.4 Fluorescence Imaging Reveals Signal Overlap Between RhB-Labeled μ TACSI Microrobots and Fibrosarcoma Cells. Brightfield and fluorescence images of fibrosarcoma cells stained for nuclei (blue) and actin filaments (red), shown alongside Rhodamine B-labeled μ TACSI microrobots (cyan). A white line indicates an example path for fluorescence intensity measurement. The corresponding normalized intensity profile reveals signal interference (blue highlight) between the RhB emission of μ TACSI microrobots and cellular fluorescence signals. Scale bars: 30 μ m.

5.2.2 Laser-Guided Locomotion and Single-Cell Pickup, Transport, and Release

Precise positioning and handling of individual cells remain a central challenge in engineering spatially defined tissue constructs. Traditional micromanipulation systems rely on solid interfaces, tethered probes, and embedded channels, which limit scalability and cell freedom. With the development of μ TACSI microrobots, we aimed to enable label-free, untethered single-cell manipulation using only laser light as a control input. By combining photothermal actuation with surface-mediated cell adhesion, μ TACSI microrobots serve as mobile tools for collecting and relocating living cells in open environments. Actuation was performed as described in Chapter 3, using a 785 nm diode laser integrated into the fluorescence microscope's optical path to enable simultaneous thermal control and Rhodamine B-based temperature feedback during imaging.

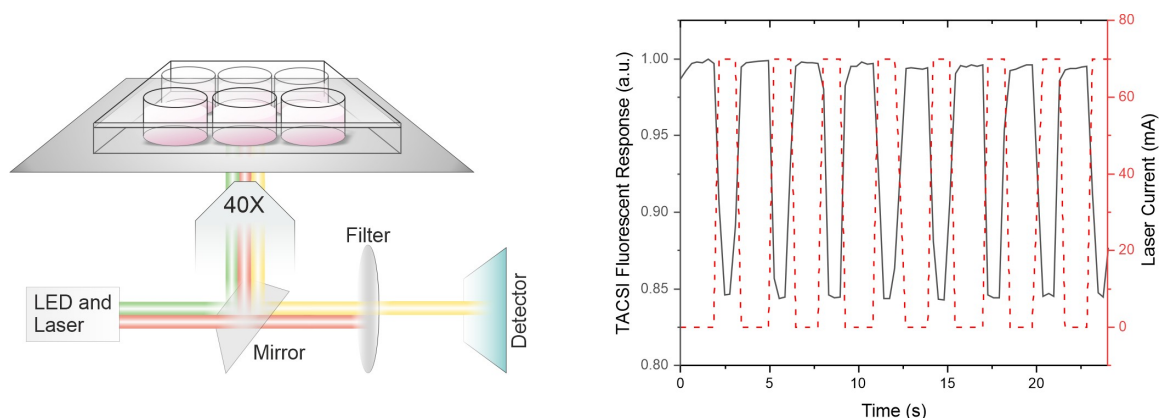


Figure 5.5 Schematic of the laser actuation system: a 785 nm laser is directed through the microscope objective onto the sample. A bandpass filter prevents reflected laser light from reaching the detector, while an LED light source enables concurrent imaging. The plot shows the fluorescence intensity of a single TACSI microrobot (black line) under cyclic laser actuation at 0.33 Hz (red dotted line), with laser power set to 70 mA. The temporal correlation demonstrates the microrobot's responsive fluorescence behavior to periodic optical stimulation.

Thermophoretic locomotion was induced by tightly focusing the laser on specific regions of the μ TACSI microrobot. When targeted at the pole, uniform convection around the microrobot led to upward propulsion. In vertical lifting mode, microrobots reached heights of up to 50 μ m within 1 second at velocities of 25 μ m/s, then slowly settled over 30 seconds due to gravity and reduced heating (Figure 5.6 a).

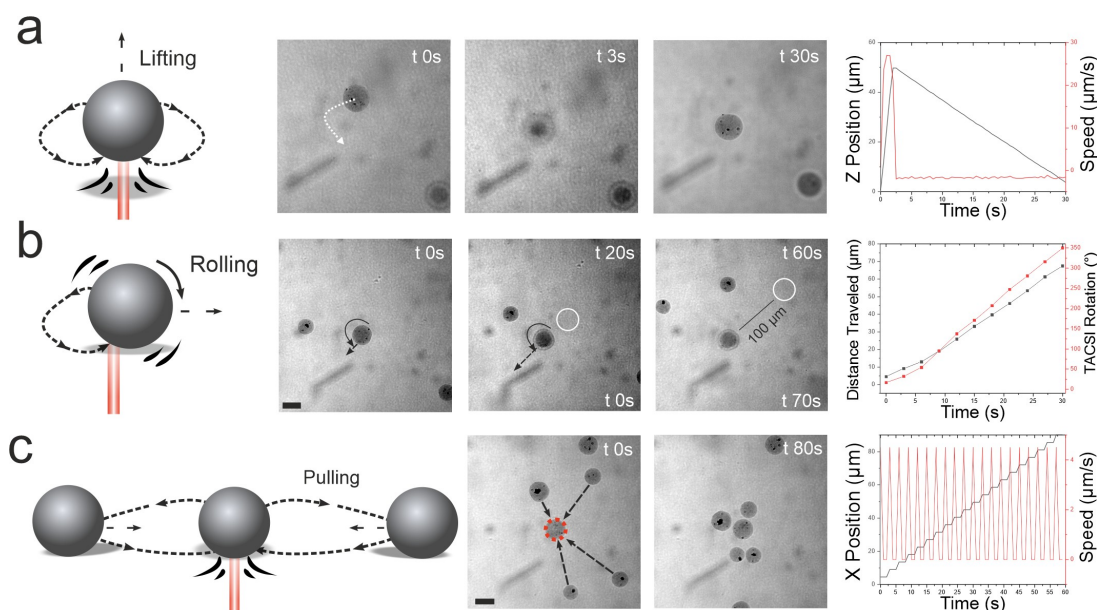


Figure 5.6 Multimodal locomotion of TACSI microrobots under laser actuation. a) Lifting motion of a TACSI microrobot shown via brightfield imaging, with corresponding speed (red line) and vertical position (black line) plotted over time. The microrobot is actuated for 1 second, rising $50\ \mu\text{m}$ above the well plate bottom at a peak speed of $25\ \mu\text{m/s}$, followed by sedimentation over the subsequent 30 seconds. Scale bar: $30\ \mu\text{m}$. b) Rolling motion visualized through brightfield images, with rotational displacement (red line) and linear travel distance (black line) of a single TACSI microrobot plotted during 1 minute of actuation at $0.33\ \text{Hz}$ laser frequency. Scale bar: $30\ \mu\text{m}$. c) Schematic depiction of the pulling mode, accompanied by brightfield images and quantitative speed analysis (red line) of multiple microrobots relative to their initial positions (black line). A single TACSI microrobot, actuated at $0.33\ \text{Hz}$ for 80 seconds, pulls four neighboring microrobots toward itself. Scale bar: $30\ \mu\text{m}$.

To induce lateral movement, the laser was asymmetrically focused on the side of the microrobot, generating directional rolling. Under pulsed laser stimulation ($0.33\ \text{Hz}$ at $70\ \text{mA}$), microrobots rotated up to $40 \pm 10^\circ$ per stimulation step, with a mean displacement of $7 \pm 2\ \mu\text{m}$ per step (Figure 5.6 b). Theoretical rolling distances based on the $30\ \mu\text{m}$ microrobot diameter would reach $10.5\ \mu\text{m}$ per full rotation; however, actual displacement was reduced by approximately 33 % due to the partial slippage. We hypothesize that the discrepancy between theoretical and observed displacement arises from a combination of factors, including transient lifting, insufficient surface adhesion, fluid drag, and local flow variations—all of which may disrupt the continuous surface contact required for efficient rolling. In addition to self-locomotion, we observed the ability to pull nearby microgels via convection fields. A stationary microrobot pulsed at $0.33\ \text{Hz}$ successfully displaced four neighboring microgels, with a peak pulling velocity of $4.1\ \mu\text{m/s}$, demonstrating indirect actuation capabilities (Figure 5.6 c).

To demonstrate the ability to manipulate live cells, μTACSI microrobots were co-incubated with HT-1080 fibrosarcoma cells in 96-well plates at a 1:1 ratio (Figure 5.7 a). In a representative experiment, a microrobot was randomly positioned $110\ \mu\text{m}$ from a target cell. Using sequential lifting and rolling maneuvers, the microrobot reached the cell within 90 seconds (Figure 5.7 b). Upon con-

tact, electrostatic adhesion occurred within one minute, resulting in stable cell capture (cell #1). To acquire additional cargo, the microrobot was rotated 180° and guided toward two additional cells. Each adhered within 1 minute, leading to the successful accumulation of three cells within 180 seconds. The presence of bound cells altered the microrobot's flow dynamics, stabilizing its motion during transport. Control over microrobot motion remained precise throughout the procedure. To evaluate release behavior, cell-laden microrobots were incubated for 14 hours under standard culture conditions. During this period, adherent cells gradually detached and reattached to the well plate substrate, confirming post-transfer viability and spontaneous release. In extended trials, up to six cells were successfully loaded onto a single microrobot. However, pairing two μ TACSI microrobots—each carrying multiple cells—exceeded the available propulsion force at the tested laser power and frequency, preventing collective locomotion of larger assemblies.

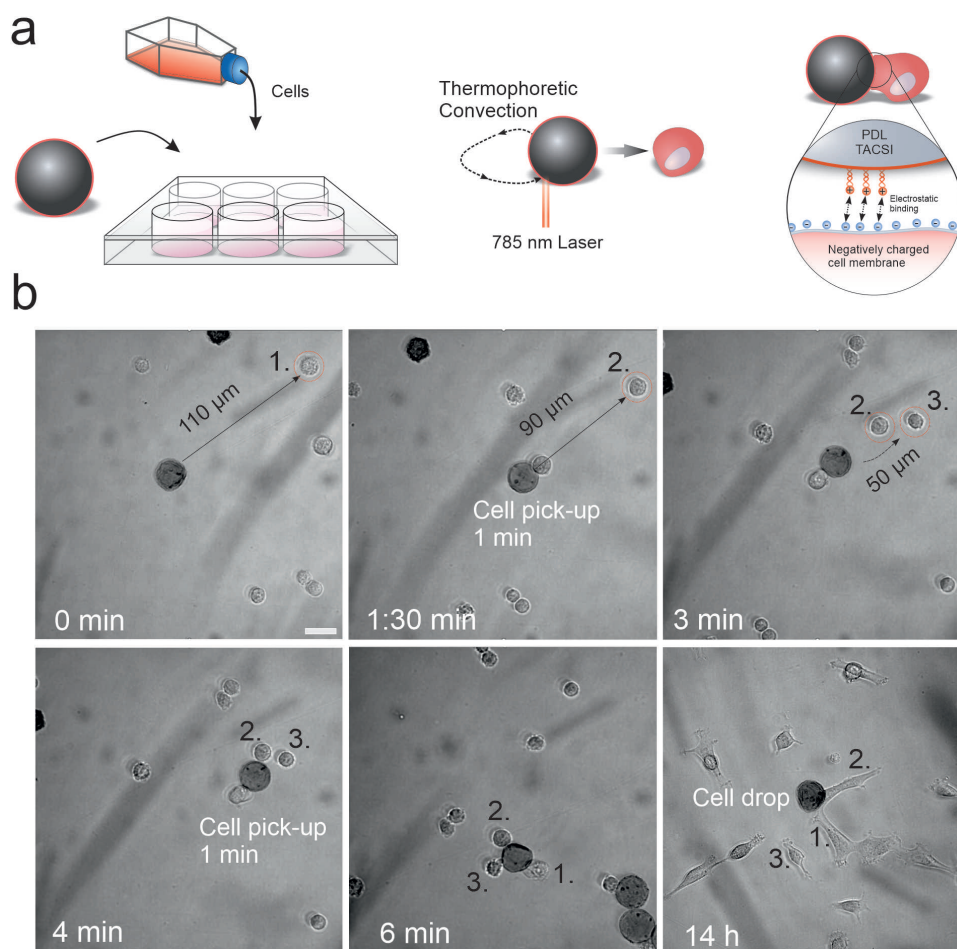


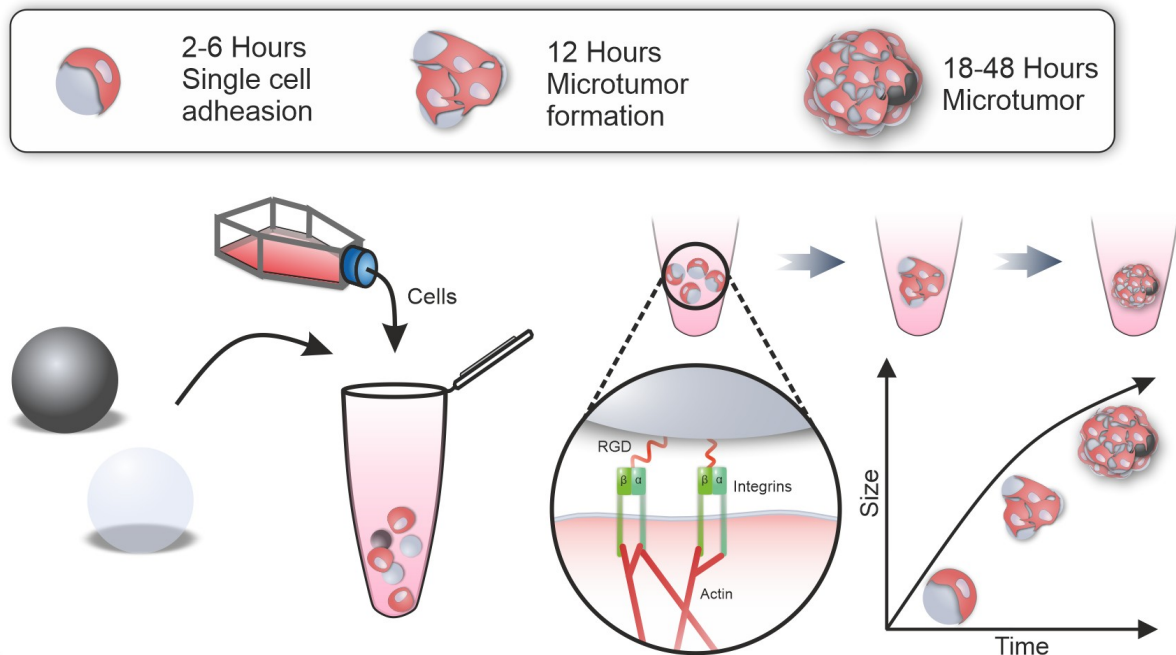
Figure 5.7 Thermophoretic Guidance and Electrostatic Cell Pick-Up by μ TACSI Microrobots. a) Schematic illustration of the single-cell pick-up mechanism. Cells and μ TACSI microrobots are co-incubated in a well plate, where thermophoretic convection enables directed movement of the microrobot toward a target cell. Electrostatic interaction between the negatively charged cell membrane and the positively charged PLL-coated microrobot surface facilitates immediate cell attachment. b) Representative brightfield time-lapse sequence showing a μ TACSI microrobot approaching and sequentially picking up three individual cells. Following the attachment of cell #1, the microrobot is guided toward cells #2 and #3. After confirming adhesion through rotation, passive release of the adhered cells occurs over a 14-hour period. Scale bar: 30 μ m.

5.2.3 High-Throughput Assembly of Microrobot-Integrated Spheroids

While laser-guided single-cell manipulation allows precise assembly of multicellular systems, it is inherently low throughput. To complement this, we developed a parallelizable, one-pot self-assembly strategy for fabricating. Three-dimensional cancer spheroids are widely recognized as more physiologically relevant models for drug screening than traditional two-dimensional cultures, as they better mimic *in vivo* tissue features such as cell–cell contact, matrix embedding, and diffusion gradients [280, 37, 281].

To initiate spheroid formation, fibrosarcoma HT-1080 cells were mixed with RGD-modified scaffold microgels (alginate with a degree of substitution of 20) and TACSI microrobots in standard microcentrifuge tubes (Figure 5.8 a). The RGD peptide supported integrin-mediated adhesion [282, 283], enabling spontaneous clustering and aggregate formation over time. No centrifugation or agitation was applied; assembly occurred through passive sedimentation and cell–matrix interaction.

a



b

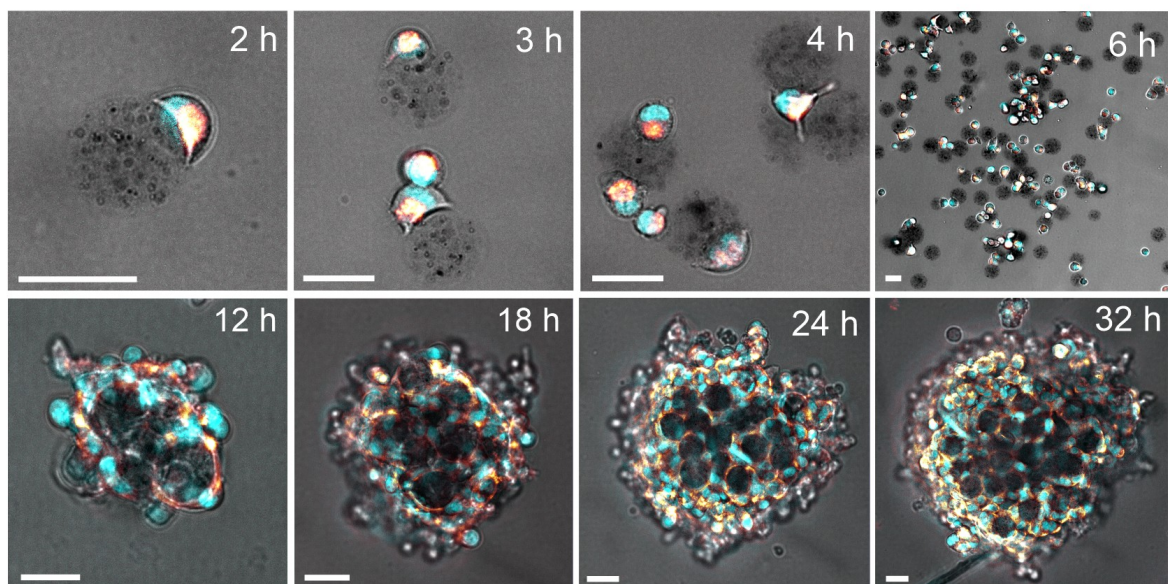


Figure 5.8 Microrobot-Assisted Self-Assembly of Cell-Microgel Spheroids: Process and Imaging. a) Schematic representation of the self-assembly process using RGD-functionalized scaffold microgels and RGD-modified TACSI microrobots. Depending on incubation time, the system enables either single-cell adhesion to individual microrobots and the formation of multicellular spheroids. b) Merged fluorescence and brightfield images of cell-microrobot assemblies at various time points. Nuclei (cyan) and actin filaments (red) are stained to visualize single cells and spheroid structures. After 2 hours, individual cells adhere to single microgels; by 12 hours, early 3D agglomerates form, followed by spheroid maturation and circularization. Scale bars: 30 μm .

Initial single-cell adhesion to microgels was detected within two hours of incubation. Fluorescence z-stacks revealed average adherence depths of $6.9 \pm 3.2 \mu\text{m}$ (Figure 5.8 b). However, RGD-integrin binding required at least 45 minutes of contact time to form stable interactions, rendering guided microrobot–cell pick-up infeasible through this mechanism alone. Over the course of 6 to 12 hours, we observed progressive clustering of cells and hydrogel particles into well-defined 3D aggregates. After 12 hours, mature spheroids with TACSI microrobots embedded near the center became apparent. Prolonged incubation further enhanced spheroid compactness and symmetry. To determine optimal conditions for spheroid assembly, we systematically varied the ratio of microgels to cells. Circularity analysis showed that spheroids required at least 20 microgels and 400 cells to form compact, symmetric structures. Below this threshold, aggregates tended to be elongated and unstable. We further tested whether mixing scaffold microgels and TACSI microrobots could improve optical properties in 3D constructs (Figure 5.9). Using a 75:25 ratio of scaffold microgels to TACSI microrobots, we achieved a balance between imaging clarity and actuation functionality. Fluorescence imaging confirmed that scaffold microgels reduced light scattering, while TACSI microrobots remained clearly visible due to their intrinsic opacity.

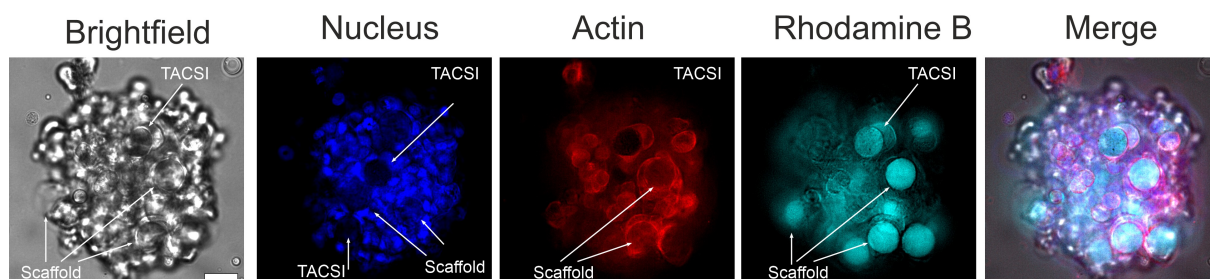


Figure 5.9 Representative brightfield and fluorescence images of a self-assembled spheroid containing TACSI microrobots. Cell nuclei (blue) and actin cytoskeleton (red) are visualized alongside Rhodamine B-labeled scaffold microgels and TACSI microrobots (cyan). Arrows highlight the locations of embedded microgels and microrobots within the spheroid structure. Scale bar: $30 \mu\text{m}$.

Interestingly, during the course of spheroid formation, a consistent reduction in microrobot diameter was observed (Figure 5.10 a). On average, TACSI microrobots shrank by $4 \pm 2.5 \mu\text{m}$ —over 10 % of their original $29 \mu\text{m}$ diameter. To determine whether this shrinkage resulted from enzymatic degradation and material breakdown, spheroids were disaggregated using trypsin buffered with calcium ions. Post-treatment, the microrobots regained their original size, indicating that the shrinkage was likely osmotic in nature, driven by local cellular stress and crowding rather than irreversible structural loss. Additionally, spheroid morphology analysis (Figure 5.10 b) revealed that circularity improved with increasing numbers of microgels and associated cells per spheroid, supporting the notion that mechanical compaction and cellular organization contribute to both structural maturation and local environmental changes affecting the microrobots [268, 284].

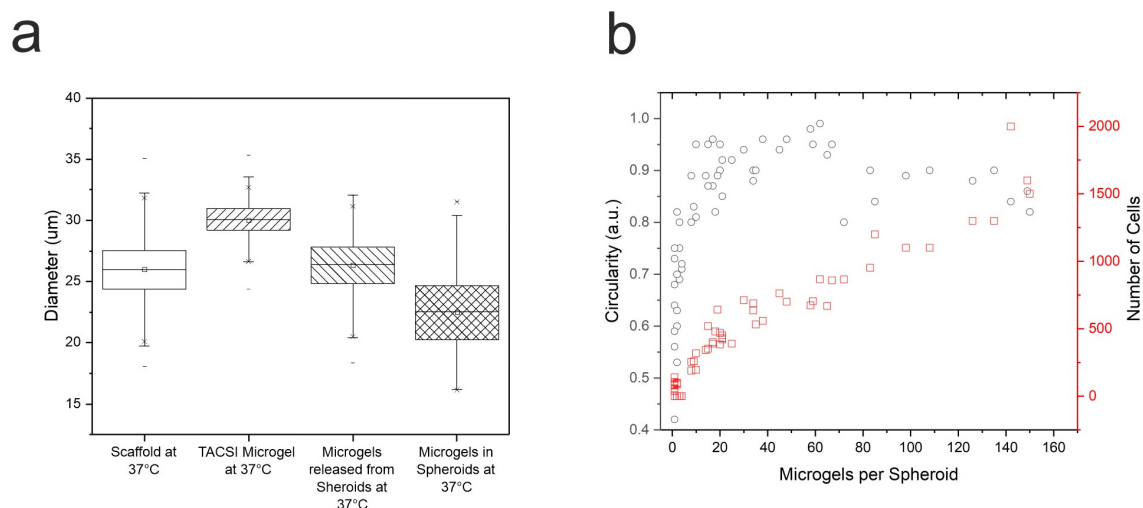


Figure 5.10 Morphometric Analysis of Microrobotic Spheroids. a) Diameter measurements at 37 °C for scaffold microgels, TACSI microrobots, microgels released from spheroids, and microgels within spheroids ($n > 3000$). b) Circularity analysis of ($n = 50$) spheroids (black circles) plotted against the number of microgels per spheroid, with corresponding cell counts per spheroid shown as red squares.

To assess biocompatibility, we performed live/dead assays on spheroids containing different combinations of scaffold microgels and TACSI microrobots (Figure 5.11). Cell viability remained above 95% across all conditions after 72 hours of incubation. No cytotoxic effects or morphological abnormalities were detected in TACSI-only, scaffold-only, and mixed spheroid populations. Finally, to demonstrate the platform's versatility, we repeated the spheroid self-assembly process using D1 mesenchymal stem cells (MSCs) (Figure A.9). These cells produced similarly structured spheroids, confirming that the workflow is applicable to multiple cell types and not limited to cancer models.

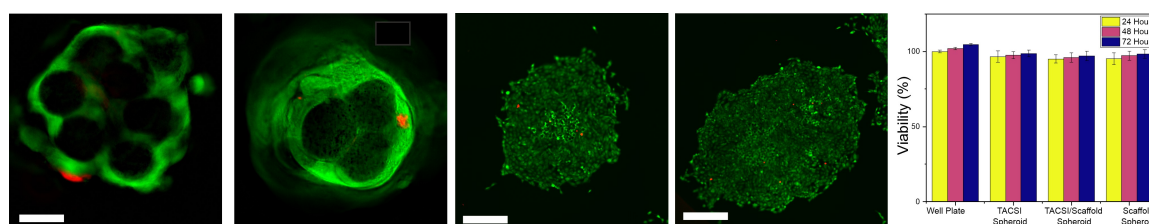


Figure 5.11 Representative fluorescence images show self-assembled spheroids composed of live (green) and dead (red) cells after 12 hours of incubation, including cells that have migrated out onto the well plate surface. Images are shown at both low and high magnification Scale bars: 30 and 300 μm. Quantitative viability data are presented for cells cultured under four conditions—on the well plate bottom, in TACSI spheroids, in hybrid TACSI/scaffold spheroids, and in scaffold-only spheroids—over 24, 48, and 72 hours ($n = 5$ per condition).

5.2.4 Laser-Guided Locomotion of 3D Spheroids

While various strategies for spheroid positioning have been explored, ranging from hydrodynamic trapping and magnetic levitation to acoustic and mechanical micromanipulation, none, to our knowledge, have demonstrated the use of light as a sole external input to drive contactless motion of spheroids. According to a recent comprehensive review of microtissue manipulation technologies

[285], existing techniques often involve direct physical contact (e.g., pipetting, aspiration-assisted bioprinting), tailored device geometries (e.g., microfluidic traps), and substrate-bound confinement, which limit spatiotemporal flexibility and real-time programmability. In contrast, the TACSI microrobot platform offers a unique, light-driven solution for actuating spheroids *in vitro*, expanding laser guidance beyond single particles to more complex, biologically relevant 3D assemblies.

Three core locomotion strategies were established for spheroid manipulation: lifting, pulling, and rolling. In the lifting mode, laser illumination induces localized convection that propels the spheroid vertically (Figure 5.12 a). Upward motion is achieved within seconds, with displacement heights dependent on laser power, exposure time, and spheroid size. This technique allows detachment from the substrate and repositioning within 3D space. In the pulling mode, a spheroid containing μ TACSI microrobots is selectively illuminated to generate horizontal convection currents (Figure 5.12 b). These flow fields are sufficient to attract nearby passive spheroids toward the activated one. Experimental observations confirmed translational movement of 800 μ m in under 60 seconds, demonstrating long-range interaction mediated solely by fluid dynamics. The third approach, rolling, relies on asymmetric laser heating to generate directed convection around an off-centered spheroid (Figure 5.12 c). This creates a net radial force that propels the spheroid laterally across the substrate. Rolling displacements of over 900 μ m were achieved within 50 seconds, enabling precise and efficient movement toward target regions and other spheroids.

Together, these modes constitute a flexible, fully optical toolbox for spheroid manipulation, offering degrees of freedom and temporal precision not achievable by current mechanical or field-based methods [285]. By leveraging localized photothermal effects through embedded TACSI microrobots, our system achieves remote-controlled, reversible motion in 3D without the need for mechanical contact, complex device integration, and medium modification. This unique approach opens new possibilities for high-resolution tissue assembly, real-time interaction studies, and the design of dynamic organoid-on-chip platforms.

5.2.5 Microrobotic Thermal Actuation and Sensing in 3D Spheroids

Controlled thermal modulation within three-dimensional cell constructs offers a powerful tool for probing heat-sensitive cellular pathways and intercellular communication. To use this capability, we first established a robust fluorescence-based calibration for temperature sensing by adapting the microthermometry method established in Chapter 3, followed by spatial mapping of thermal gradients within spheroids, and finally characterized calcium-dependent cell responses to localized heating.

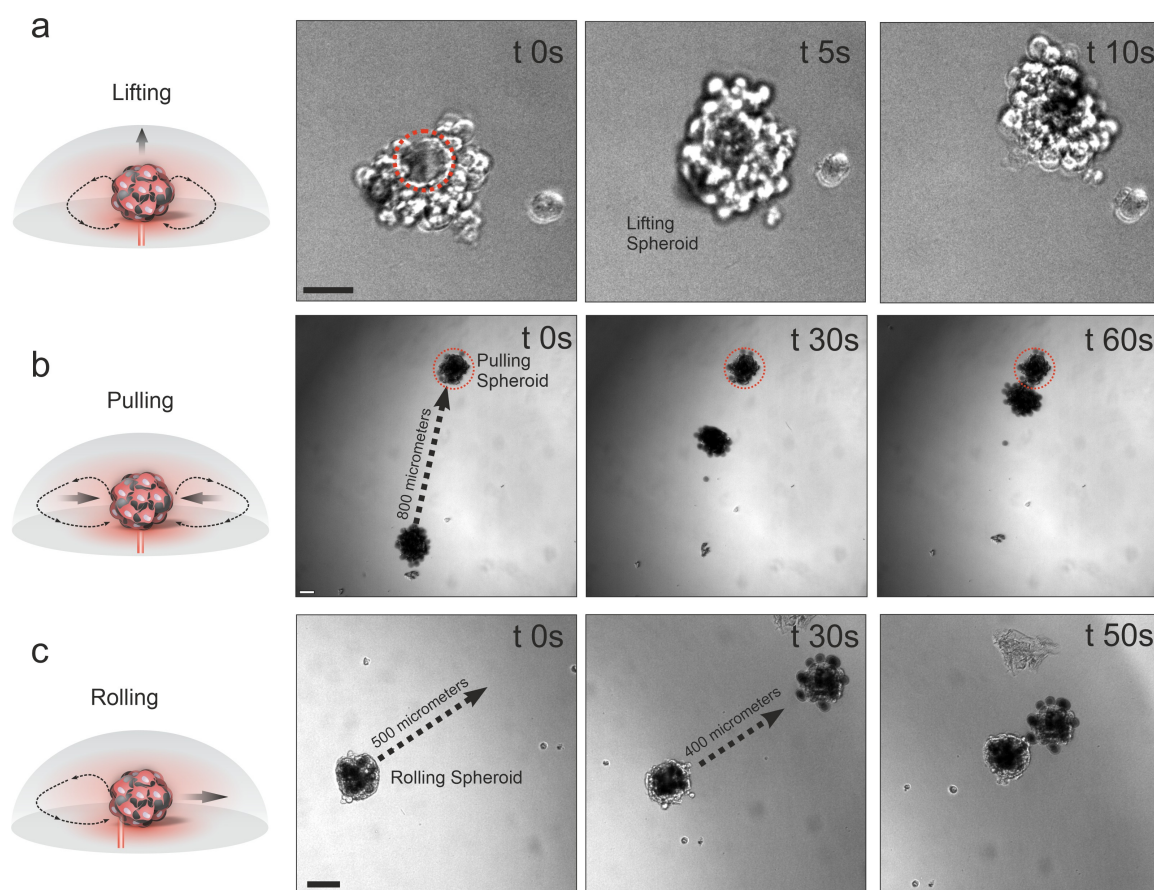


Figure 5.12 Microrobotic locomotion strategies for spheroid manipulation using TACSI microrobots. Three distinct modes of laser-actuated spheroid manipulation are demonstrated using thermally responsive TACSI microrobots. a) In the lifting mode, vertical displacement is achieved as a spheroid is raised from the substrate by a microrobot through laser-induced convection. b) In the pulling mode, a spheroid outlined in red is activated with near-infrared light, generating convective flow that draws a neighboring spheroid over a distance of 800 μm within 60 seconds. c) In the rolling mode, asymmetrical thermal convection propels a spheroid laterally by more than 900 μm in 50 seconds, positioning it adjacent to another spheroid. Scale bars: 100 μm .

To quantify the thermal output generated by photothermal stimulation of TACSI microrobots, we calibrated their fluorescence response to temperature changes using RhB as a temperature-sensitive reporter. Microrobots and scaffold microgels containing identical RhB-to-alginate ratios were placed in a temperature-controlled Petri dish heater equipped with an integrated thermometer. Fluorescence measurements were recorded across controlled thermal steps to derive a calibration curve (Figure 5.13 a–b). Unlike the initial calibration described in Chapter 3, this version uses a modified hydrogel formulation consisting of two distinct alginate types (RGD and RhB). Due to differences in matrix composition and resulting thermal and optical properties, a new calibration was required to ensure accurate temperature readout for the updated microrobot system. Both TACSI microrobots and scaffold microgels exhibited identical fluorescence behavior due to equivalent dye loading, validating their use as interchangeable microthermometers. Normalizing the RhB signal to a baseline of 37 $^{\circ}\text{C}$, we observed a linear decrease in fluorescence intensity with increasing laser-induced temperature ($R^2 = 0.98$). This calibration was used to quantify temperature outputs across a series of 20

microrobots inside assembled spheroids under increasing laser currents (50–145 mA). We used a consistent increase of approximately 1.5 °C per 5 mA increment. This allowed us to measure and induce thermal stimulation in 3D cellular environments. At maximum current (145 mA), the microrobot surface temperature reached 78 °C, demonstrating the ability to span mild (<42 °C), hyperthermic (42–48 °C), and noxious (>48 °C) temperature regimes (Figure 5.13 c).

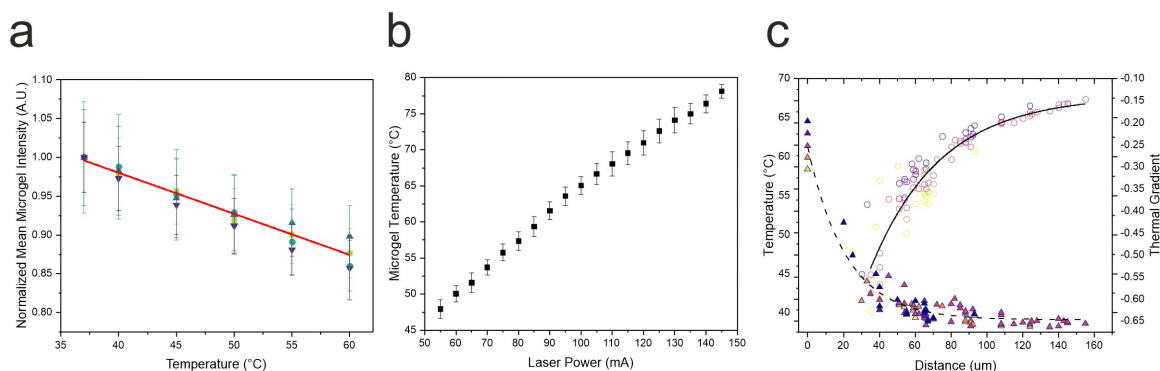


Figure 5.13 a) Normalized thermal calibration of scaffold microgels (circles and upward triangles) and TACSI microrobots (squares and downward triangles), showing a linear fit to the measured data (red solid line, $R^2 = 0.98$). All values are normalized to 37 °C; $n = 20$ per condition and temperature step. b) Core temperatures of microrobots at different NIR laser powers, ranging from 47.5 °C to 78 °C; $n = 20$ per laser power. c) Three-dimensional temperature measurements of individual microrobots and microgels (triangles and fitted dashed line, $R^2 = 0.96$), and corresponding thermal gradient profiles (circles and fitted solid line, $R^2 = 0.96$), recorded during NIR laser activation of TACSI microrobots within microtumors. Initial temperatures were 59.9 °C (yellow), 61.5 °C (orange), 62.5 °C (red), 64 °C (violet), and 65.1 °C (blue).

5.2.6 3D Thermal Mapping and Thermally Triggered Calcium Signaling in Live Cells

To investigate spatial heat propagation within 3D tissues, we embedded both scaffold microgels and TACSI microrobots into spheroids as previously described. Heating a single TACSI microrobot allowed real-time fluorescence-based temperature mapping through adjacent scaffold microgels. Controlled heating cycles were performed at setpoint temperatures ranging from 59.9 °C to 65.1 °C. Thermal decay profiles showed a steep exponential drop in temperature over distance. At 20 μm from the microrobot surface, local temperature had already decreased by approximately 15 °C, aligning well with theoretical diffusion models and prior finite element simulations. The thermal field dissipated smoothly across the spheroid, creating a radially symmetric gradient (Figure 5.13 c). To examine biological responses to localized heat, we used intracellular calcium signaling as a rapid and sensitive readout given the extensive literature on thermally activated ion channels in mammalian cells [286]. Since our goal was to establish a system capable of detecting cellular responses to microrobotic stimulation in real time, we focused on ion channel activity—specifically thermally gated calcium channels—as an ideal candidate for immediate, non-destructive feedback. For this purpose, spheroids containing HT-1080 cells were stained with Calbryte 520 AM, a live-cell calcium indicator chosen for its ease of use, strong signal intensity, and reliable fluorescence readout under

physiological conditions (see Chapter 3). Thermal stimulation was delivered to a single embedded TACSI microrobot for 5 seconds, raising nearby cellular temperatures to 48.9 °C (Figure 5.14 b). Immediately after heating, a 2.4-fold increase in intracellular calcium was observed in adjacent cells, likely mediated by activation of transient receptor potential vanilloid (TRPV) channels. Calcium levels returned to baseline within 10 seconds, suggesting reversible activation of thermosensitive ion channels. To more comprehensively assess threshold-dependent responses, this experiment was repeated under 14 thermal conditions ranging from 39.7 to 59.7 °C. Significant response thresholds were identified at 45.8 °C and 52 °C (Figure 5.14 c). The lower threshold near 45.8 °C corresponds to activation of TRPV3 and TRPV4 channels, which are known to respond to moderate heat stimuli. The higher threshold around 52 °C is consistent with activation of TRPV1, a channel associated with noxious heat. Below 45.8 °C, fluorescence increases were modest (2.0-fold), whereas temperatures above 52 °C induced more pronounced calcium uptake (4.0–13.0-fold increases). At extreme temperatures (>54 °C), calcium signaling exhibited periodic spatial propagation between adjacent cells, suggesting heat-induced paracrine signaling and stress-induced communication (Figure A.11) [287].

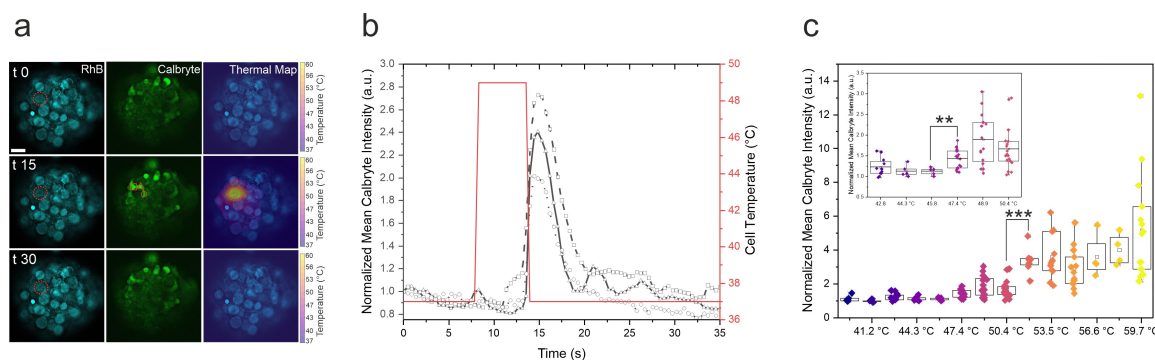


Figure 5.14 Intracellular Calcium Response to Localized Photothermal Stimulation. a) Time-lapse fluorescence images showing RhB-modified TACSI microrobots (red channel) and intracellular calcium-stained cells (green channel) over a 30-second interval during localized photothermal stimulation. The red dashed line marks the location of the heated microrobots. Scale bar: 30 μm . b) Normalized intracellular calcium fluorescence intensity in three representative activated cells (triangle, square, and circle) following a 5-second NIR laser pulse, resulting in a consistent cell temperature of 48.9 °C. c) Normalized intracellular calcium fluorescence signals recorded in microtumors under controlled temperature increments ($\Delta T = 1.5^\circ\text{C}$). Inset highlights data from 42.8 °C to 50.4 °C, with a significant increase in signal observed at 47.4 °C ($n = 5$ per measurement; $**P < 0.01$, $***P < 0.001$, Student's t -test).

Calcium recovery times were strongly dependent on the thermal load. At mild temperatures (<47.4 °C), signals normalized within 10.5 seconds (Figure 5.15). Between 47.4 and 51.9 °C, a slight acceleration in recovery was observed. However, above 52 °C, recovery durations increased rapidly, reaching 50 seconds at 54 °C and a maximum of 200 seconds at 59.7 °C. These prolonged recovery phases likely reflect a shift from channel-mediated influx to passive ion diffusion due to membrane alterations.

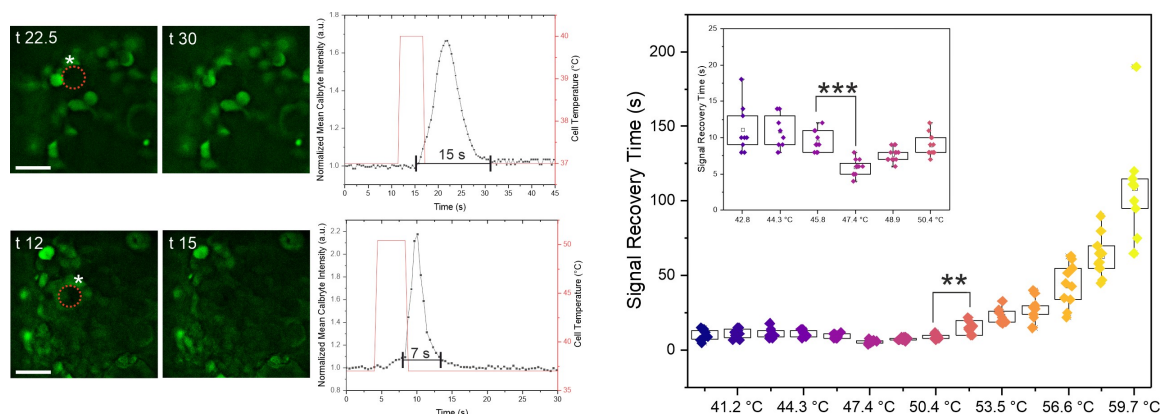


Figure 5.15 Temperature-Dependent Recovery Dynamics of Intracellular Calcium in Fibrosarcoma Cells. Representative fluorescence images of HT-1080 fibrosarcoma cells stained with Calbryte 520 AM under varying photothermal heating conditions. At lower temperatures, intracellular calcium fluorescence returns to baseline within 15 seconds, whereas higher temperatures lead to more rapid recovery. At temperatures exceeding 55 °C, signal diffusion time is prolonged up to 60 seconds. Scale bars: 50 μ m. Quantification of calcium signal recovery time in activated cells across increasing temperatures in microtumors, with 1.5 °C step increments. Inset highlights the range from 42.8 °C to 50.4 °C, showing a significant shift in recovery dynamics at 47.4 °C ($n = 5$ per measurement; $**P < 0.01$, $***P < 0.001$, Student's t -test).

Together, these findings demonstrate that TACSI microrobots can deliver tightly controlled, localized thermal stimuli within 3D constructs, induce graded calcium responses, and reveal dynamic cellular recovery behavior under heat stress. This platform opens the door for fine-scale studies of heat-regulated signaling and adaptive cellular behavior in engineered tissues.

5.2.7 Mechanical Actuation and Strain-Induced Stimulation in 2D and 3D Constructs

In native tissues, cells continuously experience and respond to compressive, tensile, and shear stresses from their environment [181]. These mechanical signals influence proliferation, migration, differentiation, and stress responses—especially in stiffened and mechanically heterogeneous disease models such as cancer [288]. Thus, to create a more physiologically relevant microrobotic platform, it is essential to integrate not only thermal actuation but also on-demand mechanical stimulation within 3D constructs.

To understand the passive mechanical behavior of TACSI microrobots and alginate hydrogel scaffolds, we first performed nanoindentation (see Chapter 4) using a calibrated nanoindenter equipped with a 10 μ m spherical tip (R_{Tip}) (Figure 5.16 a). Microgels were immobilized via pre-coating the

measurement substrate with 0.1 mg/mL Poly-L-Lysine, which prevented lateral drift during indentation and ensured consistent force application. Indentation to a depth of 560 nm revealed distinct stiffness differences between scaffold microgels and TACSI microrobots (Figure 5.16 b). The scaffold microgels exhibited a Young's modulus of 651 ± 66 Pa, while TACSI microrobots were slightly stiffer, with a modulus of 795 ± 56 Pa ($n > 25$ per group), both measured in cell culture media. This increase reflects the mechanical contribution of embedded gold nanorods and modified alginate structure, which together offer a more robust response to applied force.

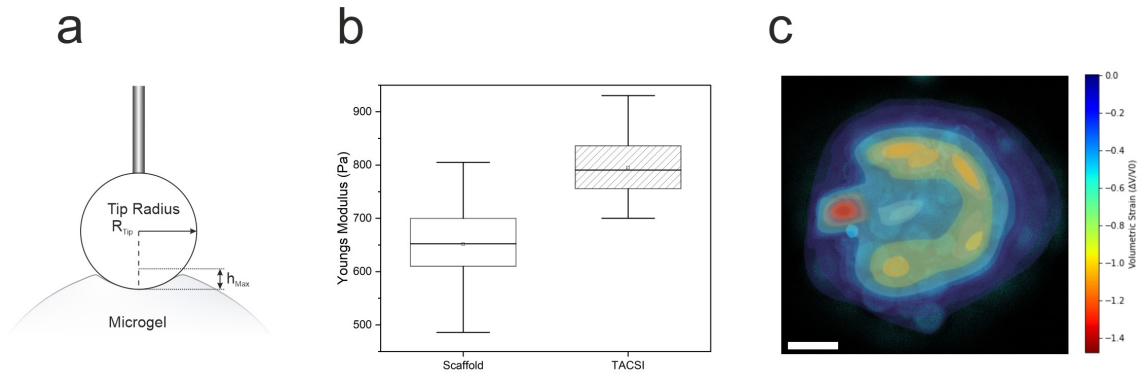


Figure 5.16 Mechanical Characterization of Alginate-Based Microgels and TACSI Microrobots. a) Schematic of the nanoindentation setup used to probe the mechanical properties of alginate scaffold microgels and TACSI microrobots, illustrating the nanoindenter tip radius (R_{tip}) and maximum indentation depth (h_{max}). b) Young's modulus (E) values obtained from nanoindentation measurements in complete cell culture media for both scaffold microgels and TACSI microrobots ($n = 50$ per condition). c) Volumetric strain map overlaid on a fluorescence image of a microtumor, visualizing deformation patterns in the scaffold and microrobot region based on Rhodamine B intensity. Scale bar: $60 \mu\text{m}$.

We then examined the extent to which microrobots deform passively when integrated into 3D tumor spheroids. Soft microgels with stiffness values ranging from 150 to 2000 Pa have previously been employed as pressure sensors within spheroids to measure internal mechanical stresses. In prior studies, alginate microgels with a stiffness of approximately 150 Pa were shown to detect endogenous stresses in the range of 10–50 Pa, while polyacrylamide beads with a stiffness around 2000 Pa were used to quantify compressive stresses up to 1.5k Pa under externally applied osmotic loading [289, 290]. We expected the TACSI microrobots to provide a similar functionality considering their Young's modulus. To assess this, the volumetric strain of the microrobot was calculated by measuring microrobot diameters before and after embedding into spheroids. Compressive stress was then estimated using generalized Hooke's law, assuming the microrobot behaves as a linear elastic material within the small-strain regime (valid up to approximately 10 % strain) [291]. The stress σ was calculated as:

$$\sigma = \frac{E}{1 - 2\nu} \cdot \epsilon_r \quad (5.1)$$

where E is the Young's modulus of the microrobot, ν is the Poisson's ratio, and ε_r is the radial strain. As expected, significant compression was observed after 24 hours of incubation, with a maximum volumetric strain of $-1.5 \Delta V/V_0$, corresponding to a mean compressive force of $1.56 \pm 0.89 \mu\text{N}$ (Figure 5.16 c). The estimated compressive stress of approximately 125 Pa falls within the lower range of previously reported values for microgel-based stress sensors in spheroids, which have detected endogenous stresses as low as 10–50 Pa and up to 1.5 kPa under external loading. These results confirmed that microgels experience biologically relevant mechanical loading when incorporated into cellular aggregates. This ability to sense and report cellular osmotic stress and traction forces may be leveraged in future designs to create mechano-responsive constructs that reveal the internal stress fields generated by proliferating and migrating cells.

In addition to acting as mechanical sensors capable of reporting strain within cell aggregates, we investigated whether microrobots could also actively deform under optical stimulation *in situ*. For this purpose, a thermoresponsive copolymer, poly(NIPAM-co-NIPAM), was introduced into TACSI microrobots. Our group has previously developed optically triggered nanorobotic microgels composed of gold nanorods and thermoresponsive polymers embedded in alginate, demonstrating reversible, light-induced deformation and force generation in the single-nanonewton range under near-infrared stimulation (see Chapter 2). These systems have been used both to characterize actuation mechanics at multiple scales and to apply controlled compressive strain to single cells in 3D, enabling the study of mechanobiological responses such as calcium signaling in mesenchymal stem cells [292, 216]. Actuating TACSI microrobots were fabricated by incorporating 4 mg of poly(NIPAM-co-NIPAM) copolymer nanoparticles—synthesized by Chen Wang via free radical polymerization and designed to exhibit a lower critical solution temperature (LCST) of 39 °C—into a 2% functionalized RGD-alginate matrix containing 10 mg/mL gold nanorods. Both components were homogeneously dispersed using tip sonication at 60% amplitude for 15 seconds. Unlike prior strategies that formed nanorobotic constructs by encapsulating gold nanorods within pre-synthesized PNIPAM structures [216, 292], our approach enabled the direct co-dispersion of PNIPAM monomers and gold nanorods into the alginate precursor solution prior to microgel formation. This one-step process simplified fabrication and ensured uniform distribution of thermoresponsive and photothermal components within the microgel matrix. This approach enabled highly consistent and reversible actuation behavior across individual microrobots. Functional performance was evaluated through localized photothermal stimulation using a NIR laser. As shown in Figure 5.17, a single microgel subjected to NIR irradiation exhibited a rapid and reversible volume change. Upon activation, the embedded gold nanorods efficiently converted light into heat, raising the local temperature to the measured 39°C—sufficient to induce the LCST phase transition of PNIPAM. This triggered a pro-

nounced shrinkage of the microgel, resulting in a 20% reduction in radius within 100 ms. Once the laser was turned off, the microgel re-swelled to its original size within 1 second, demonstrating fully reversible actuation. Importantly, neighboring microgels located only 20 μm away showed no visible deformation, highlighting the high spatial precision and confinement of the thermally induced response. This localized control is essential for single-cell applications where targeted stimulation without off-target effects is critical.

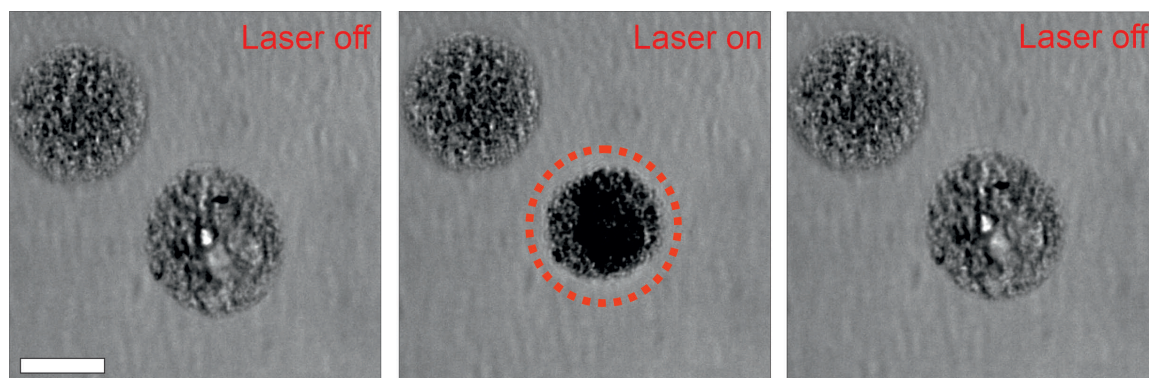


Figure 5.17 Photothermal actuation of a single PNIPAM–alginate microgel containing gold nanorods. Images show reversible shrinkage (20% radius reduction) upon NIR laser irradiation (39°C), followed by re-swelling after laser deactivation. The red outline shows the original microrobot's dimensions. Scale bar: 15 μm .

Before applying these actuating TACSI microrobots in 3D cultures, we evaluated their mechanobiological effects in 2D (Figure 5.18). HT-1080 fibrosarcoma cells were cultured in monolayers and exposed to single-microrobot stimulation for 1 hour using cyclic actuation at 0.33 Hz. Across three independent experiments, significant nuclear translocation of the mechanosensitive transcription factor, YAP, was observed. The mean nuclear to cytoplasmic YAP ratio determined by the relative fluorescence intensities increased from 1.1 in unstimulated controls to 2.25 following actuation, indicating an acute cellular response to the generated mechanical force. It is important to mention that the TACSI microrobots contained RGD-peptides at 20 degree of substitution (DS) in order to ensure mechanical coupling between the devices and the cells.

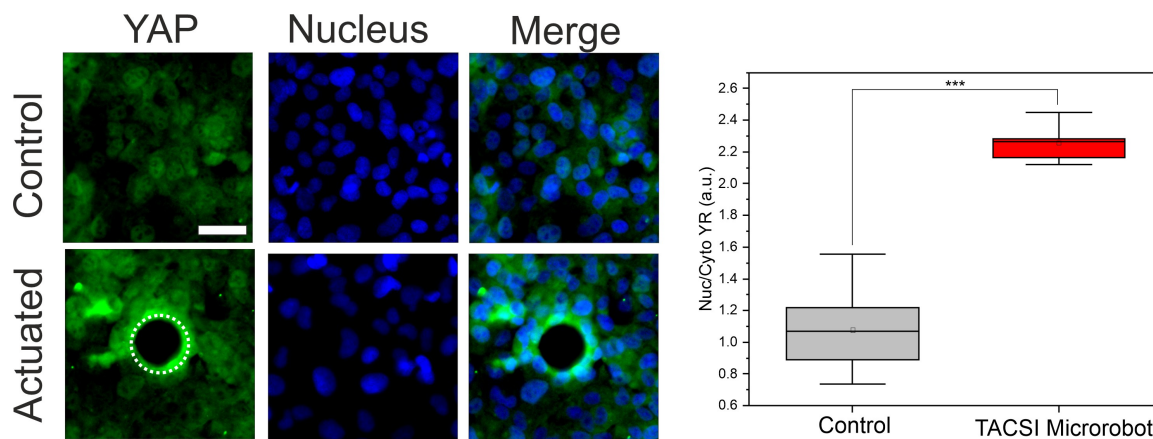


Figure 5.18 Optical actuation of TACSI microrobots induces YAP nuclear translocation in 2D-cultured HT-1080 cells. Representative fluorescence images show YAP (green), nuclei (blue, nucleus), and merged channels for control (unstimulated) and optically actuated conditions. Scale bar: 30 μm . Quantification of the nuclear YAP intensity ratio reveals a significant increase in nuclear localization following 1-hour cyclic actuation at 0.5 Hz using single microrobots. Data are presented as mean $\pm$ SD; *** $P < 0.001$, two-sample t -test, $n = 3$ independent experiments.

Encouraged by these findings, we transitioned to a 3D context by embedding actuating TACSI microrobots within HT-1080-based microtumors, stained with live-cell actin dyes to visualize cytoskeletal responses. Single microrobots were cyclically actuated at 0.5 Hz using laser stimulation. This induced rhythmic, localized deformation of the surrounding matrix and cells (Figure 5.19 a). On average, we recorded radial reduction of 10.7 ± 5.3 %, volumetric compression of 28.2 ± 12 %, and full relaxation to baseline size within 3 ± 2 seconds in the spheroids. These values were consistent across seven identically treated microtumors and demonstrated that a single optically stimulated microrobot could impart measurable, localized compression within a multicellular environment. To investigate mechanotransduction in 3D, spheroids containing thermoresponsive microrobots were embedded in collagen and stimulated using a near-infrared laser targeting a single actuating microgel (Figure 5.19 b). A single microrobot generated a total compressive force of $(1.29 \pm 0.3 \mu\text{N})$ upon activation. Although multiple actuator-containing microgels were embedded within each spheroid, only one was directly illuminated; neighboring microrobots experienced partial heating from thermal diffusion and were estimated to reach approximately one-tenth of the original force output. Immunostaining for YAP and F-actin, performed 24 hours after stimulation, revealed no statistically significant nuclear translocation of YAP compared to unstimulated controls. This differs from the behavior observed in 2D cultures and is likely due to the highly localized nature of actuation; full-spheroid mechanical stimulation may be required to elicit coordinated mechanotransductive responses in 3D environments.

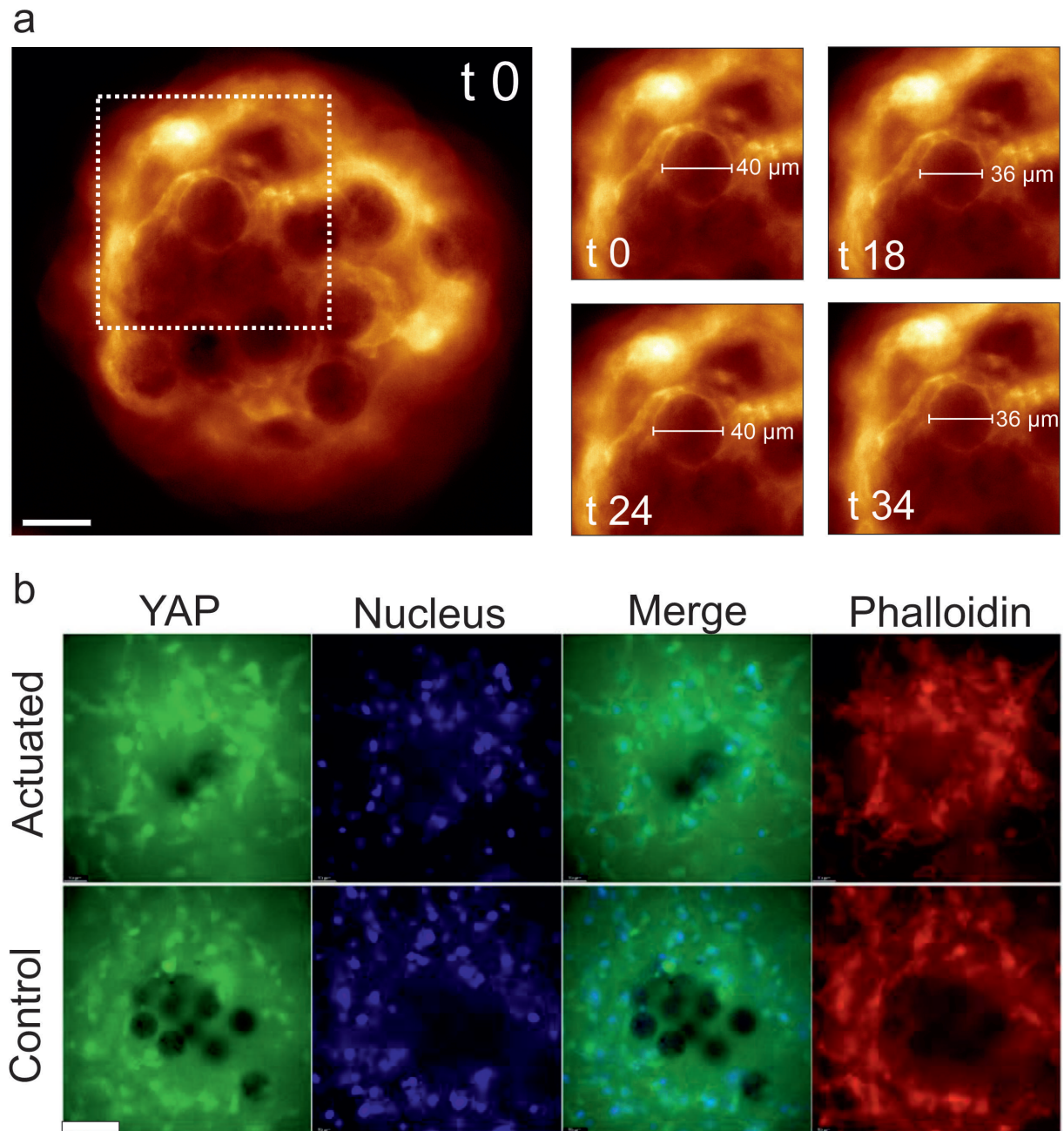


Figure 5.19 Laser-driven mechanical actuation of TACSI microrobots in 3D microtumors and YAP immunofluorescence analysis. a) Fluorescence image of F-actin in a representative microtumor undergoing mechanical stimulation by embedded TACSI microrobots. Laser actuation at 40°C induces approximately 10% radial strain. Time-lapse frames capture both deformation and relaxation dynamics over a period of 42 seconds, with two actuation events occurring at $t = 18$ s and $t = 34$ s. Scale bar: 40 μm . b) Immunofluorescence analysis of YAP and F-actin in HT-1080 spheroids following mechanical stimulation by embedded microrobots. Shown are representative images of YAP (green), nucleus (blue), merged YAP/nucleus, and Phalloidin (red) for both actuated and control conditions. Actuated: spheroid embedded in Collagen I and stimulated for 1 hour and 17 minutes (90 mA, 1 s on / 2 s off pulsed laser), then fixed 24 hours after stimulation. Control: non-stimulated spheroid cultured under identical conditions and fixed after 24 hours. Scale bar: 60 μm .

5.2.8 Dual-Mode Drug Screening and Hyperthermic Modulation in 3D Microtumors

The ability to combine thermal actuation with chemotherapeutic delivery within a single micro-robotic platform presents a significant advancement for disease modeling and functional drug screening. Solid tumors *in vivo* are not uniform entities; they exist as spatially and temporally dynamic systems characterized by gradients in oxygen, pH, drug concentration, and temperature, as well as variable extracellular matrix composition and mechanical stiffness [293, 294, 295]. Hyperthermia—mild, localized elevation of tissue temperature—has emerged as a promising adjuvant therapy to enhance drug delivery, disrupt tumor vasculature, and sensitize cells to apoptosis [296]. However, conventional *in vitro* screening models often fail to replicate these physical and chemical complexities, relying instead on static, homogeneous culture systems that overlook the dynamic microenvironment of real tumors.

To address these limitations, we employed TACSI microrobots as multifunctional tools capable of delivering both localized photothermal stimulation and chemotherapy within engineered 3D tumor spheroids. These spheroids, pre-integrated with thermoresponsive microrobots, were embedded into collagen type I hydrogels to mimic the fibrous, cell-permissive structure of the tumor extracellular matrix [297, 298, 279], thereby enabling spatially directed cancer cell invasion and matrix remodeling. Once cast, spheroids retained their structural integrity and exhibited homogeneous outward migration over a 14-hour period (Figure 5.20). This platform allowed us to apply spatially confined laser stimulation to individual microrobots embedded in the spheroids, generating defined hyperthermic zones at 53°C, 56°C, or 59°C for five minutes. In parallel, we introduced the chemotherapeutic agent doxorubicin at a concentration of 1 µg/mL— a widely used standard in chemotherapeutic assays [299, 300]. Doxorubicin accumulation within the spheroids was confirmed via its intrinsic fluorescence, and its distribution and effect were monitored throughout the experimental timeline.

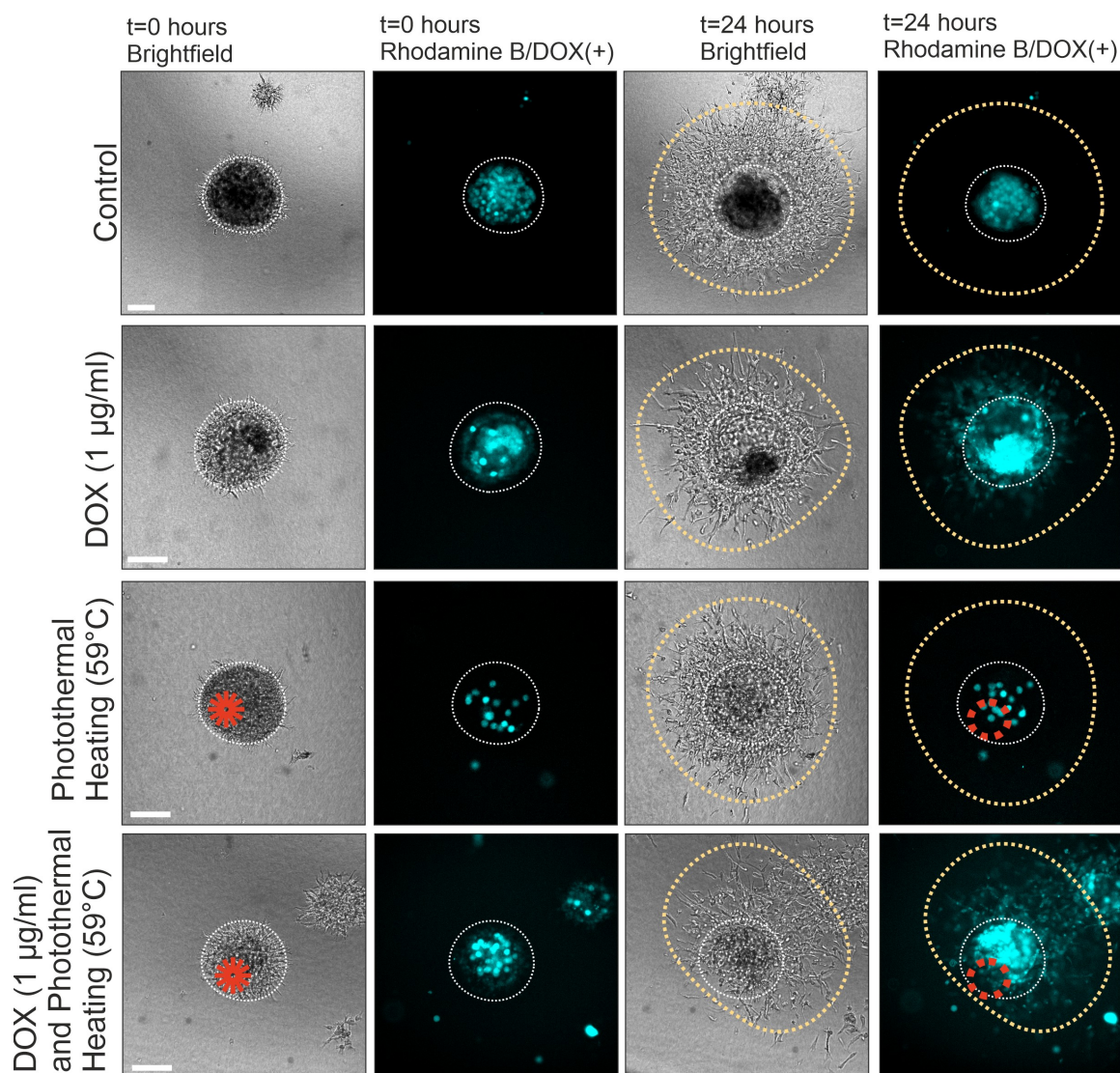


Figure 5.20 Invasion Behavior of Fibrosarcoma Microtumors After Localized Photothermal Treatment. Representative fluorescent microscopy images of HT-1080 fibrosarcoma microtumors embedded in collagen type I hydrogel. Brightfield and fluorescence channels (Rhodamine B and DOX(+)) are shown before photothermal stimulation (left, 5-minute exposure) and after 24 hours (right). White dashed outlines indicate the initial microtumor boundary, while yellow dashed outlines show the invasion area after 24 hours. Cyan fluorescence corresponds to Rhodamine B-labeled microrobots and DOX uptake. The red laser symbol and red dashed line mark the region of applied photothermal heating. Scale bars: 100 μm .

To quantify cancer cell invasion, spheroid boundaries were measured at time zero and again after 24 hours under all conditions (Figure 5.21 a). Neither hyperthermia nor DOX alone produced a significant reduction in invasion distance. However, the combination of both stressors—local heating and DOX—produced a pronounced inhibitory effect. Cells exposed to combined treatment showed decreased migration, rounded morphology, and reduced protrusive activity. These findings suggest a synergistic mechanism: photothermal heating likely enhances membrane permeability and stress signaling, amplifying the cytotoxic effects of DOX through increased DNA damage and apoptosis pathways [296]. This outcome mirrors clinical interest in hyperthermia-assisted chemotherapy as a strategy to overcome drug resistance and improve targeted delivery.

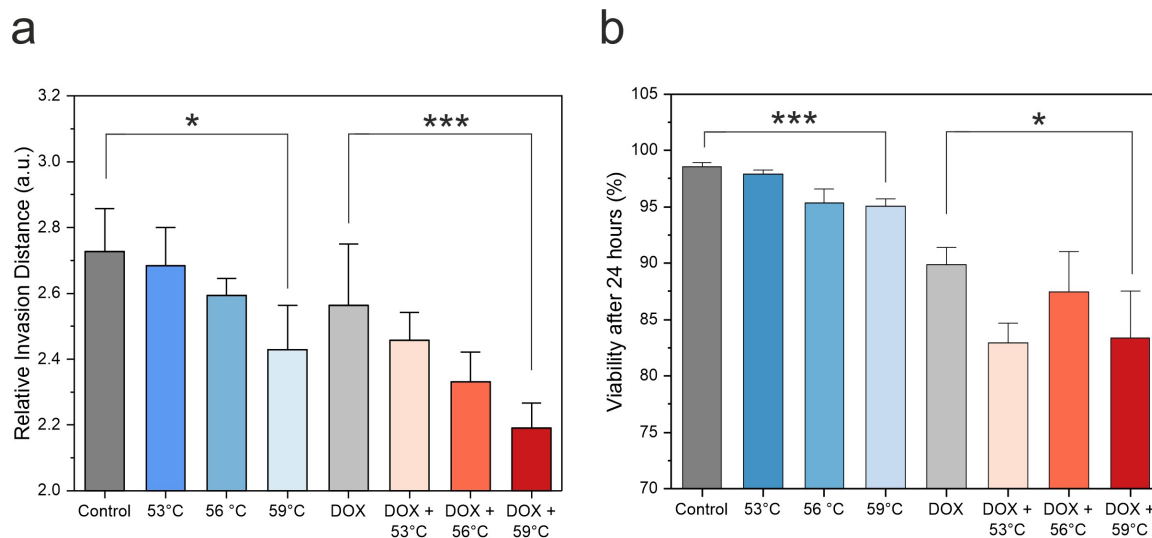


Figure 5.21 Quantification of Invasion Suppression and Cytotoxicity Following Photothermal and Chemotherapeutic Treatment. a) Quantified relative invasion distance of HT-1080 fibrosarcoma microtumors, normalized to initial spheroid size, across five conditions: control, photothermal heating (5 min), DOX alone, and combined DOX with photothermal heating (5 min). Combination therapy shows the most significant reduction in invasion distance ($n = 3$; $*P < 0.05$, $***P < 0.001$, ANOVA). b) Cell viability measured 24 hours post-treatment under the same conditions, demonstrating enhanced cytotoxicity for the combined photothermal and chemotherapeutic approach ($n = 3$; $*P < 0.05$, $***P < 0.001$, ANOVA).

Cell viability after 24 hours was assessed using live/dead staining across all conditions (Figure A.12). As expected, neither DOX alone nor heating below 59 °C significantly affected cell survival. However, the combined treatment condition produced a marked decrease in viability, confirming the cytotoxic synergy between these modalities. To probe the spatial effects of sustained heating, a separate experiment applied prolonged thermal stimulation at 53 °C for 20 minutes using a single microrobot. Rhodamine B fluorescence was used to monitor thermal distribution in real time, confirming that temperatures decreased gradually over distance, reaching 45 °C at 30 μ m from the heating site. Surprisingly, cells located beyond this thermal boundary also exhibited morphological changes, notably retraction of invasive protrusions and loss of polarity (Figure 5.22). Notably, cellular responses were also detected beyond the directly actuated microrobot, suggesting that thermal cues may propagate through secondary mechanisms such as paracrine signaling or mechanical stress redistribution. This broader activation is consistent with the involvement of thermally sensitive pathways such as mitogen-activated protein kinases (MAPK) and reactive oxygen species (ROS), which are known to amplify local perturbations into multicellular responses [301].

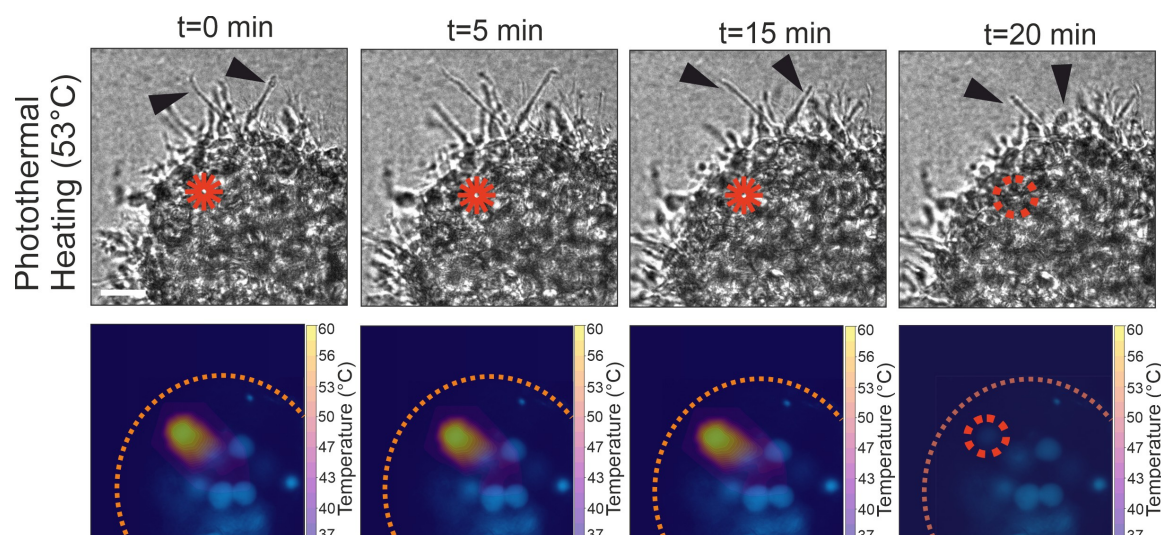


Figure 5.22 Cellular Retraction and Thermal Mapping During Extended Photothermal Stimulation. Bright-field time-lapse images of a 20-minute photothermal heating experiment, with black arrows indicating visible cellular retraction beginning after 15 minutes of continuous heating. The red laser symbol and red dashed line mark the site of photothermal stimulation. Corresponding thermal maps, generated using Rhodamine B-labeled TACSI microrobots as microthermometers, visualize heat distribution across the microtumor. The orange outline demarcates the tumor boundary. Scale bar: $30\mu\text{m}$.

5.3 Concluding Remarks

This chapter presented the TACSI and μ TACSI microrobots as a uniquely multifunctional platform, integrating cell transport, thermal sensing, and localized stimulation within a single soft hydrogel construct. While numerous microrobotic systems have demonstrated remarkable advances in specific tasks, such as targeted delivery, 3D actuation, or biosensing, few offer the combined versatility and biological relevance achieved here.

Magnetically actuated microrobots had a great impact in the field for *in vivo* steering and payload transport due to their deep tissue penetration and remote controllability. Examples include burr-shaped and porous GelMA microrobots loaded with stem cells, which exhibit rolling motion and enzymatic degradability, enabling targeted cell release and subsequent differentiation *in vitro* [302, 58]. Similarly, the scalable GelMA-based systems prioritize manufacturability and biocompatibility but are constrained by slow response times and limited functional feedback once deployed [58]. In contrast, our optically actuated microrobots offer wireless, high spatial and temporal control. Comparable to light-driven systems described in recent reviews [61], TACSI microrobots not only achieve precise locomotion but also encode stimulus-responsiveness through integrated gold nanorods for heating and Rhodamine B for temperature sensing. While other light-powered microrobots focus on propulsion for cell transport [303], few incorporate both actuation and *in situ* feedback into a single platform. TACSI microrobots instead employ reversible electrostatic adhesion using poly-L-lysine coatings, enabling stable, sub-minute cell attachment and passive release—no external biochemical or physical intervention needed. This functionality contrasts sharply with ligand-conjugated systems. Importantly, our system transitions seamlessly into 3D environments. While several platforms have been used to manipulate cells in 2D or guide them through microchannels [302, 10], integrating microrobots into spontaneously formed, viable spheroids with retained actuation and sensing capabilities remains rare. The ability to co-incubate microrobots and cells in a high-throughput, one-pot manner for spheroid assembly introduces a scalable route to construct functional, microrobot-integrated microtissues. Within these 3D constructs, TACSI microrobots act as ECM, embedded thermal actuators and microthermometers, simultaneously modulating and monitoring cellular environments. Localized photothermal stimulation was sufficient to trigger calcium influx via thermosensitive ion channels, both within and adjacent to targeted cells, suggesting activation of paracrine signaling networks. While photothermal effects have been explored for cancer ablation and gene delivery [304], real-time thermal mapping and single-cell resolution stimulation within living 3D tissues are rarely achieved in soft microrobotics.

Finally, we extend the platform's capability to testing therapeutic efficacy by embedding spheroids into collagen matrices and applying combinatorial stimulation. Our results reveal that spatially tar-

geted mild hyperthermia enhances the efficacy of low-dose doxorubicin, highlighting a synergistic interaction between physical and chemical modalities, and showcasing microrobots as local sensitizers for drug screening applications. This concept parallels recent efforts in microrobotic cancer therapy, where localized precision remains a central unmet challenge [10, 305].

In conclusion, we delivered upon the goals set out at the beginning of this chapter: to extend TACSI microrobots toward active biological manipulation in three-dimensional environments. We successfully integrated two distinct modes of cell adhesion—a fast, electrostatic interaction via poly-L-lysine coating for rapid cell pick-up, and a slower, integrin-mediated adhesion using RGD-functionalized alginate for stable biological integration. These innovations enabled selective single-cell capture and transport within 3D workspaces. Furthermore, we demonstrated high-throughput formation of structured spheroids incorporating both microrobots and scaffold microgels, facilitating simultaneous thermal and mechanical stimulation. Real-time analysis of cellular responses to localized heat, strain, and combined drug exposure underscores the multifunctionality of the system.

5.4 Materials and Methods

1. Alginate Preparation

High molecular weight alginate (I-1G; KIMICA) was used to synthesize RGD- and Rhodamine B (RhB)-functionalized alginates. Functionalization was achieved via carbodiimide-mediated coupling chemistry using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS) (Thermo Fisher Scientific). The integrin-binding peptide GGGGRGDSP (Peptide 2.0) was conjugated to alginate at a degree of substitution (DS) of 20, while RhB Lissamine was attached at a DS of 2. For both procedures, alginate was dissolved in MES buffer at 1% (w/v) and reacted with either RGD or RhB for 20 h at room temperature in the presence of EDC and sulfo-NHS (2:1 molar ratio). Following functionalization, alginate solutions were dialyzed in sodium chloride solutions for 3 days, treated with activated charcoal, filtered through a 0.22 μm membrane, and freeze-dried at -50°C for one week using a benchtop lyophilizer (FreeZone 4.5L, Labconco). The dried alginate was reconstituted in MES buffer at 2% (w/v) for subsequent microfluidic fabrication.

2. Synthesis of Thermoresponsive Nanoactuators

Thermoresponsive poly(NIPMAM-co-NIPAM) nanoactuators used for TACSI microrobot fabrication were synthesized by Chen Wang using an *in situ* free radical polymerization method. In a representative synthesis, 90 mL of ultra-pure water was purged with nitrogen gas and heated to 70°C under constant stirring at 700 rpm for 1 hour. Once thermal equilibrium was reached, 350 mg of N-isopropylmethacrylamide (NIPMAM) and 250 mg of N-isopropylacrylamide (NIPAM) were added, followed by 60 mg of N,N'-methylenebisacrylamide as a crosslinker. The stirring speed was then increased to 1400 rpm, and 480 μL of 0.1 M 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) was introduced to initiate polymerization. After 30 minutes, 60 μL of allylamine (diluted in 1 mL of ultra-pure water) was added directly to the reaction mixture, which turned cloudy—indicating nanoparticle formation. The polymerization was allowed to proceed under continuous N_2 purging and stirring at 1400 rpm for an additional 90 minutes. Upon completion, the reaction mixture was cooled to room temperature. The resulting nanoparticles were purified by centrifugation at 11,000 rpm for three cycles, with redispersion in ultra-pure water after each step. The final nanoactuators were stored at 4°C and subsequently incorporated into TACSI microrobots. These nanoparticles were designed to exhibit a lower critical solution temperature (LCST) of approximately 40°C , enabling reversible volume change upon near-infrared laser-induced heating.

3. Microgel Fabrication

Scaffold microgels, TACSI microrobots, and actuating TACSI microrobots were fabricated using 20 μm channel geometries and 4 $\mu\text{L}/\text{min}$ flow rates following the microfluidic encapsulation introduced in Chapter 3. In all cases, 50 μL of CaCO_3 was used as a cross-linker. To ensure homogeneous dispersion of Au-nanorods and PNIPAM, the TACSI precursor solution (2% functionalized alginate) was tip-sonicated at 60% amplitude for 15 s (Fisherbrand Model 120 Sonic Dismembrator). The final Au-nanorod concentration was 10 mg/mL . Actuating TACSI microrobots additionally contained 4 mg of PNIPAM, a thermoresponsive polymer (transition temperature 40°C). Microgels were stored in calcium-rich buffer (130 mM NaCl, 25 mM HEPES, 2 mM CaCl_2) at 4°C . For sterile use, scaffold microgels and TACSI microrobots were washed three times in 70% ethanol and stored in sterile DMEM at 4°C .

4. Imaging Platform and Thermal Calibration

All imaging was performed on a Leica DMI8 inverted microscope equipped with $5\times$, $10\times$, and $40\times$ air objectives, using the same setup described in Chapter 3. A 16-bit monochrome CCD camera (Leica) captured all images. A 785 nm laser was coupled through the infinity port using a dual-mirror setup and driven by an ITC4020 Benchtop Controller (Thorlabs). Laser power at the sample was measured with a PM100D meter (Thorlabs). A Chroma filter cube directed the beam to the sample stage. A 590 nm bandpass filter (Leica) enabled fluorescence capture while blocking the laser. Thermal calibration was performed in a dark room with the microscope enclosed to limit ambient light. Fluorescence excitation occurred only during image capture to minimize bleaching. Samples reached thermal equilibrium after 20 min. A 35 mm petri dish heater (Biotools) adjusted temperatures from 35°C to 60°C in 5°C increments, monitored by a wire thermometer (Okolabs). All calibrations were performed in cell media to ensure consistent optical properties. Image analysis was conducted using Leica LAS X or Fiji (ImageJ).

5. TACSI Microrobot Locomotion

Microrobots were actuated using a 785 nm laser at 70 mA with 0.33 Hz pulsing (1 s on, 2 s off). Prior to actuation, well plates were coated with Pluronic F127 (0.1 mg/mL) for 1 h to prevent cell adhesion, then washed thrice with DMEM. Laser activation at the microrobot base enabled vertical lifting, while lateral illumination induced rolling. Pulling motion was achieved via bottom-directed laser actuation. Cells were detached using trypsin and mixed with microrobots in a 1:1 ratio. Each microrobot was manually steered in 3D toward individual cells. Successful attachment

was confirmed by movement without detachment. For spheroid transport, a constant 100 mA laser power was used at 10× magnification.

6. Cell Culture and Maintenance

HT 1080 fibrosarcoma cells (ATCC) were cultured in a humidified incubator (Binder) at 37°C, 5% CO₂, and 5% O₂. Cells were maintained in DMEM (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. Medium was changed every 2–3 days. Cells were passaged at approximately 80% confluence using 0.05% trypsin (Gibco).

7. Microrobotic Spheroid Integration and 3D Fluorescence Staining

Following trypsinization, cells were resuspended in DMEM and mixed with sterile microgels or TACSI microrobots in 0.5 or 1.5 mL centrifugation tubes. A total volume of 100 µL DMEM was used. Depending on the desired spheroid density, cells and microgels were combined at 1:1 to 1:5 ratios. Incubation was performed at 37°C, 5% CO₂, 5% O₂, and 90% humidity for 2–6 h (single-cell interaction) or 12–48 h (microtumor formation). Cells were stained with BioTracker 650 Red Nuclear Dye (Merck; 2× in DMEM) and CellMask Green (Thermo Fisher; 1× for F-actin). For calcium imaging, Calbryte 520 AM (Biomol) was dissolved in DMSO at 2 mM, stored at –20°C, and diluted to 4.5 µM in isotonic buffer with 0.04% Pluronic F127. Cells were washed three times, incubated with dye for 45 min at 37°C, then washed again. The isotonic solution contained 140 mM NaCl, 2.5 mM KCl, 1.2 mM CaCl₂, 0.5 mM MgCl₂, 5 mM glucose, and 10 mM HEPES. pH was adjusted to 7.3 and osmolarity to approximately 310 mOsm using mannitol. To quantify deformation after spheroid embedding, the initial microrobot diameter (D_0) was compared to the diameter after embedding (D). The radial (linear) strain was calculated as $(D - D_0)/D_0$. Under the assumption of small, isotropic deformations, the volumetric strain was approximated as three times the radial strain.

8. Collagen Protocol and Cancer Invasion Assays

Type I rat tail collagen (5 mg/mL; Ibbidi) was prepared on ice according to vendor protocol. Gel formulation included 10× DMEM, 1 M NaOH for pH adjustment, sodium bicarbonate, sterile water (2/3 volume), and 1× medium (1/3 volume). Collagen was diluted to 4 mg/mL in 17.5 mM acetic acid and combined with microtumors. Mixtures were pipetted into 96-well plates and gelled at 37°C, 5% CO₂, 5% O₂ for 30 min. Doxorubicin (DOX; Sigma) stock was 1 mg/mL in sterile water, diluted to 1 µg/mL in DMEM. Each well received 100 µL of media or DOX solution. Cell viability was assessed with LIVE/DEAD Kit (Thermo Fisher; Calcein 2 µM, Ethidium 4 µM) after 6–72 h. Invasion distances were evaluated using brightfield images taken at 0 and 24 h.

9. Statistical Analysis

Unless otherwise indicated, all results are expressed as mean $\pm$ standard deviation from a minimum of three replicates. Normality was assessed prior to statistical testing. Student's t -test or Wilcoxon signed-rank test was used for paired samples. One-way ANOVA with Tukey's post-hoc test and two-way ANOVA for multifactorial data were applied as needed. Significance thresholds were: ns, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Graphs and statistics were generated using Origin 2021b.

6 Conclusion and Outlook

This thesis presents the TACSI microrobot—a soft, multifunctional, and untethered device designed for precise thermal and mechanical stimulation, temperature and strain sensing, and spheroid assembly at single-cell resolution under *in vitro* conditions. Addressing the need for integrated tools that combine mechanical and biochemical control within tissue-like environments, the TACSI platform employs photothermally responsive hydrogel matrices embedded with actuation and sensing capabilities. These cell-sized constructs enable targeted interactions with individual mammalian cells in both 2D and 3D cultures, establishing a foundation for advanced cellular manipulation and dynamic tissue modeling.

TACSI microrobots are fabricated via droplet microfluidics, generating monodisperse hydrogel microgels composed of calcium-crosslinked alginate, embedded gold nanorods for photothermal actuation, and Rhodamine B for localized temperature sensing. This modular architecture supports wireless, light-driven locomotion with precise thermal control and quantifiable local heating. Thermal calibration confirms delivery of biologically relevant thermal doses to nearby cells, reliably triggering intracellular calcium signaling with high spatiotemporal precision and excellent biocompatibility. TACSI microrobots thereby function as mobile microheaters and microthermometers, capable of operating on or near cell layers to selectively activate thermosensitive pathways *in vitro*.

A robust mechanical characterization framework is established for ionically cross-linked alginate microgels to optimize TACSI microrobots for biological interaction. Nanoindentation and shear rheology are used to determine how formulation parameters, calcium concentration, and media composition influence microgel stiffness and viscoelasticity. Peak mechanical stability occurs at 45 mM CaCl_2 , while additives such as gold nanorods and RGD ligands further modulate gel properties. Exposure to biologically relevant media, particularly those rich in sodium or serum, leads to pronounced and often irreversible softening. These findings highlight the critical role of ionic environments and support formulation strategies tailored to physiological conditions, providing a foundation for the deployment of TACSI microrobots as stable, biointegrated actuators and sensors in 3D cell systems.

A variant named μTACSI is developed featuring a poly-L-lysine coating for rapid electrostatic adhesion to cell membranes, enabling efficient single-cell pickup and transport within one minute. RGD-functionalized TACSI microrobots are also engineered to enable integrin-mediated cell bind-

ing and promote slower, biologically driven spheroid self-assembly. These hybrid microrobot–cell constructs are evaluated in 3D environments to study spheroid locomotion and thermomechanical interactions within tissue-like matrices. Spheroids containing TACSI microrobots demonstrate guided movement via thermophoretic convection, enabling dynamic spatial positioning in culture and facilitating studies of inter-spheroid cell migration and invasion. Photothermal actuation experiments allow quantification of temperature distribution and cellular responses under 3D thermal stimulation. Mechanical stimulation is explored using thermoresponsive microrobots embedded with poly(N-isopropylacrylamide), which contract upon light-induced heating, enabling spheroid-scale deformation both in suspension and within collagen matrices. This capability illustrates the delivery of mechanical cues in dense tissue-like environments and proves particularly useful for long-term mechanobiology assays such as cancer invasion. Furthermore, TACSI microrobots support dual-mode drug screening by combining photothermal stimulation with doxorubicin treatment. Application to collagen-embedded spheroids highlights synergistic effects on cancer cell invasion, demonstrating the platform’s potential for integrated therapeutic investigations.

The novelty of this microrobot lies in its integration of spatiotemporal control, cell-compatible actuation, and ECM-like structural functionality into a unified soft platform capable of both single-cell manipulation and autonomous tissue assembly. While various fabrication strategies for nanocomposites have been explored, achieving homogeneous dispersion of nanoparticles remains challenging, particularly in soft hydrogel systems where aggregation and poor integration limit functionality [227]. Here, this challenge was addressed through carefully optimized physical dispersion methods, ensuring consistent incorporation of functional nanomaterials. Concurrently, significant advances have been made in microrobotics: magnetic microrobots support targeted stem cell delivery [153], optically actuated systems provide high-precision positioning [303], and acoustic holography enables patterned cell assembly [106]. However, these approaches typically focus on isolated aspects of tissue engineering. In contrast, the TACSI microrobots presented here uniquely combine several functions essential for both 2D and 3D cell culture: precise transport of single cells via guided locomotion, high-throughput self-assembly into spheroids, and active locomotion of these assembled structures. Beyond their structural ECM-like role, the microrobots also deliver controlled thermal and mechanical stimulation, while simultaneously incorporating real-time sensing of temperature and mechanical strain. Together, these integrated capabilities allow monitoring of local microenvironments during and after assembly, bridging microrobotics and bioengineering, and fulfilling the original goal set for this work: developing a dynamic, scalable microtissue factory.

Building on these findings, several future directions are identified. First, spheroid locomotion is leveraged to guide the relative positioning of multicellular aggregates, enabling bottom-up assembly

of composite organoids. Spatial arrangement of spheroids comprising distinct cell populations can facilitate the construction of organoids with defined architectures. Second, while thermal stimulation yields robust results in 3D cultures, mechanical actuation requires further refinement. Optimizing force distribution, improving actuation protocols, and increasing sensing fidelity are essential for achieving reproducible mechanotransduction, particularly in stem cell differentiation assays. Additional sensing modalities of cell-secreted biomolecules, such as adenosine triphosphate (ATP), and closed-loop feedback control systems present promising extensions. In summary, this work establishes TACSI microrobots as a versatile platform for localized stimulation, manipulation, and integration within a 3D *in vitro* workspace.

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Appendix

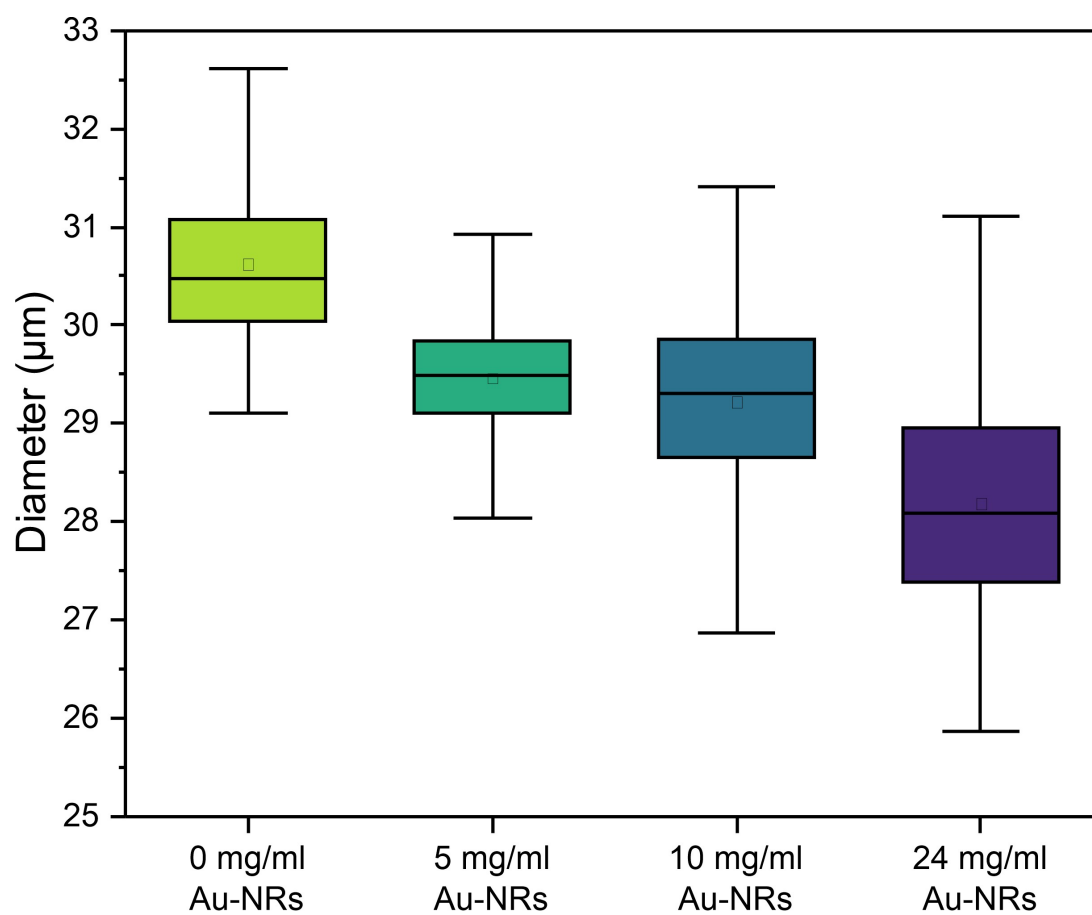


Figure A.1 Diameter change of microgels with varying amounts of incorporated GNRs. Microfluidic channel size: 30 μm. $N = 750$ per condition.

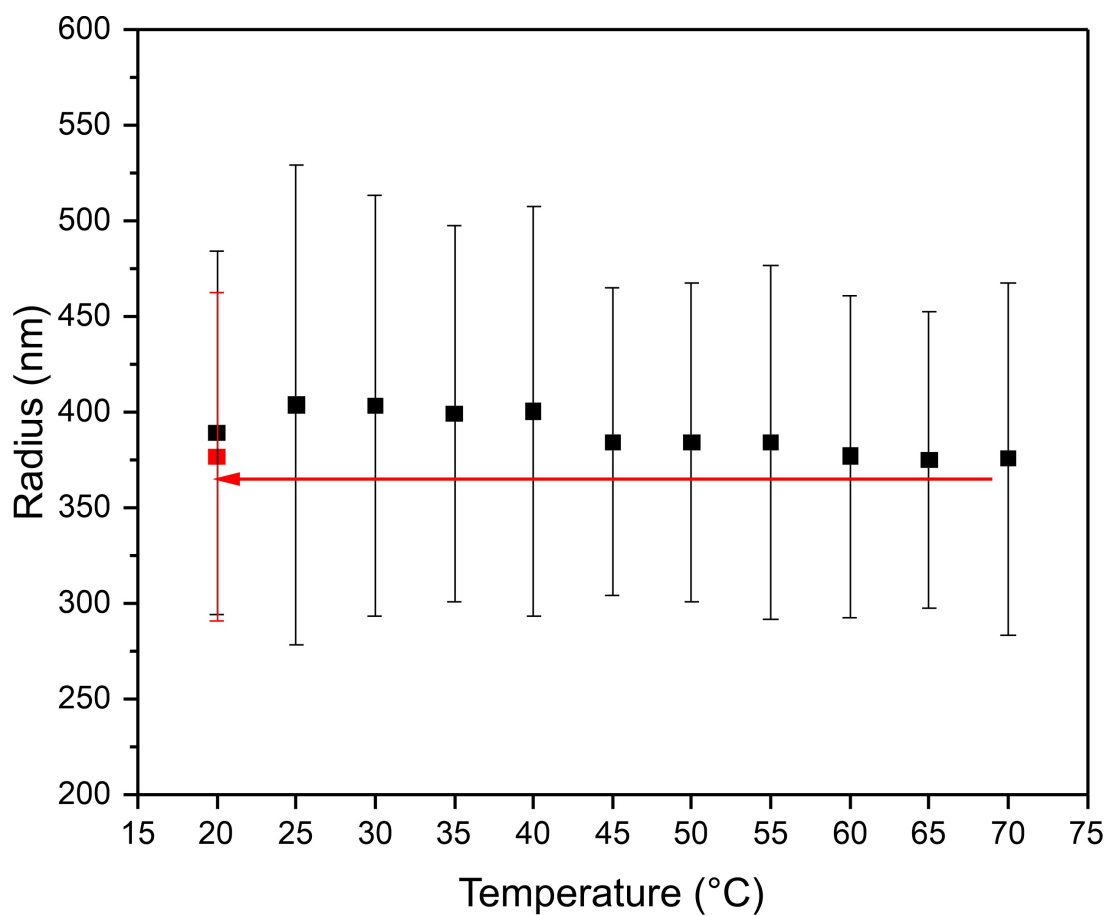


Figure A.2 Gold nanorod size measurements using DLS from 20 °C to 70 °C and back to 20 °C (red line), showing no changes in hydrodynamic radius at these stated temperatures.

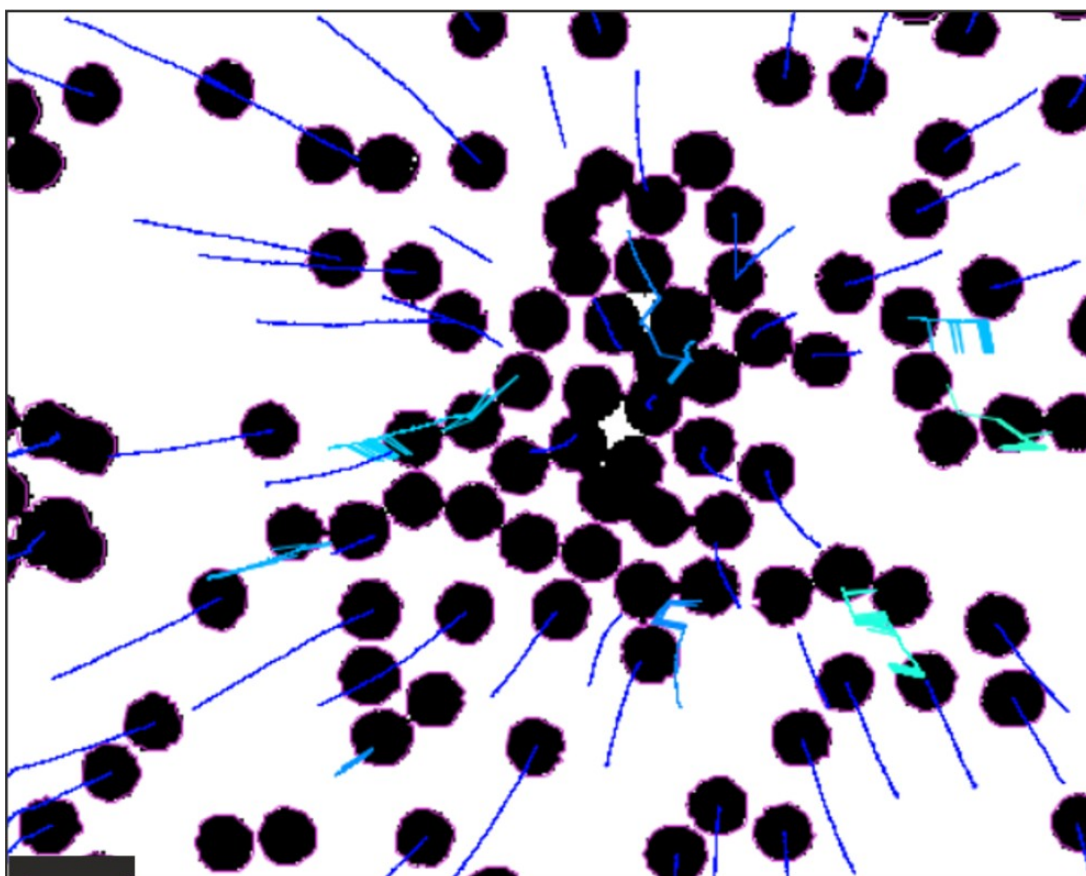


Figure A.3 Tracked motion and speed of multiple microrobots. The blue line indicates a velocity of $2 \mu\text{m s}^{-1}$ at an optical power of $3.8 \mu\text{W } \mu\text{m}^{-2}$ under $10\times$ magnification. Scale bar: $60 \mu\text{m}$.

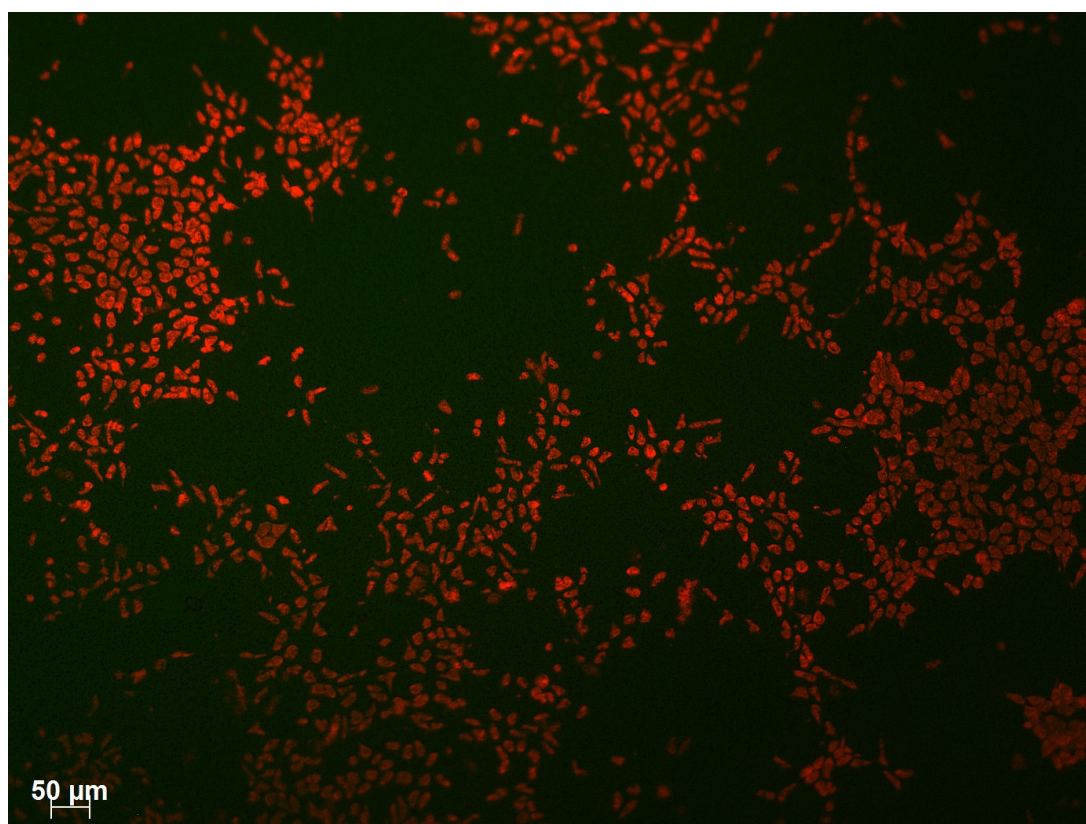


Figure A.4 Live/dead fluorescent staining of HEK 293 cells after 1 hour of incubation with CTAB-capped gold nanorods (GNRs). Live cells fluoresce green, while dead cells fluoresce red. The image shows exclusively red fluorescence, indicating complete cell death as a result of CTAB-mediated cytotoxicity. Scale bar: 50 μm .

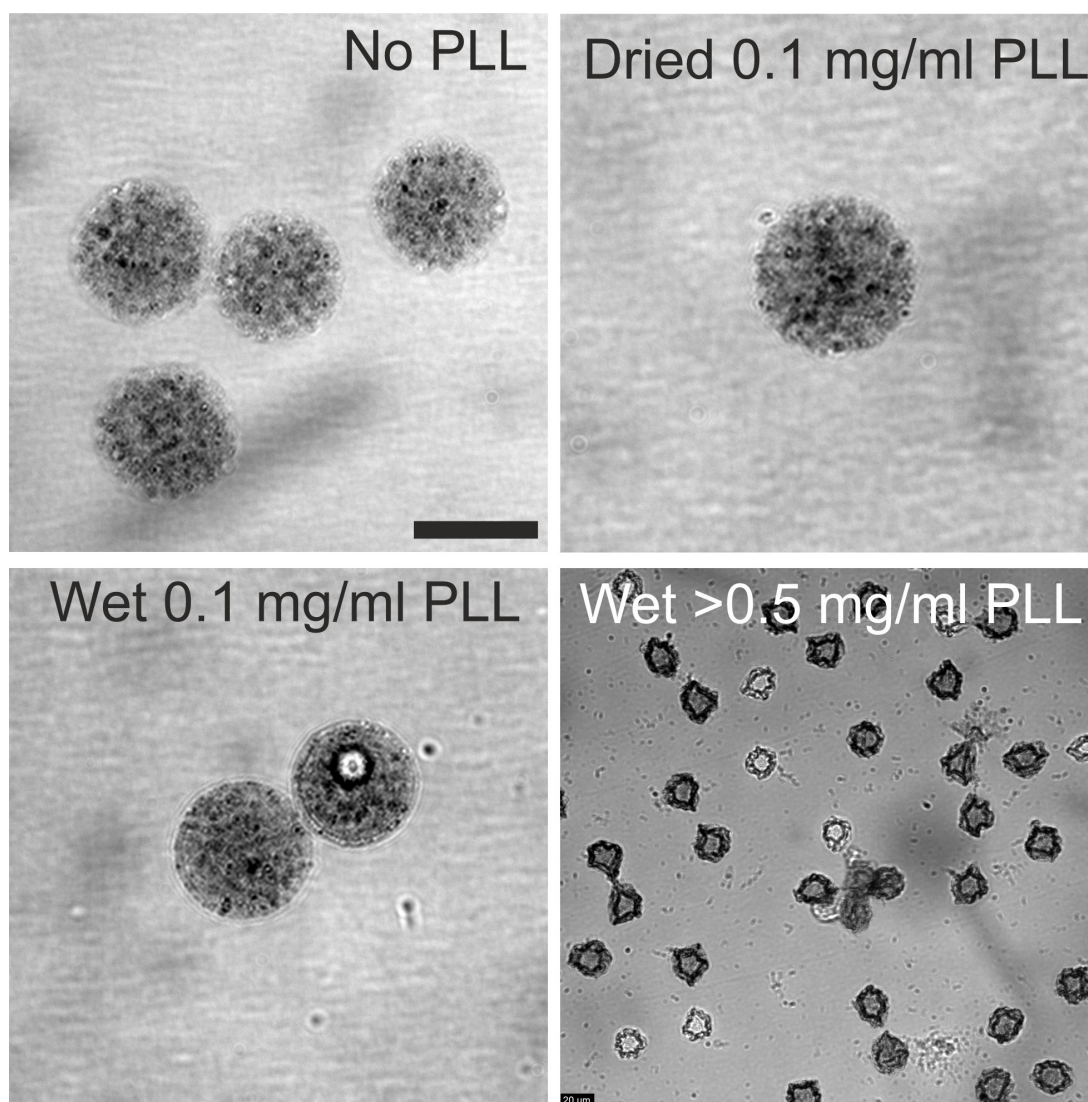


Figure A.5 Secondary cross-linking of microgels using poly-L-lysine (PLL). Representative images show microgel deformation in the absence of PLL, with 0.1 mg/mL dried PLL, 0.1 mg/mL wet PLL, and at higher PLL concentrations (> 0.5 mg/mL). Scale bars: 30 μ m.

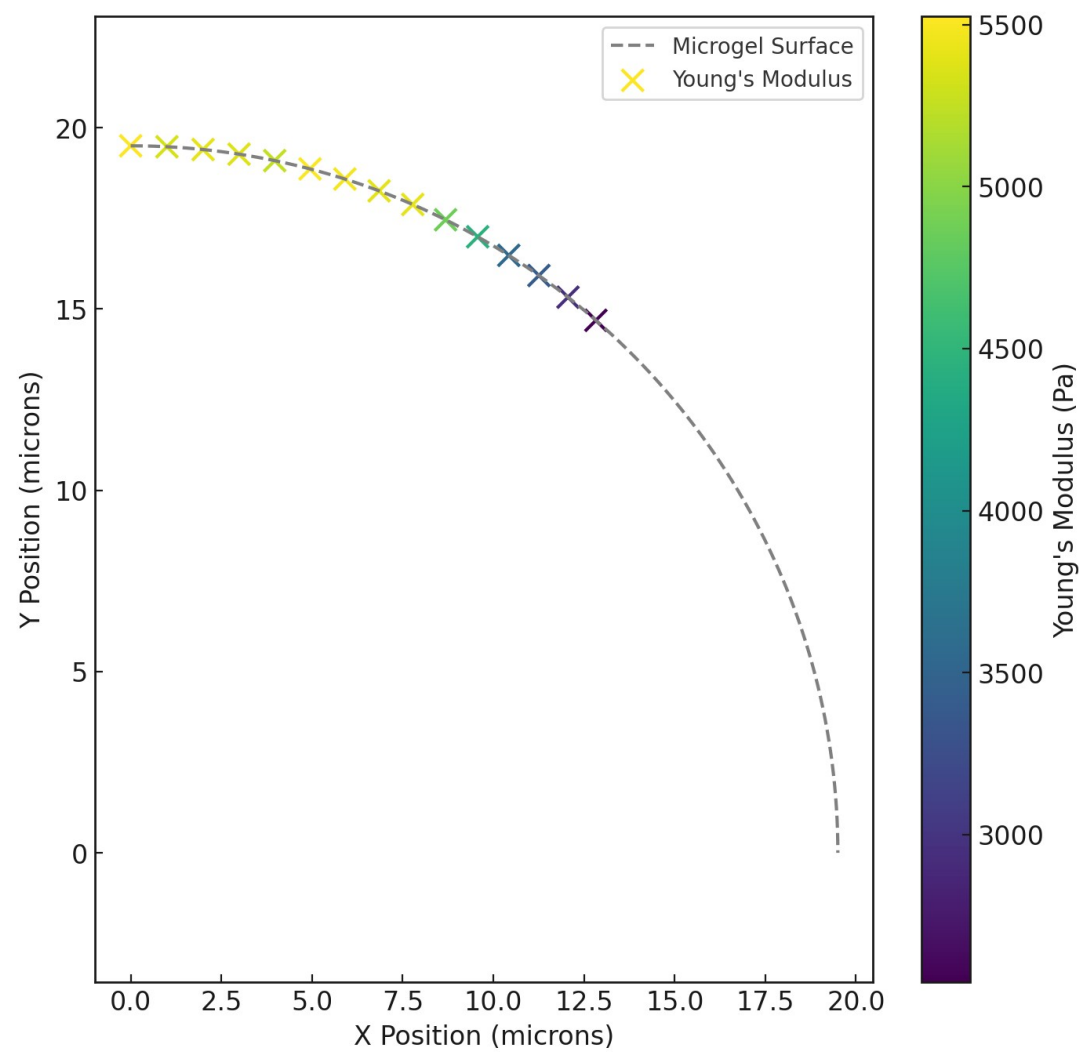


Figure A.6 Nanoindentation scan of a single microgel. The microgel was scanned with a step size of 1 μm , revealing a mean stiffness of 5.4 ± 0.08 kPa over the first eight indentation points.

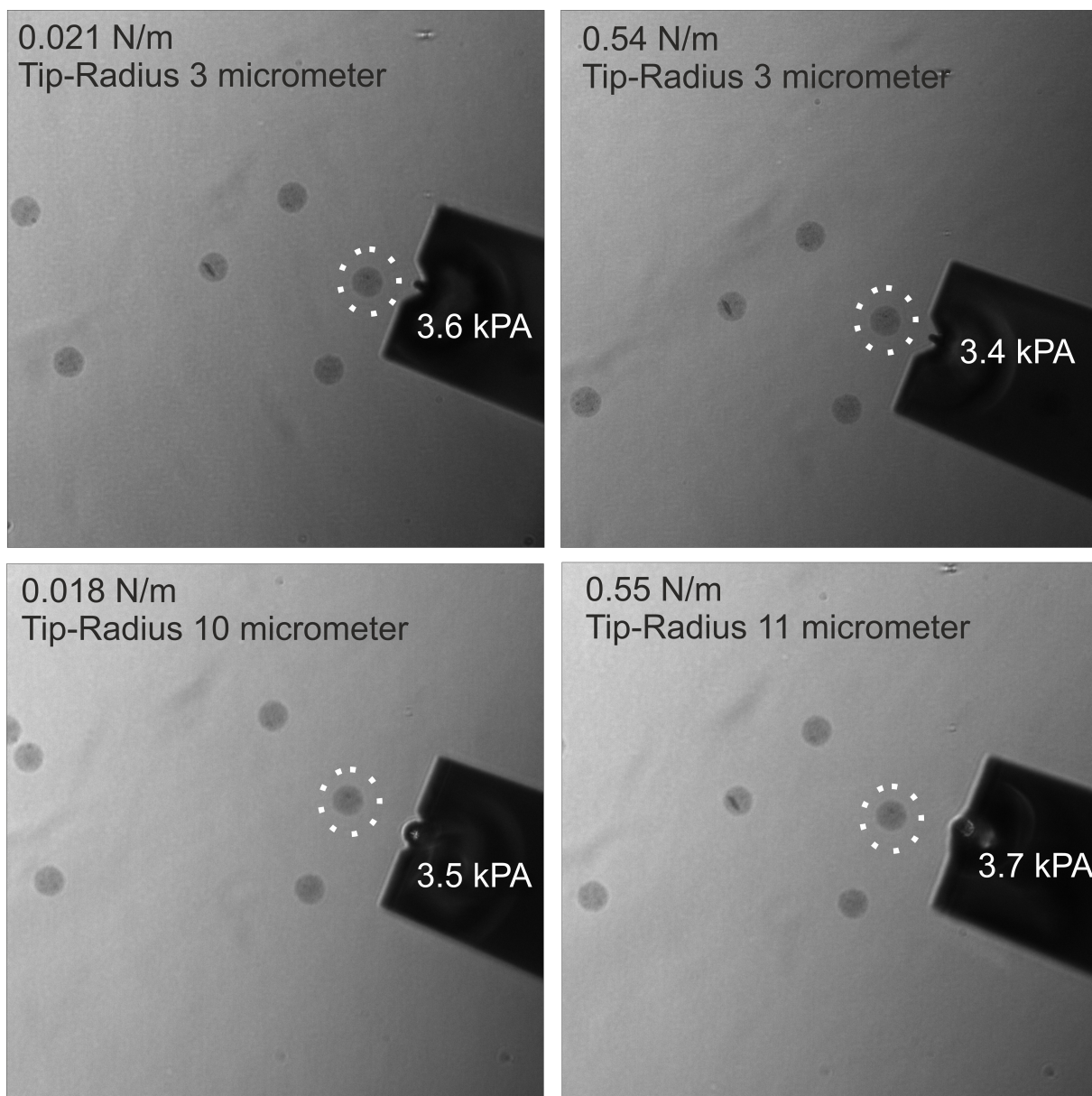


Figure A.7 Comparison of different nanoindentation probes used to characterize the same microgel. Probes with tip diameters of 3 μm (spring constant: 0.54 N/m), 11 μm (0.55 N/m), 3 μm (0.021 N/m), and 11 μm (0.018 N/m) resulted in a maximum measurement deviation of 0.3 kPa.

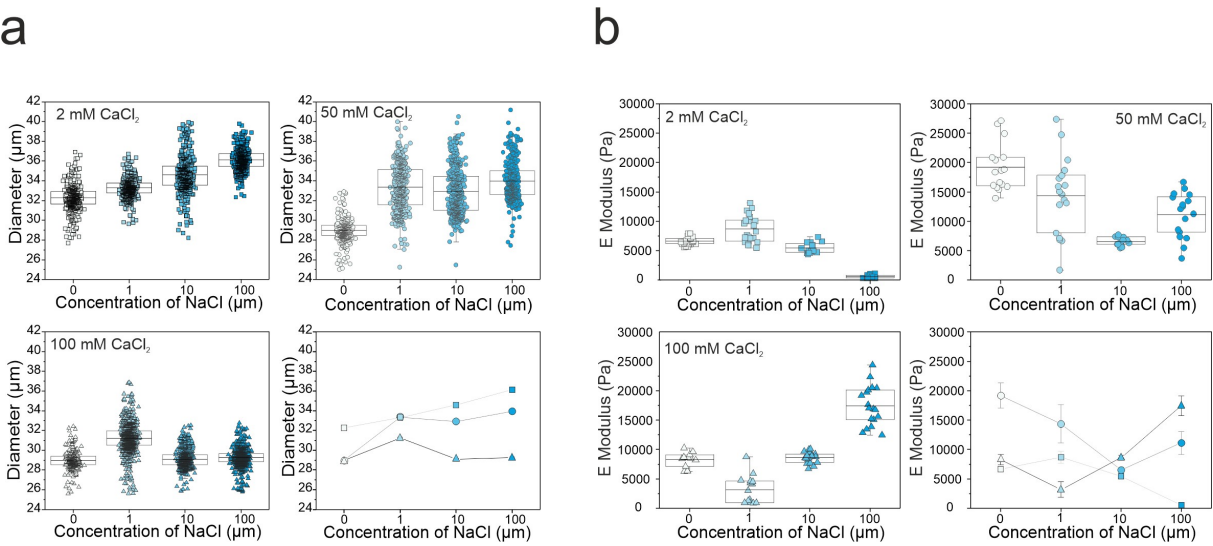


Figure A.8 Effect of NaCl and CaCl₂ concentration on microgel size and stiffness. a) Diameter of M2 microgels under varying NaCl and CaCl₂ concentrations. Data are grouped by CaCl₂ concentration: 2 mM, 50 mM, and 100 mM. Each bar represents the mean microgel diameter $\pm$ standard deviation based on particle size analysis of over 250 microgels per condition. b) Comparison of Young's modulus of M2 microgels under identical conditions. Each bar represents the mean $\pm$ standard deviation based on nanoindentation measurements.

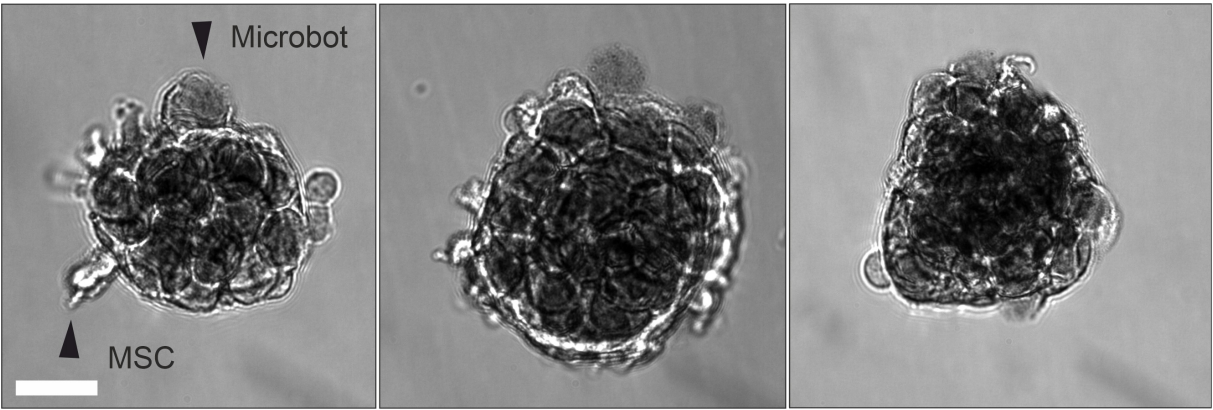


Figure A.9 Formation of Microrobotic Spheroids via Mesenchymal Stem Cell Assembly. Representative image of a microrobotic spheroid formed by mesenchymal stem cells (MSCs) over a 24-hour incubation period. TACSI microrobots act as scaffold elements within the spheroid, becoming integrated into the microtissue structure through cell-mediated assembly. Scale bar: 40 μm.

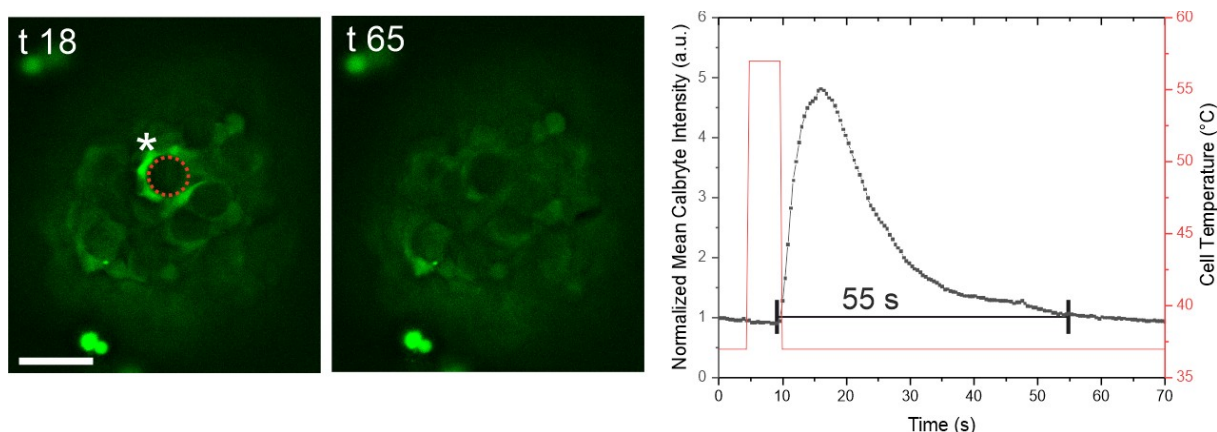


Figure A.10 Impaired Intracellular Calcium Recovery at Elevated Photothermal Temperatures. Representative fluorescence image of HT1080 fibrosarcoma cells stained with Calbryte 520 AM, temperatures exceeding 55°C lead to delayed calcium diffusion, with recovery times extending beyond 60 seconds. Scale bars: $50\text{ }\mu\text{m}$.

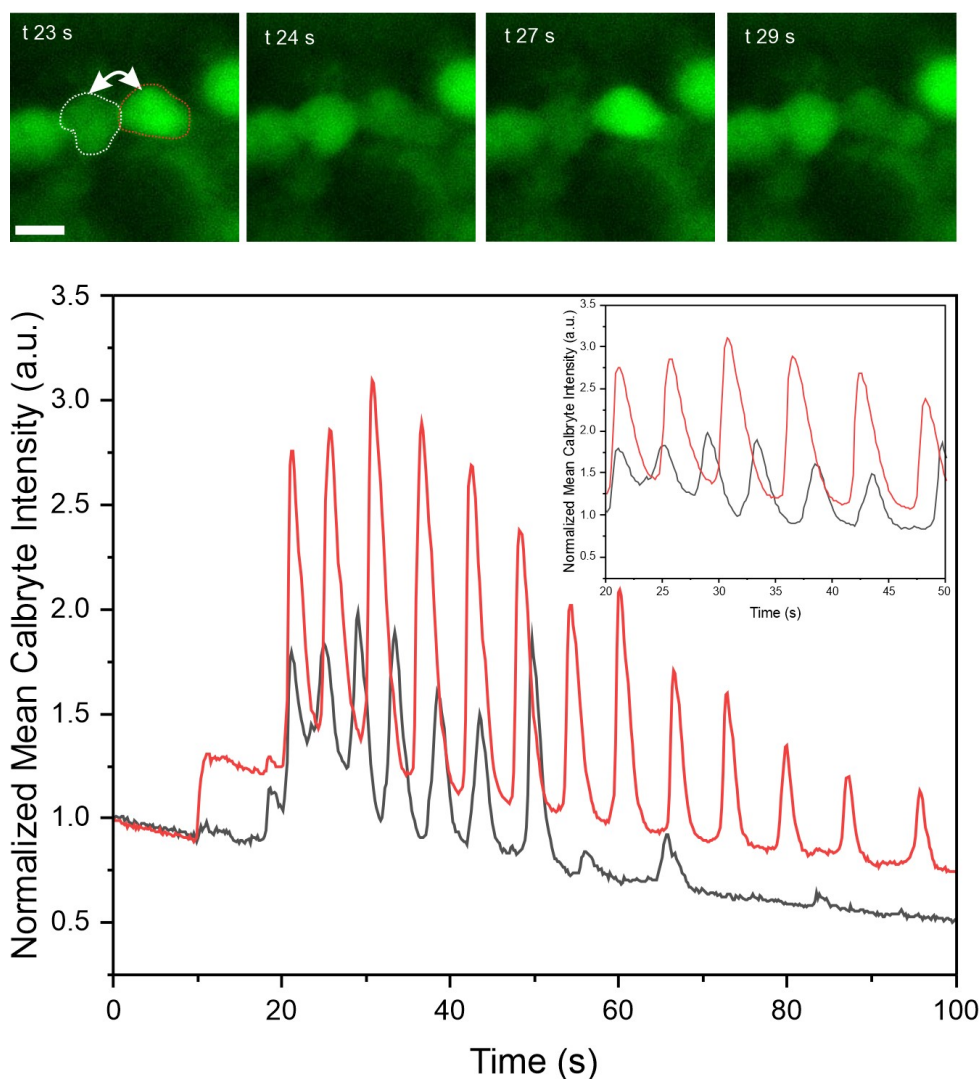


Figure A.11 Intercellular Calcium Signaling Following Localized Photothermal Actuation. Time-lapse fluorescence images of HT1080 fibrosarcoma cells stained with Calbryte 520 AM, captured within a microtumor after activation of a single TACSI microrobot. Following stimulation, intracellular calcium signals propagate periodically between adjacent cells. This dynamic is further illustrated by the corresponding graph of normalized mean Calbryte 520 AM fluorescence intensity. Scale bar: $15\text{ }\mu\text{m}$.

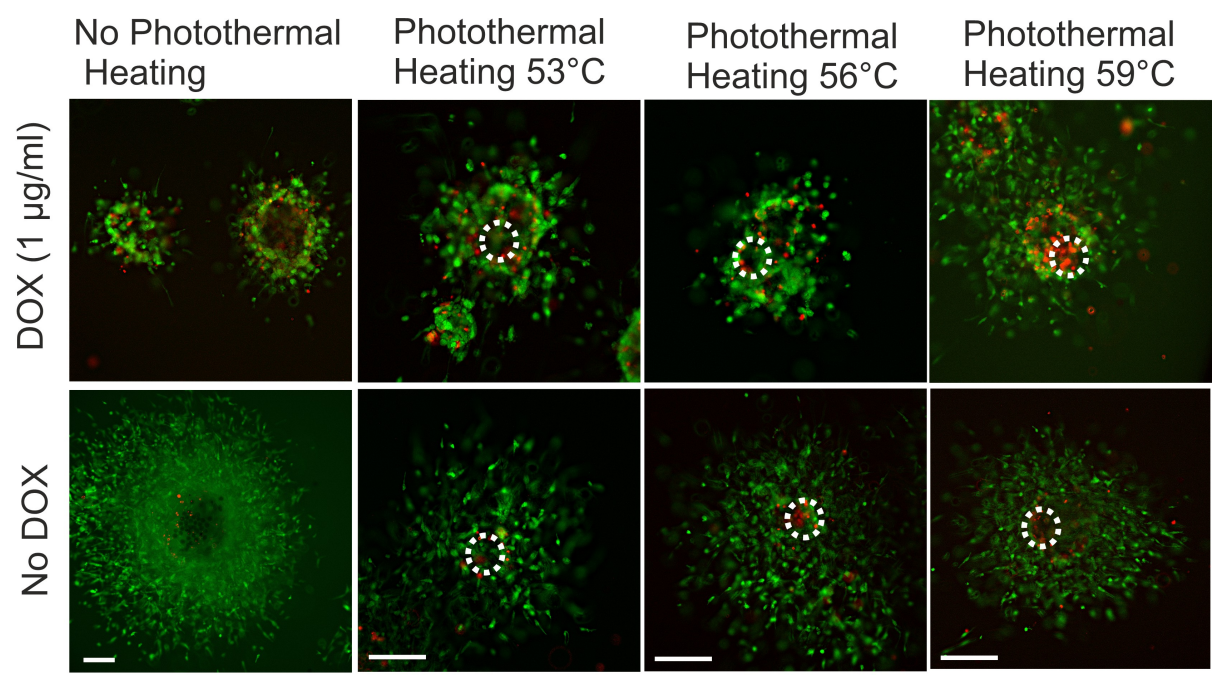


Figure A.12 Viability of HT1080 Microtumors in Collagen Hydrogels Following Photothermal Actuation. Representative fluorescence images of live/dead-stained HT1080 fibrosarcoma microtumors embedded in collagen hydrogels, acquired 24 hours after thermal actuation. Control samples did not undergo photothermal treatment. Scale bars: 100 µm.

Author's List of Publications

Journal Publications

Philipp Harder, Nergishan İyisan, Chen Wang, Fabian Kohler, Irina Neb, Harald Lahm, Martina Dreßen, Markus Krane, Hendrik Dietz, and Berna Özkale. A Laser-Driven Microrobot for Thermal Stimulation of Single Cells. *Advanced Healthcare Materials* 2023, 12 (26), 2300904. DOI: 10.1002/adhm.202300904

Philipp Harder, Leonard Funke, Jana Tamara Reh, Oliver Lieleg, and Berna Özkale. Indenting at the Microscale: Guidelines for Robust Mechanical Characterization of Alginate Microgels. *ACS Applied Materials & Interfaces* 2025, 17 (9), 13513–13526. DOI: 10.1021/acsami.3c17898

Chen Wang, Philipp Harder, Nergishan İyisan, Bolin Li, Lukas Hiendlmeier, Bernhard Wolfrum, and Berna Özkale. A Multiscale Approach to Assess Thermomechanical Performance and Force Generation in Nanorobotic Microgels. *Nanoscale* 2024, 16 (10), 5222–5231. DOI: 10.1039/D3NR06834C

Nergishan İyisan, Oliver Hausdörfer, Chen Wang, Lukas Hiendlmeier, Philipp Harder, Bernhard Wolfrum, and Berna Özkale. Mechanoactivation of Single Stem Cells in Microgels Using a 3D-Printed Stimulation Device. *Small Methods* 2024, 8 (12), 2400272. DOI: 10.1002/smtd.202400272

Unpublished / in Preparation

Philipp Harder, Nergishan İyisan, Yukun Wang, and Berna Özkale. A Soft Microrobot for Single-Cell Transport, Spheroid Assembly, and Dual-Mode Drug Screening. Received by *Advanced Materials*.