

Reconstituting Cellular Intelligence in Nonliving Biomolecular Matter

Thesis by
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The oldest book in the Chinese canon is, fittingly, about change. In the *Yijing* we read: “穷则变，变则通，通则久”—when something is exhausted, it changes; when it changes, a way opens; when a way opens, it endures.

I suspect the ancient sages who inscribed those lines had once tried to finish a doctorate.

This thesis never offered the cinematic arc of hardship blooming into glory. It offered something quieter and more stubborn: the slow realization that profound depletion—intellectual dead ends, repeated failures, crushing doubt—can itself become the turning point. With the right people beside me, exhaustion slowly gave way to transformation, and transformation, against the odds, became something that lasts.

Looking back, I can no longer tell the moments that nearly broke the work (and me) from the moments that ultimately remade it. The fractures have fused into the foundation; the river is never the same twice, and neither am I. I have come to believe that this blurring is not a failure of memory, but the signature of genuine change.

What stands out with perfect clarity now is who made that change possible.

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nothing but my old scRNA-seq lineage tracing work and zero clue what active matter even was. Instead of laughing me out of the room, he lit up and treated it like real science. He later let me TA the PBOC microscopy sessions even though I wasn't in his group, opened his lab to me completely, and was so welcoming that people kept assuming I was co-advised. That generosity meant everything. Simone Weil was right: attention really is the rarest and purest form of generosity.

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ABSTRACT

Living cells convert environmental and internal information into physical and biochemical action. This dissertation focuses on two layers of cell-like control that can be rebuilt in nonliving biomolecular systems: active physical machinery, where cytoskeletal architecture turns molecular force generation into mechanics and transport, and molecular sense-and-response, where compartment architecture turns environmental cues into biochemical output. The common problem is control. Active cytoskeletal materials remodel while generating force, so force transmission must remain predictable in a scaffold that also acts as a machine. Synthetic compartments face a related boundary problem: a compartment must preserve internal state while admitting inputs, sustaining production, and releasing functional output.

Study 1 asks how nanoscale motor forces become network-scale mechanical work. We use PRC1-mediated microtubule crosslinking to tune architecture in kinesin-driven active materials and show that connectivity alone does not guarantee mechanical output. Low-connectivity networks dissipate motor activity locally. Connected but mechanically floppy networks reorganize collectively but fail to sustain long-range force propagation or perform substantial work. Mechanically persistent networks support coherent contraction and extractable work. Velocity correlations reveal a transition from local motion to collective organization, while work measurements and a semiflexible-network model separate connectivity from rigidity. The result is a force-to-work rule for active matter: motors generate force, but crosslinkers determine whether force dissipates locally or becomes coherent, extractable mechanical work.

Study 2 asks how spatial patterning can make active matter predictable enough for task execution despite nonlinear dynamics. Motor-microtubule active fluids generate autonomous motion, but useful engineering requires flow fields specifying where material moves, which objects separate, and how fluids mix. We use light to pattern contracting motor-filament networks and use predictive modeling to compose hydrodynamic outputs into micrometre-scale flow fields. Programmed flows move and separate cell clusters, impose extensional deformation on polymers and giant vesicles, and mix fluids at low Reynolds number. The result is a force-to-flow rule: optical geometry converts internally generated active forces into predictable transport functions.

Study 3 builds an acellular sense-and-response system. A giant unilamellar vesicle, or GUV, supplies a cell-sized lipid boundary for preserving internal biochemical state. Cell-free transcription–translation, or TX-TL, supplies a nonliving biochemical engine for reading DNA and synthesizing proteins outside a cell. We engineer input-output separation across the compartment boundary: small-molecule access admits lactate and nutrients, lactate sensing switches internal productive state, TX-TL synthesizes a bispecific T-cell engager and a genetically encoded membrane gate, and controlled release exports macromolecular output. Lactate-conditioned GUVs recruit T cells to kill CD19-positive target cells. Study 3 brings selective input, productive internal state, controlled output, and target-cell killing into one nonliving compartment.

Across the three studies, we rebuild cellular functions outside living cells. Molecular architecture controls force-to-work conversion, optical patterning controls force-to-flow conversion, and compartment architecture controls cue-to-output conversion. In each case, nonliving biomolecular matter uses molecular, spatial, or compartmental structure to link input with output.

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Chapter 1

INTRODUCTION

Living cells convert environmental and internal information into physical and biochemical action. Chemical gradients bias motility, mechanical loads remodel cytoskeletal architecture, metabolic cues regulate gene expression, and immune recognition triggers effector release. Each example links input, internal state, energy consumption, and output. In the dissertation, *cellular intelligence* names nonequilibrium biomolecular control: sensing context, encoding state, consuming energy, reorganizing structure or transport pathways, and generating condition-dependent outputs. The phrase does not imply consciousness, intention, or human-like cognition. Cellular intelligence, as used here, refers to measurable control implemented by molecules, membranes, and active materials (Bray, 1995; Hartwell et al., 1999; Barkai and Leibler, 1997; Yi et al., 2000).

A dissertation on reconstituted cellular intelligence must narrow from whole-cell behavior to experimentally separable layers of cell-like control. A complete living cell integrates metabolism, repair, growth, division, inheritance, adaptation, and evolution inside a self-maintaining body. Rebuilding every layer at once would turn the project into an artificial-life problem. This dissertation instead isolates capacities central to cellular action and asks how far each capacity can be rebuilt outside living cells. A neutrophil pursuing a bacterium makes the split concrete: the cell senses a target, polarizes internally, remodels its cytoskeleton, generates traction, navigates obstacles, deforms its membrane, and engulfs prey (Rogers, 1950s). The behavior combines two experimentally separable layers. The first layer is **physical action**: cytoskeletal architectures convert internally generated molecular forces into structure, deformation, mechanical work, and transport. The second layer is **molecular sense-and-response**: compartmental biochemical systems convert environmental cues into internal regulatory state, newly produced macromolecular outputs, release, and biological effect.

The dissertation therefore asks two questions:

1. **How can active cytoskeletal architecture and spatial activation program the conversion of internally generated motor stresses into mechanical**

structure and transport?

2. How can synthetic-cell architecture couple molecular input, internal regulation, cell-free production, and macromolecular release into a measurable biological response?

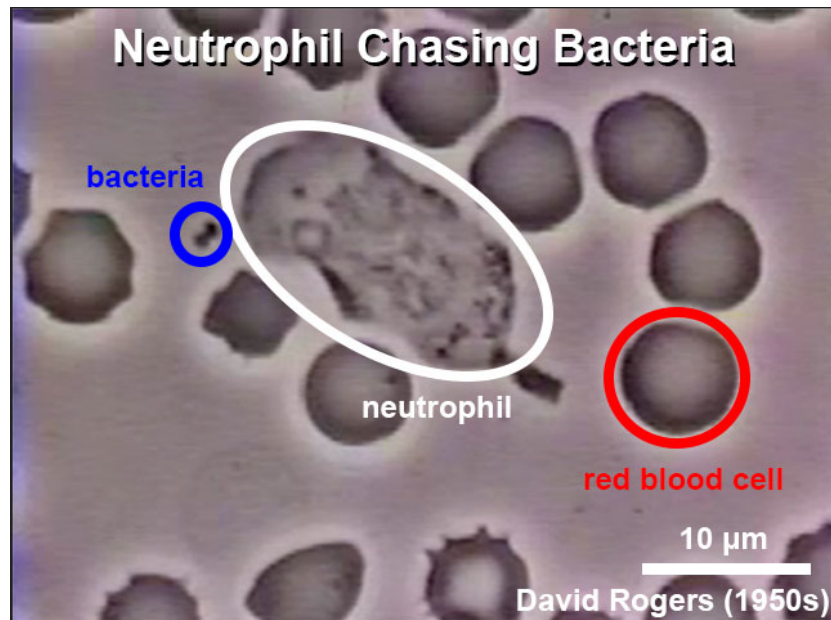


Figure 1.1: **Neutrophil chemotaxis as cellular sensing and action.** Still image from David Rogers's classic movie of a human neutrophil pursuing *Staphylococcus aureus*. Blue ring, bacterium; white ring, neutrophil; red ring, red blood cell. Scale bar, 10 μm . Image credit: David Rogers/Vanderbilt University.

Bottom-up reconstitution provides the experimental strategy for answering both questions. Intact-cell biology reveals how living cells perform state-dependent behavior in native context, engineered living-cell systems show how sensing and response can be programmed into viable organisms, and computational models formalize cellular state and predict cellular transitions. Bottom-up reconstitution asks a different question: which defined molecular components are sufficient to produce a selected cellular function under controlled physical and biochemical conditions? Reconstitution gives direct control over composition, geometry, energy supply, boundary conditions, and interfaces. The limitation is equally important: reconstituted systems usually lack full autonomy, repair, metabolism, growth, and evolution. This incompleteness becomes a design constraint, not a defect. The goal is to rebuild selected functions with enough control to expose design principles behind cell-like behavior (Shimizu et al., 2001; Vincent Noireaux and Albert

Libchaber, 2004; Adamala et al., 2017; Niederholtmeyer, Chaggan, and Devaraj, 2018).

Cell-like physical action is difficult to engineer because active cytoskeletal materials are simultaneously structures and machines. Passive materials such as elastic solids or ordinary fluids primarily deform in response to externally applied forces. Cytoskeletal active materials generate force internally through ATP-consuming molecular motors. In a motor–filament network, the architecture bearing load is also the architecture producing, transmitting, storing, dissipating, or redirecting stress. Kinesin consumes ATP to slide microtubules, microtubules provide polar structural tracks, and crosslinkers define load paths through which motor-generated stresses propagate. Architecture and actuation therefore cannot be designed separately. Motor activity remodels the network, while network connectivity determines whether motor activity produces contraction, deformation, rupture, persistent structure, or flow (M. Cristina Marchetti et al., 2013; Ramaswamy, 2010; Daniel Needleman and Zvonimir Dogic, 2017; Banerjee, Gardel, and Schwarz, 2020).

Active cytoskeletal materials make the physical-action question experimentally tractable. Microtubules provide stiff, polar structural tracks; kinesin motors convert ATP hydrolysis into directional stepping; crosslinkers determine which filaments couple, how stresses transmit, and whether sliding becomes local rearrangement or network-scale mechanics. Reconstituted microtubule–motor systems have shown how purified cytoskeletal proteins can self-organize into asters, bundles, contractile networks, active gels, and flowing materials. Yet force generation alone is not a design principle. The same motor activity can produce extension, contraction, aster formation, collapse, or persistent flow depending on polarity, connectivity, filament length, motor valency, confinement, and boundary conditions. The physical-action question is therefore architectural: which structural parameters govern functional behavior in materials whose constituents actively generate forces while responding to external inputs? (Nédélec et al., 1997; Surrey et al., 2001; Sanchez, Daniel T. N. Chen, et al., 2012).

The central gap in active cytoskeletal engineering is the link between molecular architecture and functional output. Motor–filament systems can generate active stresses, but engineering requires control over where stresses go and what stresses produce. In a connected network, motor-generated sliding can be stored, transmitted, frustrated, or dissipated depending on the available load paths. In a fluid, the same active stresses can drive chaotic motion or designed flow depending on spatial orga-

nization and hydrodynamic coupling. The first two projects address complementary forms of the same physical problem. The first project asks how molecular connectivity controls stress-to-structure conversion in a contracting network. The second project asks how spatial activation controls stress-to-flow conversion in an active fluid. Together, the two projects ask how active matter can be used as programmable physical hardware, beyond its spontaneous self-organizing motion (Tyler D. Ross et al., 2019b; Zijie Qu et al., 2021; Yang et al., 2025).

The first project answers how molecular connectivity programs mechanical response in an active cytoskeletal network. The system combines microtubules, kinesin-1, ATP, and PRC1, a MAP65-family crosslinker enriched at antiparallel microtubule overlaps. PRC1 is not passive glue. PRC1 changes overlap geometry, connectivity, sliding resistance, and force transmission. By varying PRC1 concentration, the project tunes the architecture through which kinesin-generated stress propagates. Weakly connected networks collapse into sparse, highly deformed cable backbones. Strongly connected networks retain dense, straighter load-bearing structures. Post-contraction morphology becomes an image-based mechanical readout: cable survival reports load-bearing connectivity, and cable deformation reports mechanical compliance. The project shows how active stress depends on architecture. Network connectivity selects whether motor stress becomes compliant collapse or mechanically coherent structure (Subramanian et al., 2010; Lansky et al., 2015; Gaska et al., 2020; Alfieri, Gaska, and Forth, 2021).

The second project answers how spatial activation programs active matter into transport. Reconstituted motor–microtubule systems can generate autonomous flows, but autonomous motion alone does not create an engineering platform. Flow fields must be spatially specified, predictable, and composable. The light-controlled active-fluid project patterns contracting motor–filament networks in space, limits hydrodynamic interactions between active units, and uses a predictive model to design micrometre-scale flow fields. The active material supplies the energetic flow source, the optical pattern supplies spatial instruction, and the model links geometry to output. The resulting flows transport and separate cell clusters, probe extensional rheology of polymers and giant vesicles, and generate mixing at low Reynolds number. The project converts active cytoskeletal matter from a self-organizing phenomenon into a programmable microfluidic actuator. In the language of the physical-action question, light controls how internally generated motor stress becomes spatial transport (Tyler D. Ross et al., 2019b; Zijie Qu et al., 2021; Yang et al., 2025).

Cell-like sense-and-response is difficult to engineer because a nonliving compartment must preserve internal state while exchanging signals, nutrients, and outputs. A sealed vesicle can protect internal chemistry, but complete isolation prevents sensing, feeding, and communication. A highly permeable vesicle can exchange material, but uncontrolled permeability destroys compartmental state. Living cells solve the boundary problem with membranes, channels, transporters, secretion pathways, metabolism, and repair. Synthetic cells must solve a reduced version with far fewer parts. A useful sense-and-response compartment requires at least six coupled functions: a boundary, an input path, a molecular sensor, an internal regulatory state, an energy-consuming production system, and an output path. Failure at any interface can break the response chain. Input may not enter, sensor leak may activate expression without cue, TX–TL resources may be depleted, output proteins may misfold, pores may damage the membrane, and vesicle heterogeneity may obscure function. The synthetic-cell problem is therefore not module availability alone. The problem is integration (Vincent Noireaux and Albert Libchaber, 2004; Lentini et al., 2014; Adamala et al., 2017; Hilburger et al., 2019; Monck, Elani, and Ceroni, 2024).

GUV synthetic cells provide a membrane-bounded platform for building compartmental biochemical response. Giant unilamellar vesicles create cell-sized lipid compartments compatible with microscopy, encapsulated reactions, and membrane-protein interfaces. Cell-free transcription–translation supplies a biochemical engine capable of converting genetic programs into newly synthesized macromolecular outputs. Membrane pores define communication with the environment. Small pores such as alpha-hemolysin can support nutrient exchange and small-molecule input, whereas larger inducible pores are needed for macromolecular release. The membrane is therefore part of the control architecture, not a passive wrapper. In a sense-and-response synthetic cell, the membrane determines which signals enter, which molecules remain trapped, and when output reaches the environment. The relevant field gap is the coupling of input transport, transcriptional regulation, energy-dependent production, release actuation, and biological function inside one finite compartment (Vincent Noireaux and Albert Libchaber, 2004; Garenne et al., 2021; Van de Cauter et al., 2023; Song et al., 1996; Tweten, 2005).

Cell-free TX–TL changes a synthetic cell from a delivery container into a productive response system. A passive liposome can release preloaded cargo after chemical or mechanical disruption. A TX–TL-containing vesicle can synthesize new output after receiving an input. The difference matters for cellular intelligence because pro-

ductive response requires energy-dependent biochemical work rather than simple cargo escape. TX–TL encodes internal state in promoter occupancy, RNA abundance, protein production, and resource allocation. A sensor can therefore gate de novo production of a payload and an actuator. In therapeutic synthetic cells, productive architecture is especially important: environmental context can trigger synthesis of a biologically active macromolecule in addition to any release of stored drug. The challenge is resource competition, since payload and actuator consume the same finite transcriptional, translational, and energetic capacity (Shimizu et al., 2001; Shin and Vincent Noireaux, 2012; Takahashi et al., 2015; Garenne et al., 2021).

The third project answers how synthetic-cell architecture converts molecular context into therapeutic action. We build nonliving synthetic cells that sense lactate, a tumor-microenvironment metabolite, and autonomously express and release a therapeutic T-cell engager. The system uses GUVs as nonliving compartments, alpha-hemolysin pores for constitutive small-molecule input and nutrient exchange, the ALPaGA/LldR circuit for lactate-responsive transcriptional control, cell-free TX–TL for protein production, and cholesterol-dependent cytolysin pores for macromolecular output. Lactate functions as a tumor-associated metabolic cue rather than a tumor-specific marker. Lactate input derepresses production of the T-cell-engaging antibody fragment and a pore-forming release actuator. The release actuator opens an output route for macromolecular product, allowing the synthetic cell to communicate with living immune cells. The endpoint is functional rather than purely fluorescent: lactate-conditioned synthetic cells are evaluated for the ability to trigger T-cell-mediated killing of CD19-positive Raji cells. The project reconstructs a biochemical sense-and-response chain: metabolite input, internal regulatory state, energy-dependent production, macromolecular release, and biological effect (Zúñiga et al., 2021; Colegio et al., 2014; Z.-H. Wang et al., 2021; Tweten, 2005; Goebeler and R. C. Bargou, 2016; Newman and Benani, 2016).

The three projects answer the dissertation questions through three control variables in nonliving biomolecular systems. PRC1 concentration changes active-network mechanics by changing connectivity and force transmission. Light controls active-fluid transport by defining where contracting motor–filament networks form and how hydrodynamic outputs combine. Lactate-responsive gene regulation controls therapeutic actuation by converting a metabolic cue into protein production and release. The control variables differ, but the logic is shared: input defines system

state, energy-consuming biomolecular machinery performs work, and the system generates an output in mechanics, transport, or biochemical function. The dissertation therefore treats cellular intelligence as a spectrum of reconstitutable control functions, not as a property exclusive to complete living cells.

The dissertation rebuilds selected cellular functions without rebuilding a whole cell. Chapter 1 develops molecular programming of active-network mechanics and shows how PRC1-controlled connectivity selects between compliant cable collapse and dense load-bearing architecture. Chapter 2 develops optical programming of active-fluid transport and shows how patterned motor–microtubule contraction can generate designed flow fields for transport, separation, mixing, and extensional rheology. Chapter 3 develops biochemical programming of synthetic-cell response and shows how lactate-responsive GUVs can produce and release a T-cell-engaging therapeutic output. In these chapters, nonliving biomolecular systems perform selected functions normally associated with living cells: mechanical organization, spatial transport, and context-dependent biochemical actuation.

The central claim of the dissertation is simple: architecture can program action in nonliving biomolecular matter. In active cytoskeletal networks, architecture means filament polarity, overlap geometry, crosslinker occupancy, connectivity, and spatial activation. In synthetic cells, architecture means membrane permeability, input transport, gene regulation, TX–TL resource allocation, release actuation, and external biological coupling. Across both branches, function emerges from organization of components into input–state–energy–output chains. Reconstituting cellular intelligence therefore does not require claiming artificial life or human-like cognition. In this dissertation, reconstitution means showing how defined biomolecular systems convert context into action. The three experimental systems do so through molecularly controlled mechanics, optically controlled transport, and metabolite-controlled therapeutic response.

Chapter 2

FORCE PROPAGATION IN ACTIVE CYTOSKELETAL NETWORKS

Abstract

Conventional materials science establishes clear structure–function relationships. Active matter complicates those relationships because its components consume energy while bearing load. This material–machine duality raises a central question: which structural parameters convert molecular-scale motor activity into micrometre-scale force propagation and mechanical work? Here we answer this question in a reconstituted microtubule–kinesin active material by tuning PRC1, a microtubule crosslinker that bridges neighbouring filaments. PRC1-mediated crosslinking sets network architecture and routes the same motor input into distinct mechanical outputs. Low-connectivity networks dissipate activity locally. Connected but mechanically floppy networks reorganize collectively but fail to sustain long-range stress transmission or generate substantial work. Mechanically persistent networks support long-range force propagation, coherent contraction and extractable work. Velocity correlations reveal a PRC1-dependent transition from local motion to long-range collective organization, while work measurements show that connectivity alone does not guarantee mechanical output. A semiflexible-network model explains this gap: connectivity and mechanical persistence arise at distinct thresholds, and, in the model, bending elasticity is required for a connected sub-isostatic network to support shear rigidity. These results give a structure–function rule for active matter: motors generate force, but crosslinkers set network connectivity and thereby determine whether that force is dissipated, transmitted or converted into work.

Introduction

Conventional materials science links structure to function. Grain size, defect density, lattice architecture and crosslink density serve as design variables because they predict how passive materials bear load, conduct energy or deform (Hall, 1951; Petch, 1953; Meyers, Mishra, and Benson, 2006; Borca-Tasciuc et al., 2000; Lakes, 1987; Bertoldi et al., 2017; Broedersz and Fred C. MacKintosh, 2014). Active materials complicate this rulebook. Their components consume energy and inject forces, so the material architecture both sets mechanical structure and changes under

internally generated stress (M. Cristina Marchetti et al., 2013; Doostmohammadi et al., 2018; Sanchez, Daniel T. N. Chen, et al., 2012). In cytoskeletal active matter, molecular motors generate local stresses that can remain near their origin, reorganize nearby filaments, or propagate over micrometre length scales to perform mechanical work (Mizuno et al., 2007; Koenderink et al., 2009; Sanchez, Daniel T. N. Chen, et al., 2012). The central problem is to identify the structural parameters that determine which of these outcomes occurs.

This structure–function problem appears across cytoskeletal systems. Actomyosin networks switch between elastic, fluid-like and contractile states as motor activity and crosslinking change (Koenderink et al., 2009; Alvarado et al., 2013; Murrell and Gardel, 2012). Mitotic spindles use connected microtubule networks to transmit local motor-generated stresses into coherent long-range flows (Benjamin A. Dalton et al., 2022; Forth and Kapoor, 2017). Reconstituted active microtubule gels also change their deformation modes as the balance between motor forcing and passive crosslinking changes (Najma et al., 2022). Across these systems, motors do not act on a fixed passive scaffold. Motor-generated stresses remodel the scaffold that transmits them, so the same molecular forcing can produce different mechanical outcomes. A general rule remains missing: how do internally generated motor stresses act through cytoskeletal architecture to produce local dissipation, collective organization or mechanical work?

Reconstituted active matter provides a way to isolate this architectural control from the biochemical complexity of cells. External-control strategies have shown that active materials can be organized by boundaries, passive templates and spatiotemporal light patterns (Kun-Ta Wu et al., 2017; Tyler D. Ross et al., 2019b; Sciortino et al., 2023; Yang et al., 2025). These approaches specify where active forces are generated, confined or guided. They do not, by themselves, reveal how a material internally routes a fixed motor input through its own network architecture. The complementary problem is internal architectural control: how does changing cytoskeletal network connectivity, while holding motor input fixed, route identical motor stresses toward local dissipation, collective organization or mechanical work? PRC1 provides a direct molecular handle on this question. As a nonmotor microtubule crosslinker, PRC1 bridges neighbouring, preferentially antiparallel, microtubules (Mollinari et al., 2002; Subramanian et al., 2010). Varying PRC1-mediated crosslinking changes the network that receives motor-generated stress without changing the kinesin motor that supplies force.

Here we use a PRC1-tuned microtubule–kinesin system to identify the structural requirements for force propagation and work in active cytoskeletal matter. PRC1 first drives a connectivity transition: low-connectivity networks dissipate motor activity locally, whereas connected networks organize motion over micrometre length scales. Connectivity alone, however, does not guarantee mechanical output. Connected but floppy networks organize motor-driven motion but produce little work, whereas connected and rigid networks support long-range force propagation, coherent contraction and extractable work. A semiflexible-network model explains this separation by showing that increasing crosslink fraction first creates a connected giant component and only later produces a rigid network with finite shear modulus (Head, Levine, and F. C. MacKintosh, 2003; Broedersz and Fred C. MacKintosh, 2014). The resulting structure–function rule is simple: connectivity organizes motor activity, but rigidity turns motor activity into work. This rule suggests a route to programmable cytoskeletal materials in which molecular crosslinkers tune network connectivity and rigidity, while optical activation controls when and where motors apply force.

Results

PRC1-mediated crosslinking switches active microtubule contraction from local dissipation to global force propagation.

We used a defined and optically controlled in vitro system to ask whether PRC1-mediated microtubule connectivity determines the length scale over which motor-generated forces propagate. The system consists of stabilized fluorescent microtubules, light-activatable kinesin motors and PRC1, a nonmotor microtubule crosslinker that bridges neighbouring, preferentially antiparallel, microtubules (Tyler D. Ross et al., 2019b; Subramanian et al., 2010). Blue-light activation dimerizes the kinesin motor module, allowing motors to engage neighbouring microtubules and drive contraction within a prescribed illumination pattern. By varying PRC1 concentration while holding motor input and light geometry fixed, we asked whether changing network connectivity alone could switch the active material between local and global contraction states.

Throughout the paper, we use force propagation in an operational sense: motor-generated stresses produce coordinated motion across the microtubule network and mechanical response beyond the local contraction site. We quantify force propagation with three linked readouts: the velocity correlation length ξ , tracer-bead displacement and extractable work estimated from bead motion.

First, we observed two distinct contraction behaviours across PRC1 conditions. At low PRC1 concentration, microtubule contraction remained confined to the illuminated region and produced many small, weakly coupled structures. We refer to this state as the local force-dissipating phase. At higher PRC1 concentration, contraction recruited material over substantially larger distances and produced a system-spanning response within and around the light-activated region. We refer to this state as the global force-propagating phase (Figure 1A). Consistent with this visual transition, the average microtubule density in the illuminated region increased more strongly in the global phase than in the local phase, indicating long-range recruitment and concentration of material into the active network (Figure 1B).

To quantify the spatial scale of coordinated motion, we computed velocity fields from the microtubule image sequence and measured the two-point velocity correlation function, $C(r, T)$, as a function of separation distance r (Figure 1C). This correlation function measures how far velocity directions and magnitudes remain coordinated across the active material. For local contractions, $C(r, T)$ decayed rapidly over tens of micrometres, consistent with many disconnected or weakly coupled contracting structures. For global contractions, $C(r, T)$ persisted over hundreds of micrometres and often became negative at large separation distances, consistent with symmetric material recruitment toward the contracting network.

The extracted correlation length, ξ , showed an abrupt PRC1-dependent transition. As PRC1 concentration increased, ξ jumped from approximately $57 \mu\text{m}$ to approximately $248 \mu\text{m}$, separating a local phase from a global force-propagating phase (Figure 1D). We interpret this jump as an operational connectivity transition: below the transition, motor-generated motion remains local; above it, PRC1-mediated crosslinking organizes microtubules into a network capable of coordinating motion over micrometre length scales. This result establishes PRC1 as a molecular control knob for network connectivity in active cytoskeletal matter.

Network connectivity organizes motor-driven motion but does not guarantee mechanical work.

The PRC1-dependent increase in correlation length marks the transition from local force dissipation to collective motor-driven organization. A connected active network, however, does not necessarily function as a work-performing material. To test whether connectivity alone predicts mechanical output, we tracked tracer beads during contraction and estimated the minimum mechanical work performed against

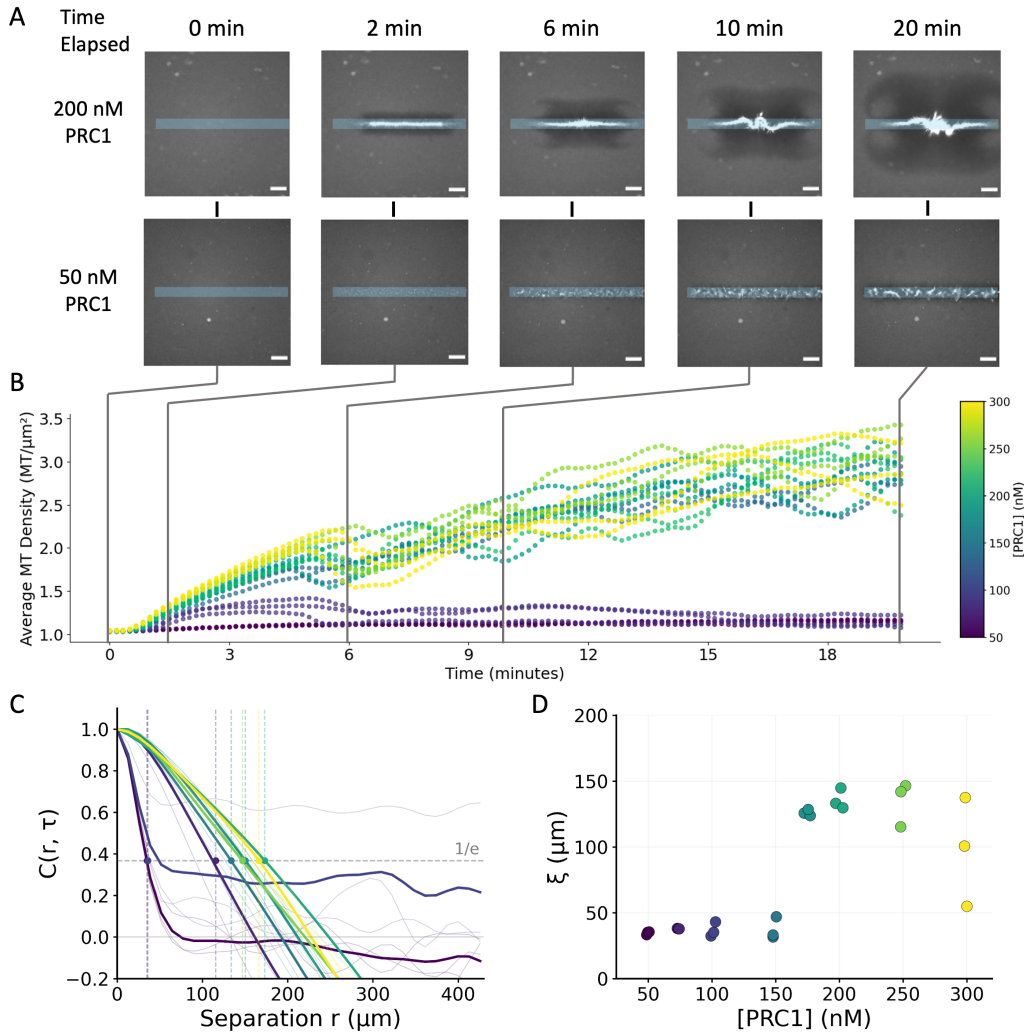


Figure 2.1: PRC1-mediated crosslinking switches active microtubule contraction from local to global. **A**, Time-lapse images of fluorescent microtubules during light-activated contraction at low and high PRC1 concentration. Low-PRC1 networks remain confined to the illuminated region and form many small contractile structures. High-PRC1 networks recruit material over longer distances and form a global contractile structure. Blue shading indicates the illuminated region. Scale bar, 100 μm . **B**, Microtubule density in the illuminated region during contraction, color-coded by PRC1 concentration. High-PRC1 networks show stronger material recruitment into the activated region than low-PRC1 networks. **C**, Spatial velocity correlation function $C(r, T)$ as a function of separation distance r for different PRC1 concentrations. Low-PRC1 networks show rapid correlation decay over tens of micrometres, whereas high-PRC1 networks maintain correlated motion over hundreds of micrometres. **D**, Correlation length ξ as a function of PRC1 concentration. The jump in ξ from approximately 57 μm to approximately 248 μm marks the transition from a local force-dissipating phase to a global force-propagating phase.

viscous drag using Stokes' law.

Mechanical work increased with PRC1 concentration, but the increase did not follow connectivity alone. Low-PRC1 networks produced little work, consistent with their short correlation lengths and local contraction phenotype. Intermediate-PRC1 networks also produced low work, despite correlation lengths in the global range. In contrast, high-PRC1 networks produced an approximately threefold increase in work output (Figure 2A). Plotting work against correlation length showed the same separation: networks with similar ξ produced either low or high work, demonstrating that connectivity alone does not predict mechanical performance (Figure 2B).

The time evolution of ξ separated the connected low-work and connected high-work states. At 100 nM PRC1, ξ remained low throughout the recording. At 200 nM PRC1, ξ remained high and stable during contraction. At 185 nM PRC1, ξ began in the global range but decayed during motor-driven coarsening (Figure 2C). The intermediate-PRC1 network therefore organized motor-driven motion over long distances at the start of contraction, but did not maintain the architecture required for sustained work output.

Tracer-bead trajectories provided an independent readout of force transmission. At 100 nM PRC1, beads showed only short, uncoordinated displacements. At 185 nM PRC1, bead motion remained concentrated near the illuminated region, indicating that motor-generated stresses did not propagate efficiently into the surrounding material. At 200 nM PRC1, beads displaced from distances greater than 400 μm from the illuminated region, showing long-range force transmission through the active network (Figure 2D). The transition from boundary-confined bead motion to long-range bead displacement coincided with the jump in work output.

High-work connected networks preserve imposed geometry during motor-driven contraction.

We used a rectangular light pattern as a geometric template for motor activation to identify the structural difference between the low-work and high-work states. We then imaged microtubule fluorescence over time and extracted one-dimensional intensity profiles along a fixed line through the activated region. A broad plateau with sharp boundaries indicates material recruitment across the imposed geometry, while isolated peaks indicate remodeling localized to sparse paths. This assay tests whether a connected network can maintain the imposed geometry while motors drive contraction.

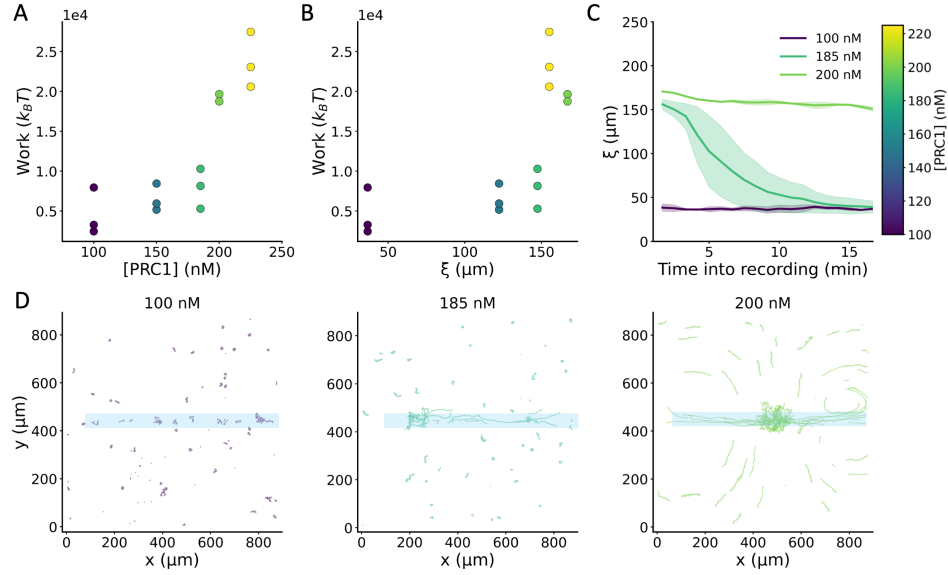


Figure 2.2: Connectivity is not sufficient for mechanical work. **A**, Mechanical work, reported in units of $k_B T$, as a function of PRC1 concentration. Work remains low at low and intermediate PRC1 concentrations and increases at higher PRC1 concentrations. **B**, Mechanical work as a function of velocity correlation length ξ . Networks with similar ξ can produce either low or high work, showing that correlation length and network connectivity alone do not determine mechanical performance. **C**, Correlation length ξ over recording time for 100, 185 and 200 nM PRC1. At 100 nM PRC1, ξ remains low throughout the recording. At 200 nM PRC1, ξ remains high and stable. At 185 nM PRC1, ξ begins in the global range but decays during motor-driven coarsening, separating connected low-work networks from connected high-work networks. **D**, Tracer bead trajectories during contraction at 100, 185 and 200 nM PRC1. Low-PRC1 networks produce only short bead displacements. Intermediate-PRC1 networks move beads primarily near the illuminated region. High-PRC1 networks displace beads from distances greater than 400 μm , indicating long-range force propagation through the active network.

Disconnected networks showed little structural response to the rectangular pattern. Microtubule fluorescence remained diffuse throughout the recording, and intensity profiles along the center line stayed nearly flat. The absence of material accumulation across the illuminated region matched the short correlation length and low work output observed in low-connectivity networks (Figure 3A, B).

Connected low-work networks responded to motor activation but did not preserve the rectangular geometry. Microtubules accumulated into sparse branched structures within the illuminated region before collapsing into a compact aggregate. The corresponding line profiles developed localized peaks rather than a broad plateau across the pattern. The network therefore organized motor-driven remodeling over a larger length scale, but the remodeling remained concentrated along sparse paths instead of spreading across the full activated region (Figure 3C, D).

Intermediate networks partially preserved the imposed geometry. Microtubule fluorescence increased across the rectangular region, and the line profiles broadened relative to the connected low-work state. The contraction remained heterogeneous, placing these networks between sparse-path remodeling and shape-preserving contraction (Figure 3E, F).

Connected high-work networks preserved the imposed geometry before contraction. Microtubule fluorescence filled the rectangular region, the line profiles developed a broad plateau with sharp boundaries, and the network subsequently contracted into a compact central structure. The shape-preserving intermediate state indicates that high-work connected networks distribute motor-driven remodeling across the activated region before focusing material into the final contracted state (Figure 3G, H).

A semiflexible-network model separates connectivity from rigidity.

We built a spring-network model to test whether connectivity and rigidity can emerge at different crosslinking thresholds in a cytoskeletal-like network. Following models of crosslinked semiflexible polymer networks (Head, Levine, and F. C. MacKintosh, 2003; Broedersz and Fred C. MacKintosh, 2014), we placed fibers randomly in a periodic domain and treated geometric intersections as potential crosslinks (Figure 4A). We then activated a fraction p_{xl} of those intersections, applied a small shear strain, relaxed the network and measured two outputs: the giant component fraction S , which reports network connectivity, and the shear modulus G , which reports rigidity. In this model, p_{xl} represents effective crosslink

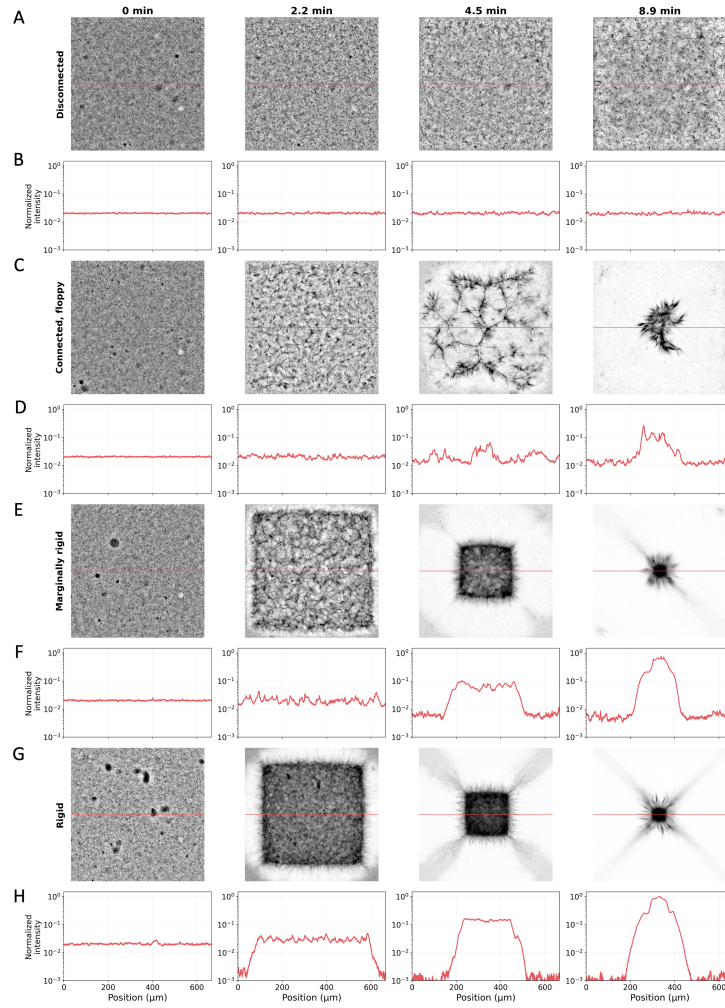


Figure 2.3: High-work connected networks preserve imposed geometry during motor-driven contraction. **A, B**, Disconnected networks show little structural response to rectangular light activation. Microtubule fluorescence remains diffuse over time, and normalized line profiles along the red line remain nearly flat, indicating minimal material recruitment across the imposed geometry. **C, D**, Connected low-work networks respond to motor activation but do not preserve the rectangular geometry. Microtubules accumulate into sparse branched structures inside the illuminated region before collapsing into a compact aggregate. The corresponding line profiles develop localized peaks rather than a broad plateau across the pattern. **E, F**, Intermediate networks partially preserve the imposed geometry. Microtubule fluorescence increases across the rectangular region, and the line profiles broaden relative to connected low-work networks, but contraction remains spatially heterogeneous. **G, H**, Connected high-work networks preserve the imposed geometry before contraction. Microtubule fluorescence fills the rectangular activation region, the line profiles develop a broad plateau with sharp boundaries, and the network subsequently contracts into a compact central structure.

density and serves as the model analogue of increasing PRC1-mediated microtubule crosslinking, rather than a direct calibration of PRC1 concentration.

Increasing p_{xl} produced two distinct transitions. At low p_{xl} , the network remained fragmented into disconnected clusters. At intermediate p_{xl} , the giant component fraction S increased sharply, indicating that the network had become connected. The shear modulus G , however, remained near zero. Thus, the model produced a connected-but-floppy regime: a spanning network existed, but the network did not yet behave as a rigid material. At higher p_{xl} , $G/G_{\max}$ increased, and the participation ratio rose (Figure 4B). The increase in participation ratio indicates that elastic energy became distributed across the network rather than concentrated along sparse paths.

The bending-stiffness control identifies why connectivity alone is insufficient in the model. The parameter κ_b denotes the bending stiffness of the modeled fiber segments. It penalizes changes in angle along the fiber backbone; it is not a measurement of geometric curvature in the fluorescence images. With finite fiber bending stiffness, increasing p_{xl} produced both the connectivity transition and the later rigidity transition. When fiber bending stiffness was removed by setting $\kappa_b = 0$, the giant component still formed, but the shear modulus remained zero across all crosslink fractions (Figure 4C). Thus, central-force connectivity creates a connected network, but the connected network becomes rigid only when the fiber backbone can resist bending.

This bending-stiffness control is important for interpreting the PRC1 experiments. PRC1-mediated crosslinking can create paths through which motor-driven motion becomes organized over micrometre length scales. A connected path alone, however, does not guarantee that the network can bear stress. In the model, the connected-but-floppy regime provides a mechanical analogue of the connected low-work state observed experimentally: the network spans the system, but deformation localizes along sparse paths and the material produces little elastic resistance. The connected-and-rigid regime provides a mechanical analogue of the connected high-work state: the network spans the system and distributes elastic response across the material.

We next asked whether experimental microtubule/PRC1 architectures showed the same separation between connectivity and rigidity. We extracted skeleton networks from fluorescence microscopy images across PRC1 concentrations and computed their mechanical response using the same spring-network framework. The resulting quantity is an image-derived spring-network modulus, not a direct bulk rheology

measurement. The model-predicted modulus increased across the PRC1 series, whereas the giant component fraction alone did not monotonically predict the mechanical response. A second, higher-recall network extraction method preserved the same ordering. These image-derived networks support the model interpretation: PRC1-mediated crosslinking first creates connected networks, but only networks that cross a second rigidity threshold convert motor-driven organization into substantial work.

Force-propagating active matter powers cell transport and droplet motility.

The active network's ability to generate force and transport material suggests its potential as an engine and transport agent for biological processes and future bio-robots. To demonstrate this capability with biological materials, we investigated the movement of suspended human Jurkat cells within the active cytoskeleton network. Upon light activation, a contracting global-phase aster formed and began capturing cells. Using dynamic light patterns, we directed the aster to move 1 mm while collecting cells along its path (Figure 5A, B). As the aster moved, its cross-sectional area increased linearly, aiding cell retention (Figure 5C). When approaching a cell, aster inflows generated power in the range of 10–59 nW, driving the cell toward the aster center (Figure 5D).

The global-phase aster captured multiple cells simultaneously. Initially, it captured only cells within the light-activated region. As incubation time and aster size increased, material outside this region was rapidly recruited, expanding the capture range. Of 32 captured cells, only 2 were released during transport, both from the aster arms. Cells in the aster core were consistently transported throughout the experiment, suggesting structural differences between the arms and core. The randomly crosslinked structure of the core may provide greater carrying capacity by forming a more rigid scaffold that retains cells more effectively than the arms. Cells that were not captured showed minimal movement compared with captured cells (Figure 5E). The precise control and localized force generation of this active matter system, operating in the 10–59 nW range, make it well suited for delicate tasks such as single-cell manipulation, enabling gentle transport or positioning of individual cells within microfluidic devices without the risk of photodamage or electrical disruption associated with other methods (Xie and Jian, 2020; Eriksson et al., 2007).

In the biological context, cytoskeleton-generated active fluids have been proposed to

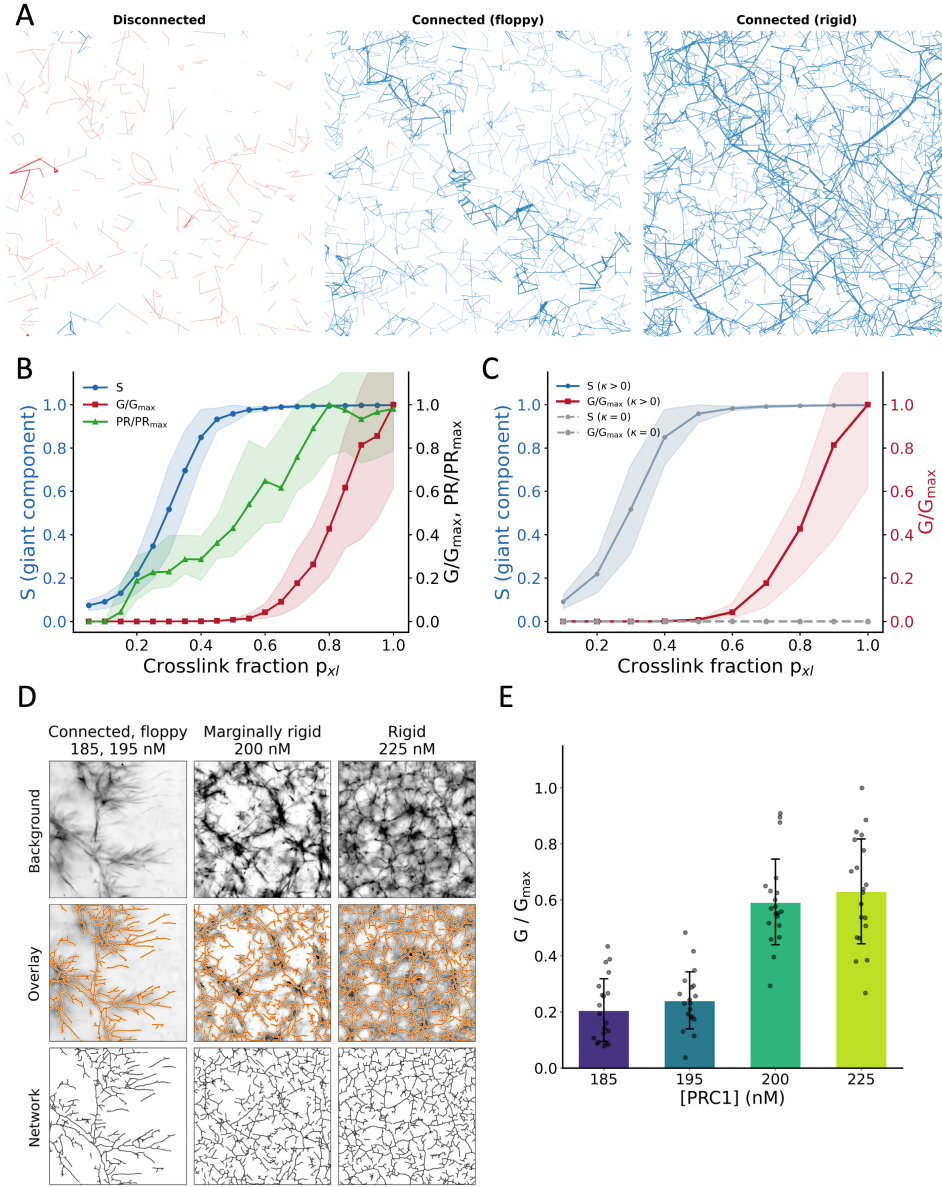


Figure 2.4: A semiflexible-network model separates connectivity from rigidity.

A, Representative spring-network configurations at increasing crosslink activation fraction p_{xl} . Fibers are placed randomly in a periodic domain, and geometric intersections define potential crosslinks. A fraction p_{xl} of potential crosslinks is activated. The model transitions from disconnected clusters to a connected-but-floppy network and finally to a connected-and-rigid network. **B**, Giant component fraction S , normalized shear modulus $G/G_{\max}$ and normalized participation ratio $PR/PR_{\max}$ as a function of p_{xl} . The giant component forms at lower p_{xl} than the onset of finite shear modulus, producing a connected-but-floppy regime. The rise in participation ratio marks the redistribution of elastic energy across the network after rigidity onset. **C**, Fiber bending stiffness is required for rigidity in the model. With finite fiber bending stiffness $\kappa_b > 0$, increasing p_{xl} produces separated connectivity and rigidity transitions. With $\kappa_b = 0$, the giant component still forms, but G remains zero across all p_{xl} . Thus, central-force connectivity alone does not make the model network rigid.

contribute to cytoplasmic streaming, which can enhance cellular transport. To mimic this biological process, we encapsulated our active cytoskeleton network in aqueous droplets emulsified in oil. When activated by light, the active cytoskeleton network did not propel the aqueous droplet in either the global phase or the local phase (Figure 5F). However, outside our currently defined phases, we were able to generate directed droplet movement by light activation, and the movement quickly stopped when the light was turned off. In this phase, the active network did not contract into global or local asters but instead exhibited a highly crosslinked state with minimal contraction. We hypothesize that the crosslinked network created localized tension at the water–oil interface, resulting in Marangoni flow that propelled the droplet forward.

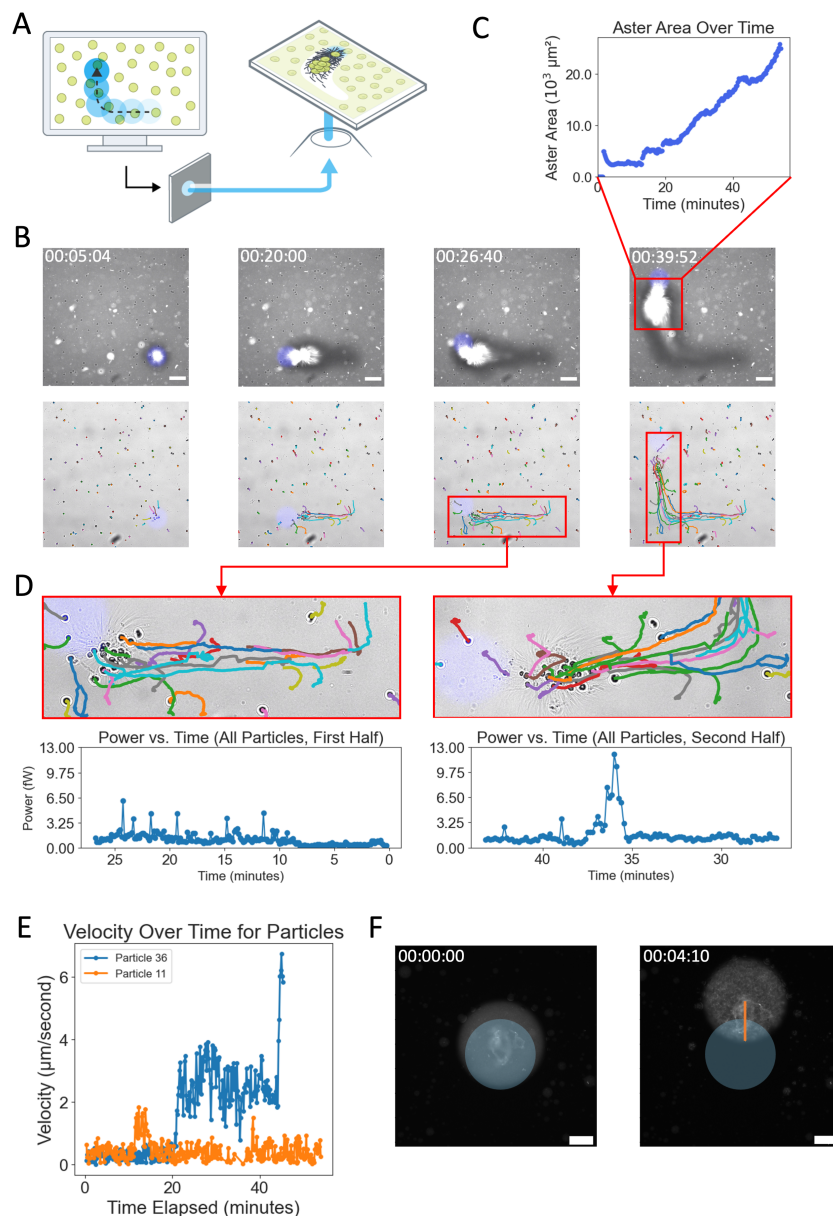


Figure 2.5: Aster-based active cell transport. **A**, Schematics of cell transport using active matter. Briefly, in a field with active matter and cells, we used a dynamic light pattern similar to the one in Figure 3B to recruit and transport cells using global-phase asters. **B**, Time-lapse images of an aster (top) concentrating and transporting Jurkat T cells (bottom). Scale bar, 100 μm . **C**, Aster area increases as aster displacement increases due to constant recruitment of microtubules. **D**, Power generated by moving cells with respect to time. Briefly, we calculated the force using the same method as in Figure 2B by using the Stokes equation and cell tracking. Power is calculated as $P = W/\Delta t$. **E**, Example cell velocity of a stationary cell (orange) versus a cell captured by the aster (blue). **F**, Proof-of-concept motile droplet powered by a contracting active cytoskeleton. Blue light indicates the illuminated region, and the orange line represents the trajectory of the droplet. Scale bar, 100 μm .

Discussion

In this paper, we used PRC1 to tune network connectivity in a light-controlled microtubule–kinesin active material. Increasing PRC1 concentration shifted the system from a local force-dissipating phase to a global force-propagating phase, as measured by microtubule recruitment and velocity correlation length. The same PRC1 series also revealed a second threshold. Intermediate-PRC1 networks organized motor-driven motion over micrometre length scales but displaced beads weakly and produced little work. High-PRC1 networks preserved the imposed activation geometry, displaced beads hundreds of micrometres from the activation region and produced substantially more work. The resulting rule is simple: connectivity organizes motor activity, but rigidity turns motor activity into work.

This rule refines the material–machine picture of active cytoskeletal matter. Kinesin supplies local ATP-driven sliding forces, but the network determines whether those forces dissipate, reorganize nearby filaments or propagate through the material. In a passive material, crosslinks determine how an externally applied load is distributed. In an active material, the load is generated internally, so every motor event is both an actuation and a perturbation to the network that receives it. PRC1 therefore acts not as a force generator, but as an architectural control parameter that changes how kinesin-generated stress is routed through the microtubule network.

The semiflexible-network model explains why connectivity alone is insufficient. Increasing crosslink fraction first creates a connected giant component and only later produces a rigid network with finite shear modulus. This places the PRC1-tuned active material in the broader mechanics of crosslinked semiflexible networks, where crosslink density, filament stiffness and network geometry determine whether a connected structure deforms through floppy modes or behaves as an elastic solid (Head, Levine, and F. C. MacKintosh, 2003; Broedersz and Fred C. MacKintosh, 2014). The bending-stiffness control in the model is not a direct measurement of microtubule curvature in the experiment. It identifies the mechanical principle needed to interpret the data: central-force connectivity can create a spanning structure without creating a load-bearing material.

The same connectivity–rigidity distinction may be a general biological strategy. Cells rarely regulate mechanical output by changing motors alone. They also tune crosslinker concentration, crosslinker lifetime, filament polarity, overlap length, turnover and boundary conditions. Mitotic spindles provide one example: connected microtubule networks and gel-like states have been linked to long-range microtubule

transport and coherent flows (Benjamin A. Dalton et al., 2022). Our reconstituted system isolates a minimal version of this principle with one biologically specific crosslinker and a fixed motor input. PRC1-tuned connectivity sets the length scale over which motor activity becomes organized; rigidity determines whether the organized network can propagate force and do work.

Our result suggests a synthetic-biology route to programmable active materials. In previous active-matter programming work, light patterns controlled where active matter generated flows and enabled composition of transport, separation and mixing modules (Yang et al., 2022). Here, the programming layer is internal. Before the optical pattern specifies where motors act, PRC1 specifies what kind of material receives the motor stress. Future crosslinkers could expand this molecular control layer. Crosslinker valency could tune connectivity, binding lifetime could tune how long force-transmitting paths persist, linker compliance could shift the rigidity threshold and polarity selectivity could encode contraction geometry. Crosslinkers therefore do more than glue filaments together; they determine whether an active material behaves as a local dissipater, a collectively remodeling network or a work-producing machine.

Several limitations define the next steps. PRC1 concentration likely changes several microscopic variables at once, including overlap number, overlap lifetime, frictional resistance, bundle architecture and motor accessibility. Direct rheology or calibrated microrheology would test whether the model shear modulus corresponds to an experimental rigidity transition. PRC1 turnover measurements would determine how crosslink lifetime competes with motor forcing. Engineered PRC1 variants or synthetic crosslinkers would test whether connectivity and rigidity can be tuned independently. Three-dimensional networks and dynamic microtubules would test whether the same rule survives in geometries closer to cells. Controlling this connectivity-to-rigidity switch would help move reconstituted cytoskeletal systems beyond self-organization toward programmable biomolecular machines: motors provide the engine, crosslinkers define the transmission and light specifies the task.

Chapter 3

DYNAMIC FLOW CONTROL THROUGH ACTIVE MATTER PROGRAMMING LANGUAGE

Fan Yang, Shichen Liu, Heun Jin Lee, Rob Phillips, and Matt Thomson

Abstract

Cells use “active”, energy-consuming motor and filament protein networks to control micron-scale transport and fluid flows. Biological active materials offer a route to dynamically programmable devices that convert chemical energy into work at molecular length scales. However, reconstituted motor-microtubule systems exhibit non-linear dynamics and generate chaotic flows (K.-T. Wu et al., 2017), so they cannot be directly harnessed for engineering applications. Here, we develop a programming strategy for biological active matter that allows us to construct micron-scale fluid flow fields for transport, separation, and mixing through the modular composition of light-controlled programs. We circumvent non-linear dynamic effects within the active fluids by limiting hydrodynamic interactions between contracting motor-filament networks that we pattern with light. Using a predictive mathematical model, we design flow fields to accomplish a series of canonical microfluidic tasks and apply the flow fields experimentally to transport and separate cell clusters, probe the extensional rheology of polymers and giant lipid vesicles, and generate mixing flows at low Reynolds numbers. Our findings show that active matter can be used as an engineering material for dynamic micron-scale flow control.

The control of micron-scale transport and fluid flow is a foundation of modern technology including synthetic chemistry, DNA sequencing and single-cell genomics (Stone, Stroock, and Ajdari, 2004; Whitesides, 2006). Conventional transport control technologies including microfluidic devices generate force and flow at macroscopic length scales using pumps and vacuum manifolds, and then transmit force to microscopic length scales through channels that are fabricated in PDMS through soft lithography to control the fluid flows. Many of the challenges associated with micron-scale manipulation in conventional microfluidics stem from the fact that energy cascades down from macroscopic scales to microscopic scales. Specifically, flow fields generated by pumps are sculpted by passive channels fabricated for one task and cannot be dynamically reprogrammed for a new task. Further, flow

fields are shaped by channel geometry so that imperfections in design can lead to instabilities and intensive design and iteration cycles.

Biological systems achieve spatio-temporal control over fluid transport by inverting the flow of energy relative to current microfluidic technologies. Cells generate force and flow at molecular length scales through the hydrolysis of ATP by motor and filament proteins within “active”, energy consuming, cytoskeletal structures (Gilpin, V. N. Prakash, and M. Prakash, 2017). Motor and filament proteins generate molecular-scale motion which, then, becomes organized on macroscopic micron length scales through self-organization. By generating force on molecular length scales, cells can control the geometry of fluid flow fields with micron-scale precision for cell movement, internal transport, and foraging (Kamiya, 1981; Glotzer et al., 1997; Forrest and Gavis, 2003; D. Needleman and Shelley, 2019; Stein et al., 2021). Further, cells can modulate the geometry of active structures in time to dynamically reprogram flow fields to respond to the environment.

Thus, biological active matter could be used to program and dynamically control fluids, because purified motor-filament proteins can generate micron-scale flows in solution (Suzuki et al., 2017; K.-T. Wu et al., 2017; Stein et al., 2021). The dynamics of active matter can also be controlled with light, providing a route to optically programmable micron-scale transport (T. D. Ross et al., 2019). Active-matter-based flow control could execute dynamic micron-scale tasks including transport, separation, sorting and mixing. However, active fluids are difficult to control because non-linear active stresses couple active matter to solvent flows (K.-T. Wu et al., 2017). Previous work (K.-T. Wu et al., 2017) demonstrates that active fluids can exhibit “active turbulence”, where generated flow fields contain vortices and other transient structures with similarities to macroscopic turbulence. Active flows can be controlled through microfluidic chambers with designed boundaries (K.-T. Wu et al., 2017), but prefabricated geometries limit the application of geometrically controlled active fluids and cannot capture the dynamic spatio-temporal modulation of flows induced by active matter in biological systems (Gilpin, V. N. Prakash, and M. Prakash, 2017; Mathijssen et al., 2019). New control strategies for active fluids could therefore provide insight into the physical principles underlying force-generating active fluids.

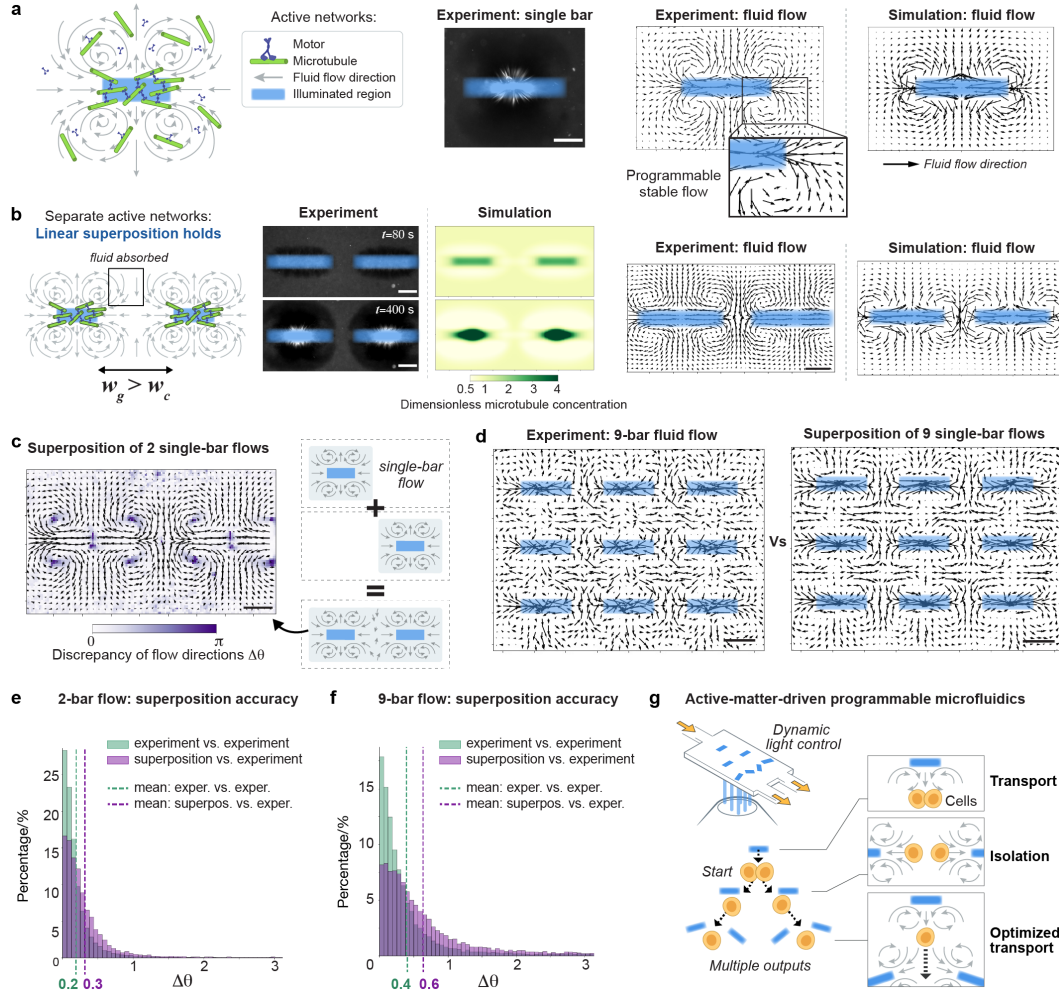


Figure 3.1: Linear superposition quantitatively predicts fluid flow fields induced by optically-controlled active matter. **a**, Under illumination, light-activatable motors can link to form tetramers and crosslink microtubules into a network. The contractile active network generates flows in the surrounding fluid which is absorbed lengthwise into the light bar and pumped out widthwise. **b**, Above a critical spacing w_c , the flow field generated by two light bars is a linear superposition of two single-bar flows. **c**, Comparison of the measured two-bar flow field with superposition of two single-bar flows shows that the linear superposition holds quantitatively. The heatmap represents the angle $\Delta\theta \in [0, \pi]$ between measured and superposed flows. **d**, Measured 9-bar flow fields and superposition of 9 single-bar flows to assemble a 9-bar-array flow. **e** and **f**, Superposition provides high-accuracy flow field prediction for both 2-bar (**e**) and 9-bar (**f**) compositions, with errors comparable to variations in experimental replicates. **g** Linear superposition enables construction of active-matter-driven programmable microfluidics. The sketch shows an conceptual application using assembly of light bars to transport and separate cells. All scale bars are $100 \mu\text{m}$ and all flow fields are time-averaged over 240 s.

Here, we develop a spatio-temporally flexible method for modular design and construction of micron-scale flow fields using light-controlled biological active matter. We use an engineered system in which motor protein activity is modulated by light (T. D. Ross et al., 2019). Although active-fluid dynamics are generally non-linear, active-matter-powered flows can be composed through superposition when illuminated regions are sufficiently isolated. By composing “primitive” flow fields generated by a single static or moving rectangular light bar, we generate flow fields that transport, stretch and separate micron-scale particles. We apply superposition-based flow programming to extensional rheology of polymers and giant lipid vesicles, and micron-scale manipulation tasks on primary human cells, such as separating an unconstrained cell cluster into individual cells *in situ*. The advantages of our system are: there are no requirements on precise channel design and microfabrication, PDMS lithography or pressure-pump control; our system can generate local flows around objects of interest without disturbing other regions in the channel; different programming modules can be additively assembled for specific transport tasks, enabling streamlining operations and multi-tasking in a single channel; the system also allows us to move and control primary human cells, providing a potential platform for programmable manipulation of particles in biology and chemistry.

Flow programming through linear superposition of light bars

To harness the capabilities of biological active matter for technology applications, we seek to develop a modular framework where we can compose a basic set of primitive light patterns, such as rectangular bars, to generate flows that can achieve functions including transport, stretching and mixing. Our inspiration is Stokes flows, where inertial effects can be neglected and fluid dynamics can be described by a linear partial differential equation (PDE) known as the Stokes equation, $\mu \nabla^2 \mathbf{u} - \nabla p = \mathbf{0}$, with $\mathbf{u}$ the flow velocity, μ the viscosity and p the pressure. The amazing power of the dynamic linearity in Stokes flows is that, if we know the flow field generated by a single point source, then we can compose points sources and the resulting flow field can be simply predicted through addition of flow fields generated by these point forces individually (Leal, 2007). Superposition provides a substantial simplification for predicting and programming flows through simple addition of point sources. However, in our system we are sculpting flows using active-matter-generated forces, and in general, linear superposition does not hold for active fluids due to their inherent non-linearity and apparent disorder (M. C. Marchetti et al., 2013; K.-T. Wu et al., 2017).

Our experimental system consists of stabilized microtubules, and kinesin motor proteins that have been engineered to reversibly “link” in the presence of blue light by fusing motors to optically dimerizable iLID proteins (Guntas et al., 2015a; T. D. Ross et al., 2019). The kinesin K401 in our system is a homodimer that can bind on one microtubule in the dark state. Upon illumination with blue light, the kinesin motors form tetramers and can bind on two microtubules. To avoid confusion, we term the homodimer and tetramer motors “unlinked” and “linked” motors, respectively. In previous work (T. D. Ross et al., 2019), we have demonstrated that light induction generates contractile motor-filament networks that induce spontaneous fluid flows within the system. In the Supporting Information, we formulate a continuum model that can quantitatively predict dynamics of active matter and solvent flows. Our model is a three-phase complex fluids model, and the three phases are crosslinked microtubules, freely-moving microtubules and solvent fluid. The crosslinked microtubules are modeled as a viscoelastic gel, which self-contracts driven by its internal active stresses; the freely-moving microtubules are passive particles carried by both the gel and the solvent flow; the solvent flow is generated by the contraction of the active gel and balanced by the hydrodynamic resistance in the flow cell (Supplemental Information).

In our model, the solvent flow is governed by the Stokes equation with a driving force applied by the motion of crosslinked microtubules:

$$\gamma c(\mathbf{v} - \mathbf{u}) + \mu \nabla^2 \mathbf{u} - \nabla p = 0, \quad (3.1)$$

where c and $\mathbf{v}$ are the spatially varying concentration and velocity of the crosslinked microtubules, respectively, and γ is the drag coefficient between the microtubules and the solvent. In equation (1), $\gamma c(\mathbf{v} - \mathbf{u})$ acts as a field of point forces applied upon the fluid. However, unlike the body-force-free Stokes equation, which is a purely linear, time-independent PDE, active fluids are transient and non-linear. Furthermore, the microtubule concentration field c is also carried by the solvent flow $\mathbf{u}$ (Supplemental Information), so that the microtubule network at a position $\mathbf{x}_i$ experiences dynamics due to the long-range flows induced by microtubule network at positions $\mathbf{x}_j$ in the system. In general c is therefore both time-dependent and also an implicit function of the ambient flow field, $\mathbf{u}$. Fundamentally, the problem is that microtubule networks activated at different locations within the system interact through fluid flows, and flow-induced interactions lead to transport of the microtubule network giving rise to non-linear stresses and transport phenomena within the active fluids.

We find that superposition can be restored in the model by restricting interactions among spatially isolated regions of light signals. Mathematically, we show that the flow field $\mathbf{u}_i$ generated by a single network i decays as a power law $x^{-3.5}$ with x the distance to the bar center. When another network j is placed beyond a cut-off distance such that $c_j \mathbf{u}_i = 0$ (Materials and Methods), superposition is recovered, and the flow field $\mathbf{u}$ generated by a system of spatially isolated light patterns can be predicted through simple linear superposition of individual forces in equation (1), that is, $\gamma c(\mathbf{v} - \mathbf{u}) = \gamma \sum_i c_i(\mathbf{v}_i - \mathbf{u}_i)$, where the subscript i indicates the microtubule concentration, velocity and the solvent velocity induced by a single network i , in the absence of other networks (Materials and Methods). This tells us that to maintain linearity in a system with multiple active agents at low Reynolds number (Re), the flow fields induced by each agent should decay to very small at the locations of other agents comparing to their self-generated velocities. Therefore, we develop a modular programming strategy where we minimize long-range interactions by positioning the isolated light patterns far enough from each other so that the activated networks only weakly interact.

Linear superposition principle enables quantitative prediction of experimental flow fields

Consistent with our theory, we find experimentally that the flow field generated by two rectangular light bars can be predicted by superposition when the bars are separated above a critical spacing. We use a rectangular light bar as the basic unit of programming design, which dynamically functions as a microfluidic pump: the active network absorbs fluid lengthwise and pumps it out widthwise generating four counter-rotating vortices (Fig. 3.1a). We test our principle for linear superposition with two light bars placed side by side. When their gap width w_g exceeds a critical spacing w_c , the active networks self-contract within their respective illuminated regions (Fig. 3.1b). The resultant microtubule and flow fields (Fig. 3.1b) are, at least qualitatively, linear superposition of two single-bar fields (Fig. 3.1a).

We find that linear superposition of single-bar flows can quantitatively predict flow fields generated by multi-bar compositions. We additively assemble the single-bar flow fields (Fig. 1a) to construct 2-bar (Fig. 1c) and 9-bar (Fig. 1d) fluid flows, and plot the discrepancy of flow directions with measured flows in Fig. 3.1e and Fig. 3.1f, respectively. There are mainly two sources of discrepancy: the errors induced by superposition and the experimental variations from inherent thermal fluctuations and variations in concentration and activity of the proteins

during the preparation process in independent experiments. To distinguish them, we compare the distributions of discrepancy from superposition with the distributions of experimental variations (Figs. 1e and 1f), which show that the errors induced by superposition are comparable to the intrinsic experimental variations. Similarly we also compare the discrepancy of flow magnitudes in superposition of 2-bar flows with experimental variations (Fig. B.7, Supplemental Information). The mean fractional change of magnitudes induced by superposition is 0.31, only slightly above the mean in experimental variations, which is 0.23. The small superposition-induced errors in both flow magnitudes and directions demonstrate that linear superposition can be achieved quantitatively in multi-bar compositions. Linear superposition is long thought to be impossible in active matter systems, while in our experiments it is made possible by confining the active matter within the illuminated regions. Outside the light regions, microtubules and unlinked motors do not crosslink and dynamic linearity of Stokes flows still holds. Linear superposition is the foundation of constructing a modular programming language for microfluidic control (Fig. 1g). In our control strategy, only fluid flows outside the illuminated regions are utilized for transport tasks (Fig. 1g).

Hydrodynamic interactions between active networks determine superposition length scales

The linear superposition fails when the spacing between light bars are below a critical length. The active networks move outside the light regions and eventually merge (Fig. 2a). Our continuum model allows us to determine how the critical spacing originates from hydrodynamic interactions between active networks. The rectangle active networks absorb fluid flow along the long axis (x -axis in Fig. 2b), and therefore, can attract neighboring networks through hydrodynamic interactions. The flow decaying with distance provides a mechanism for whether the two active networks will merge. In the 2-bar composition (Figs. 1b and 2a), each network generates flow that propagates to the neighboring network. The flow field generated by one network, therefore, exerts force on the neighboring network leading to flow-induced drift that is also counteracted by activity-induced self-contraction. When bars are placed close enough, within a critical length w_c , the flow generated by one network is sufficient to move the other network out of the illumination region against self-contraction (Fig. 2a).

How flow decays with the distance to the bar center is essential in determining the critical spacing, which is found to follow a power law. The intensity of a flow field

generated by a given rectangular network determines the network spacing required for superposition to hold. We first conduct simulations and experiments with two light bars of different sizes and similar aspect ratios (Fig. 2b). The scaling of the flow magnitude is the same for both bars, which grows linearly and decays as $(x/w)^{-3.5}$ within and outside the illuminated region, respectively, where w is the bar length. The simulations are in quantitative agreement with experiments, except for a small region near the edge of the larger light bar. The flow magnitude drops slightly in this region, which may suggest that a boundary layer is developed here when the light bar is large enough. The flow data for two sizes of bars collapse onto each other when the distance x is normalized by the bar length w (Fig. 2b), demonstrating that w is the length scale of flow variation. In practice, a spacing of half of the bar length $w/2$ is usually enough to avoid merging of networks as the flow already decays by more than 98% at $x = 3w/2$. Note that the estimate $w_c \sim w/2$ shows that the critical distance grows linearly with the bar length. However, the factor $1/2$ is an overestimate and can guarantee linear superposition in a wide range of bar scales and aspect ratios in practice. The exact values of w_c also depend on the flow conditions, such as the solvent viscosity and the flow chamber height as discussed later. To test the generality of superposition, we also compute the effects of aspect ratios of light bars on the flow decay. Numerical results reveal that the flows outside the illuminated regions always decay in a power law, with the exponents ranging from -4 to -3.5, regardless of the aspect ratios (Fig. B.2, Supplemental Information). This demonstrates that linear superposition can be generalized to different bar sizes and shapes. Additionally, we perform superposition of experimental flow fields generated by pentagonal light patterns in Fig. B.8 in the Supplemental Information to show that the principle of linear superposition is not limited to rectangular light shapes.

To test how the flow intensity affects the critical spacing, we compute a phase diagram of two networks with different gap widths and flow intensities (Fig. 2c). The flow intensity is tuned by a dimensionless parameter $\bar{\zeta} = c_0\gamma h^2/12\mu$, where c_0 is the initial microtubule concentration and h is the height of the flow cell. The parameter $\bar{\zeta}$ is found to be the most important dimensionless number governing the flow intensity in our model (Eqn B.28, Supplemental Information). The physical meaning of $\bar{\zeta}$ is the ratio of the driving force, $c_0\gamma$, and the hydrodynamic resistance in the flow cell, $12\mu/h^2$. The phase diagram (Fig. 2c) shows that the critical spacing required for superposition increases with the flow intensity. The reason is that as the flow intensity generated by network one increases, network two, its neighboring

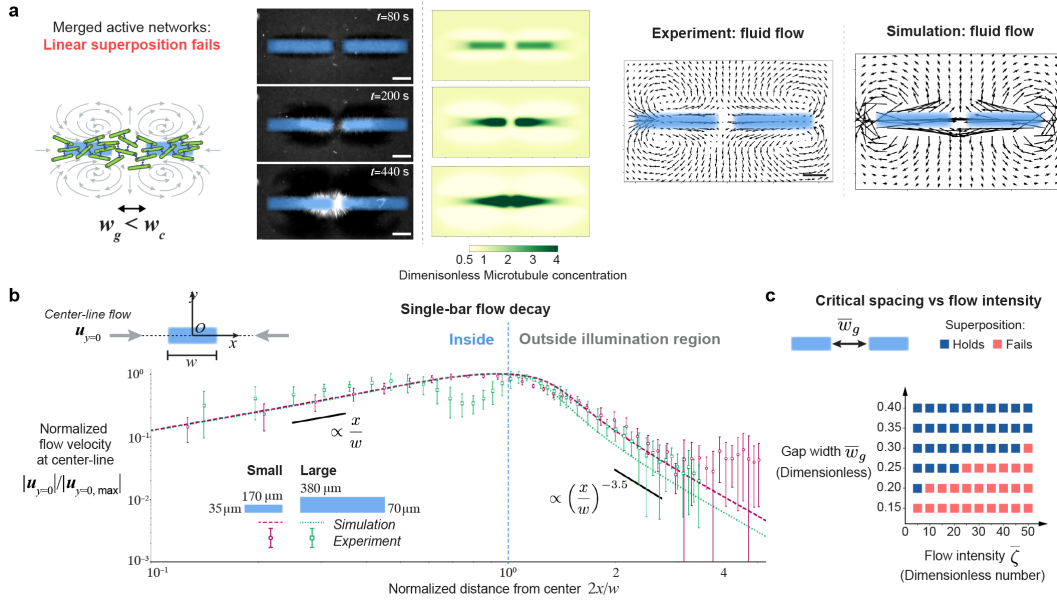


Figure 3.2: **Superposition length scale is set by hydrodynamic interactions between active networks.** **a**, Experimental and simulated results of microtubule and solvent flow fields. The simulated microtubule concentration is normalized by its initial value. Below the critical spacing, linear superposition of flow fields fails. The active networks move towards each other and eventually merge. Scale bars, 100 μ m. **b**, The center-line flow profile induced by a single bar. **c**, Simulated phase diagram of superposition shows that the critical spacing increases with the fluid flow intensity. All flow fields are averaged over 240 s.

network, should be placed farther away. This ensures that the flow induced by network one decays to be no longer sufficient to drag network two outside the illuminated region.

Design of multi-bar flow fields for transport and stretching with numerical optimization

Superposition opens up a convenient route to design of flow fields using optimization via translation, rotation and summation of single-bar flow data. We study an optimization problem of using three light bars to transport particles along a line segment AB (Fig. 3.3a). The objective function f is defined as the line integral of fluid flows along AB (Fig. 3.3a). To ensure the particle transport along a straight path, the line segment AB should be placed at the axis of symmetry of the bar configurations (Fig. 3.3b). Using simulated flow data, we can plot the optimization landscape in Fig. 3.3c. Each point in Fig. 3c is already optimized over the orientation angle (Supplemental Information). The optimal solution, consisting of

the optimal x -, y -coordinates and the optimal orientation angle of each light bar, can be found by the maximum value in the optimization landscape, labeled by a star in Fig. 3.3c (Supplemental Information). We test this optimal solution in experiments by comparison with non-optimal three-bar configurations, and find that the optimal solution transports particles both fastest and furthest (Fig. 3.3d and 3.3e). The results show that active matter programming language is capable of optimizing practical design using only linear transformation of single-bar flow fields.

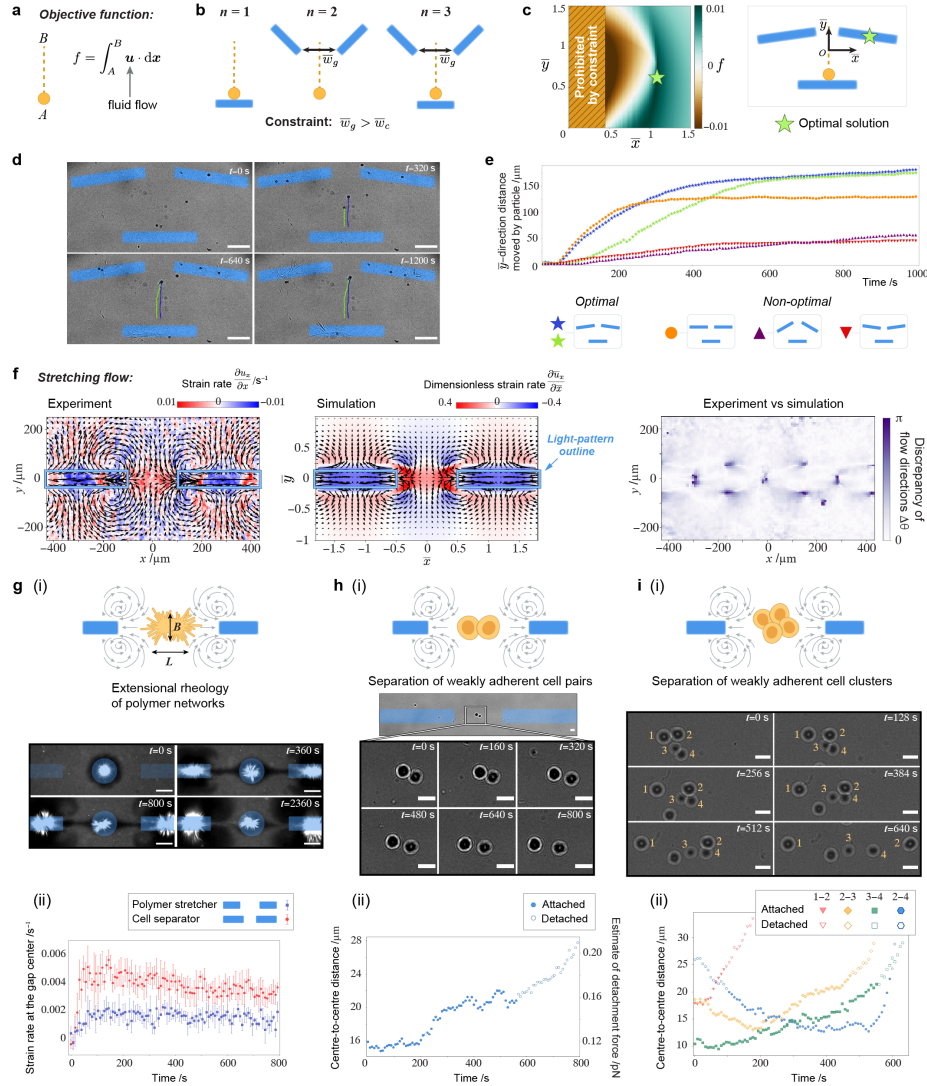


Figure 3.3: Superposition provides a simple design principle for optimized transport of particles, microrheology of polymer and isolation of cells. **a**, The design goal, to move a particle from point A to B, and its corresponding objective function. **b**, The particle path AB should coincide with the axis of symmetry in the composition of light bars. **c**, The optimal 3-bar configuration is determined by the maximum value in the optimization landscape and displayed on the right. **d**, Experimental images of using the optimal light bar configuration (blue) to transport two particles. **e**, Time course of particle displacement in optimal vs non-optimal designs. **f**, Measured and simulated stretching flows generated by two light bars and their quantitative discrepancy. Both flow fields are averaged over 240 s. **g**, (i) Experimental images of a pre-formed microtubule aster being stretched by the elongational flow, which is used to measure rheological properties of the aster; (ii), Measured flow strain rates over time in the polymer-stretching and cell-separating experiments. The strain rates are averaged over a $140 \times 50 \mu\text{m}$ region at the gap center. Error bars represent s.d. of at least 4 measurements. **h**, (i) Snapshots of detaching two cells; (ii), Centre-to-centre distance of the two cells and estimated detachment force over time. **i**, (i) Snapshots of separation of a cell cluster in the stretching flow. (ii) Centre-to-centre distances of cells over time. Due to constraints of image resolution, the time of detachment has an error of ± 30 s. The scale bars

Programming flows for microrheology and cell isolation

To show that active matter programming language can be flexibly applied and motivate new applications, we utilize stretching flows induced by two light bars (Fig. 3.3f) for microrheology of polymers, division of giant lipid vesicles, and manipulation tasks of cells. Rheological properties of polymer networks can be inferred from its deformation parameter $D_f = (L - B)/(L + B)$ and flow strain rates in an extensional flow, where L and B are the length and breadth of the deformed aster respectively (Fig. 3.3g). We use the two-bar flow to stretch a microtubule aster pre-formed with a circular light pattern (Fig. 3.3g). Measurement of strain rates at the gap center shows that the flow is quasi-steady since $t = 100$ s, and the average value is $\partial u_x / \partial x \approx 0.0015 \text{ s}^{-1}$ (Fig. 3.3g). Consequently the shear modulus of the microtubule aster is calculated to be $1 \times 10^{-7} \text{ Pa}$ (Materials and Methods). This shows that our system can be potentially used in extensional rheology where viscoelastic properties of materials are deduced from their deformation profiles under straining (Squires and Mason, 2010), and also in single polymer dynamics (Schroeder, 2018) where single polymers, such as DNA strands (Perkins, Smith, and S. Chu, 1997), need to be stretched *in situ*. To perform these functions, traditional microfluidics usually relies on channel geometry to generate stretching flows (K. M. Foster, Papavassiliou, and O’Rear, 2021), such as sculpting channel contraction (Ober et al., 2013; Mancuso and Ristenpart, 2017) or cross-slot geometries (Perkins, Smith, and S. Chu, 1997). However the former method cannot fix particles in the channel because the flow constantly carry them downstream; the cross-slot or Taylor’s four-mill geometries (Taylor, 1934) can fix the particle at an equilibrium point but it is difficult to move the particle to this point since it is mechanically unstable. By contrast, active matter programming language can be flexibly implemented to stretch particles *in situ*, by projecting light patterns around objects of interest. We also note that our method is not meant to replace state-of-the-art high-end methods like optical tweezers but provides a low-cost and simple strategy for microrheology.

To further demonstrate the versatility of our system in extensional rheology, we show using the stretching module to stretch and divide a giant lipid vesicle (GV) into daughter vesicles in Fig. 3.6 and Video S3. GVs are extensively studied as the basic model for artificial cells (Walde et al., 2010). Division of GVs can be used to probe their mechanical properties and is also crucial to mimic cell division (Miele et al., 2022). At the initial stage ($t \leq 88$ s), we observe the fusion of two giant unilamellar vesicles (GUVs) and an oligovesicular vesicle (OVV) into a larger GV (Fig. 3.6b). OVVs represent the structure of “vesicles in vesicle” (Šegota and

Težak, 2006) and are common byproducts during GV preparation (Materials and Methods) (Giuliano et al., 2021). The solvent flow is relatively small at the initial stage (Fig. B.4, Supplemental Information) and the mechanism of GV fusion is unclear to us. The fused GV then undergoes stretching due to the flow and becomes elongated along the axis of the maximum strain rate (Fig. 3.6c). Between $t = 584$ s and 616 s, the GV is divided into 3 daughter vesicles (Fig. 3.6d). The daughter vesicles are stable in the solvent flow (Fig. 3.6e).

We now show using active matter to separate an unconstrained weakly adherent cell cluster into isolated cells. Separation of cells are central in cell sorting applications (Shields, Reyes, and López, 2015). Systematic study of cell-cell detachment are usually conducted through either atomic force microscopy (AFM) (Benoit et al., 2000) or pulling two cells apart using two micropipettes (Y.-S. Chu et al., 2004; Maître et al., 2012). Both methods require highly-skilled operators and also could harm the cells when constraining them with solid instruments. In contrast our system can generate extensional flows in the vicinity of cell clusters and separate them (Fig. 3.3h and Video S1). The cells used are Jurkat cells, which are a human T cell line and known to express cell adhesion molecules such as integrins and CD2 receptors (Tözeren et al., 1992; Zhao et al., 2022). The local flow strain rates near the cells are around 0.004 s^{-1} (Fig. 3.3g), and the application of flows for 500 – 800 s leads to cell detachment. The detachment force applied by the flow is approximately $3\pi\mu a\Delta l\partial u_x/\partial x$ (Materials and Methods), where $a = 10 \text{ }\mu\text{m}$ is the cell radius, and Δl is the center-to-center distance of the cell pair (Fig. 3.3h). Using the average strain rate 0.004 s^{-1} , the detachment force is proportional to Δl and calculated in Fig. 3.3h. The detachment force generated in our setup, around 0.1 pN, is much smaller than the adhesion force measured by AFM on Jurkat cells, at the scale of 10 pN. This suggests that strong adhesive bonds are not formed in the cell pair in Fig. 3.3h and the cells have the morphology of weakly adherent cells with surface interactions but not cortical associations (van der Merwe and Barclay, 1994; Beri et al., 2020). To confirm whether our setup can separate strongly adherent cells and also to test the viability of cells in the solution, we stain the cells with viability indicators, Ethidium Homodimer-1 (EthD-1) for dead cells and Calcein AM for live cells, as shown in Fig. 3.7. We find that both Jurkat and Raji cells (Methods and Materials) can stay alive in our solution, but the stretching module cannot separate live-cell clusters, due to the weak force that it can provide. This confirms that our setup can only separate weakly adherent cells. However, this “small force” generated by the stretching module can be used for new applications such as isolating dead cells from

live cells. In Fig. 3.7a, we isolate one live cell from a dead-cell pair that are initially close with each other, at a distance of $30\ \mu\text{m}$. Both the live and dead cells follow the solvent flow and move to opposite directions. In Fig. 3.7b, we show isolating a pair of dead cells from a cluster of at least 7 live cells, initially separated by $20\ \mu\text{m}$. The dead cells move to the left while the live-cell cluster is undisturbed because it is too big to be transported by the flow. These demonstrate that the small-force feature of our setup can be also used to separate weakly adherent cells from strongly adherent cell clusters at the micron-scale.

Weakly adherent cell clusters consisting of more than two cells can also be separated *in situ*, revealing more complex collective behaviors. Fig. 3.3i and Video S2 show separation of a four-cell cluster in the extensional flow. In addition to three cell-cell separation events (1-2, 2-3 and 3-4 in Fig. 3.3i), we also observed two initially separated cells (2 and 4 in Fig. 3.3i) first coming into contact and then being separated, through sliding along the membrane of cell 3. New applications like these can be motivated because our system allows for flexible sculpting of local flow fields near particles of interest, as opposed to traditional microfluidics which usually relies on pumps to generate a global flow throughout the entire channel.

Active mixing with rotating bars

Our programming framework can also be extended to dynamic light patterns. Mixing at low Re is a major challenge in microfluidics because flows are laminar in this regime and molecular diffusion is slow (Stroock et al., 2002). We show that a rotating light bar can generate stirring flows to mix the circular region swept by it. Both the crosslinked microtubule gel and the flow field, consisting of four vortices, rotate following the rotating light bar, as shown in Figs. 3.4a and 3.4b, which can also be predicted by our simulations. As the light bar rotates, the microtubule gel dynamically forms and self-contracts into a core. As the light bar is shed on a new location from one pulse to the next, two plumes of newly-crosslinked microtubules are formed at the ends of the gel, which transport both mass and momentum of microtubules to the network core, as well as rotates the more densely-crosslinked network center (Fig. 3.4a). After one cycle, the circular region swept by the light bar will be mixed by the rotating vortices (Fig. 3.4b).

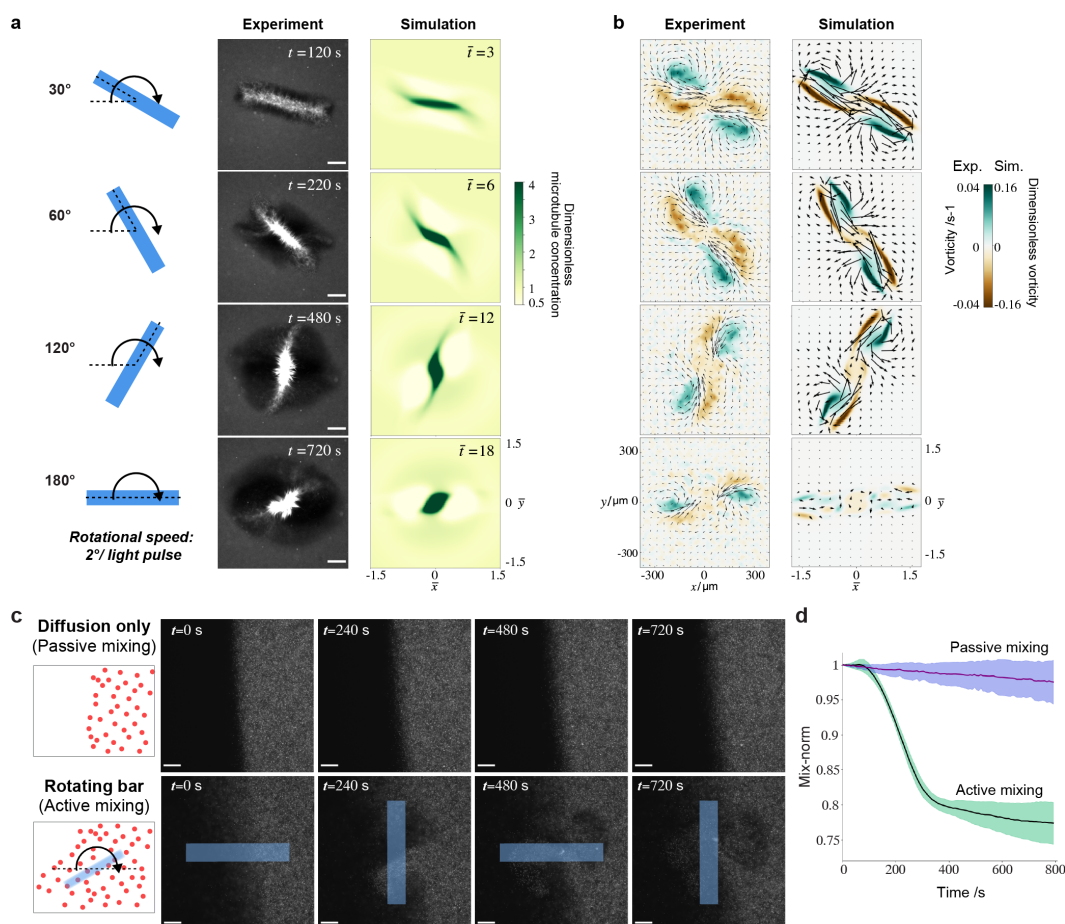
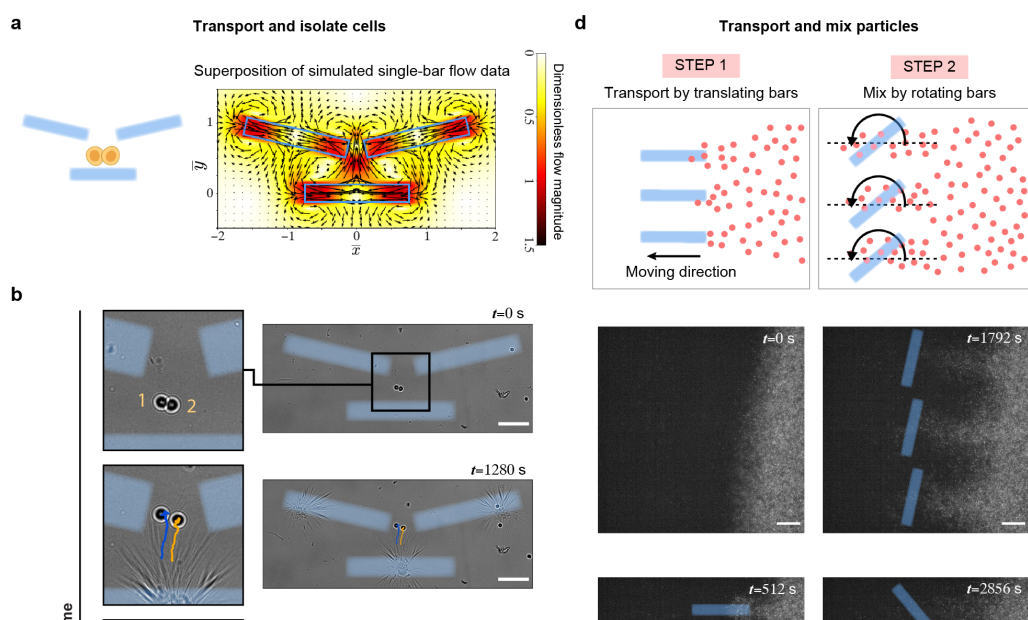


Figure 3.4: **Rotating light bars enables active mixing in microfluidics.** **a**, Experimental and simulated images of microtubules under a rotating light bar. **b**, Measured and simulated flow fields in **a**. The measured flow fields are averaged over five experiments. **c**, A rotating bar can mix particles in the circular region swept by it. The controlled experiment on the top shows that the passive mixing by diffusion is much weaker than the active mixing. **d**, Time courses of mix-norms in active and passive mixing. Data are presented by the mean values $\pm$ s.d. of 5 experiments. Scale bars, $100\ \mu\text{m}$.



We quantify the mixing efficiency of the active mixer made from a rotating bar on fluorescent particles, and find its mixing efficiency significantly higher than the passive mixing by pure diffusion (Fig. 3.4c). The active mixing is mainly accomplished by three flow effects at different length scales. At the length scale of the light bar, w , the rotating bar can push particles from one side of the interface to the other. At the length scale of the vortices, $w/2$, each region occupied by one of the four vortices is mixed by the vortex. At the length scale of the thermal motions of the active microtubules, the small-scale flows can also help randomly mix the particles (K.-T. Wu et al., 2017). To quantify the mixing efficiency at all length scales, we adopt a multi-scale mix-norm (Thiffeault, 2012). For a particle fluorescence intensity field $I(\mathbf{x}, t)$ with a Fourier expansion $I(\mathbf{x}, t) = \sum_{\mathbf{k}} I_{\mathbf{k}} e^{i2\pi\mathbf{k}\cdot\mathbf{x}}$, where $\mathbf{k}$ and $I_{\mathbf{k}}$ are the wave vector and its corresponding Fourier coefficient, respectively. The norm is defined by $\|I(\mathbf{x}, t)\|_H = (\sum_{\mathbf{k} \neq \mathbf{0}} |\mathbf{k}|^{-2} |I_{\mathbf{k}}|^2)^{1/2}$ (Thiffeault, 2012). We further rescale $\|I(\mathbf{x}, t)\|_H$ by its initial value, and define the mix-norm to be $\|I(\mathbf{x}, t)\|_H / \|I(\mathbf{x}, t = 0)\|_H$. A lower mixer-norm represents a better mixing result. The time courses of mix-norms in passive and active mixing are plotted in Fig. 3.4d. At $t=800$ s, the mix-norms in active and passive mixing drop 22% and 2%, respectively, which demonstrates that active mixing is tenfold more effective than passive mixing by diffusion. Another advantage of our system is that it can create localized mixing in a designated region without disturbing its surroundings. The size and location of the mixing region can be programmatically controlled by optical signals. Mixing is notoriously difficult in microfluidics, where current techniques usually require textured surfaces (Stroock et al., 2002) or external energy input (Lee et al., 2011) to stir the flow. The stirring flows then need to pass through a long channel to ensure enough time for mixing. Our system provides a promising method for locally mixing arbitrary regions without passing through a long channel.

Superposition of primitives to construct multi-step flow programs

Superposition enables assembly of programming modules in space and time to streamline multi-step tasks. We first show a module combination to transport and separate cells at the same time (Fig. 5a). The cells are propelled by the outflow generated by the bottom bar, and isolated by the two bars at the top. The flow fields can be easily predicted from linear superposition of simulated single-bar flow data (Fig. 5a), which can also be used for adjustment of the bar positions and inclinations. For example, we design the two top bars to tilt downwards to facilitate the cell transport. Experiments verify that the module combination can transport

and isolate cells at the same time (Fig. 5b and 5c).

We also sequence two dynamic light signals in time to transport and mix particles at a larger scale (Fig. 5d). We first transport the particles from right to left using three translating bars. The inflows of light bars can attract particles that form into three stripes following the bars' movement. We then use three rotating bars to mix the stripes of particles, which disperse in the region originally devoid of particles.

Discussion

In this paper, we use biological active matter to build optically controlled microfluidic flows. Active materials generate force at molecular scales and can be used as hardware in technology (Omar et al., 2019), but systematic control of active matter has been a challenge. Here, we control active-matter motion with a programmable external field, light. The dynamics of active fluids can also be quantitatively predicted by a continuum model, allowing model-driven design and optimization of flow fields.

Linear superposition of local coherent flows allows methodical design of complex flow fields and scale-up of our system. Although active fluids are generally thought to be chaotic and difficult to control, we find that nonlinear interactions between active networks decay as a power law with separation length. We therefore first construct local regions of organized flow and then isolate them above a critical spacing, so that the networks interact weakly and the local flow fields can be additively assembled across the channel. This modular strategy also gives a physical route for maintaining order and performing work in active fluids. Our theory gives a general framework for microtubule-motor-solvent systems, which may be extended to study other out-of-equilibrium cytoskeletal structures, such as mitotic spindles. All experiments in this paper are controlled to be at similar conditions and all simulations share the same physical parameters (Supplemental Information), demonstrating the versatility of our model.

A future device could integrate and automate diverse micron-scale transport tasks in a single channel, with applications in chemistry and biology. Further improvements include expanding the toolbox of basic flow units for more adaptable flow design by incorporating flow fields generated from different light shapes, such as polygons, ellipses and concave shapes. Our setup could also be developed into a closed-loop control system by integrating a computer-vision program to analyze channel images and a decision-making program to compute the optical input from real-time

feedback and task goals. To overcome limitations in the proteins' sensitivity towards temperature and solute conditions, we are extending our current system to build a double-layer structure, where the top layer contains the solvent and objects of interest and the bottom layer is the active fluids. The two layers are immiscible and the idea is to program the flow in the bottom layer that in turn controls the motion of the top layer through the force transmission at the interface.

There has been growing interest in using optical control as a non-invasive method to generate micron-scale flows. It has been shown that laser-induced heat can generate a thermoviscous flow inside embryos, which can be applied to probe biological functions of cytoplasmic flows and active microrheology in cells (Mittasch et al., 2018). We also note that an electrically-programmable microfluidic device has been proposed recently using artificial cilia to stir flows near the chamber walls (W. Wang et al., 2022), while our setup is more efficient in driving flows near the center. Different programmable-control mechanisms might complement each other towards a fully-automated future of microfluidics.

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Materials and Methods

Kinesin Purification, Microtubule Polymerization. Kinesin purification, microtubule polymerization and chamber construction were described in previous work (T. D. Ross et al., 2019). In short, we constructed and purified two kinesin K401 with light-induced hetero-dimer system of iLID and SspB-micro: K401-iLID and K401-micro. For protein expression, we transformed the plasmids into BL21pLysS cells and induced the cells with IPTG. For protein purification, we lysed the cells and used Ni-NTA agarose resin to pick up His tags that were provided by the base plasmids. MBP domain was used and subsequent cleaved off in K401-micro expression to ensure the micro domain remains fully functional during expression. Tubulin was polymerized with the non-hydrolysable GTP analog GMP-CPP (Georgoulia, 2012).

Labeled and unlabeled tubulin were palleted and then incubated at 37°C to form GMP-CPP stabilized microtubulues. We then characterized the microtubule length distribution by immobilizing them onto cover glass surface using poly-L-lysine. The median microtubule length is 1.0 μm , much smaller than the critical length w_c at the scale of 100 μm (Supplemental Information).

Flow chamber treatment and construction. The chambers were made from microscope slides and cover slips that were passivated against non-specific protein binding with a hydrophilic acrylamide coating (Lau, Prasad, and Z. Dogic, 2009). In brief, microscope slides and cover glass are first cleaned by sonication in 2% Hellmanex III solution for 15 minutes. Excess Hellmanex III are then washed out with ddH₂O and then ethanol sonication. The glass is then incubated overnight in 0.1M HCl to remove any trace metal and finished in 0.1M KOH sonication. After cleaning and etching, the glass us unnersed in a silanizing solution of 98.5% ethanol, 1% acetic acid, and 0.5% 3-(Trimethoxysilyl)propylmethacrylate for 10-15 minutes. After rinsing, the glass is baked at 110° C for 30 minutes. The glass is than immersed overnight in a degassed 2% acrylamide solution with 0.035% TEMED and 3mM ammonium persulfate. The glass is rinsed in ddH₂O and air dried just before use. A flow cell made with pre-cut parafilm was used to seal between the microscope slides and coverslips making a channel that is about 70 μm in height. After the addition of reaction mixture, the flow cells were sealed with dental silicone polymer.

Energy Mixture and Reaction Mixture. An energy mix consisting an energy source (ATP), a crowding agent (glycerol), a surface passivating reagent (pluronic acid), oxygen scavengers (glucose oxidase, glucose, catalase, Trolox, and DTT) and ATP-recycling reagents was made on ice prior to combining the motor proteins and microtubules. After equilibrating the energy mix to ambient temperature, K401-micro, K401-iLID and microtubules were combined with the energy mix into a reaction mix. Concentrations for protein monomers for the K401-micro and K401-iLID constructs were 1 μM , and for microtubules, 1.5 – 2.5 μM . To minimize unintended light activation and non-specific protein binding, the sample was prepared under dark-room conditions with filters to block wavelengths below 580 nm. For all experiments conducted for this study, the reaction mixture consisted of 59.2 mM K-PIPES pH 6.1, 4.7 mM MgCl₂, 3.2 mM potassium chloride, 2.6 mM potassium phosphate, 0.74 mM EGTA, 1.4 mM MgATP (Sigma A9187), 10% glycerol, 0.50 mg/mL pluronic F-127 (Sigma P2443), 0.22 mg/ml glucose oxidase (Sigma G2133), 3.2 mg/mL glucose, 0.038 mg/mL catalase (Sigma C40),

5.4 mM DTT, 2.0 mM Trolox (Sigma 238813), 0.026 units/ μ L pyruvate kinase/lactic dehydrogenase (Sigma P0294), and 26.6 mM phosphoenolpyruvic acid. K401-micro, and K401-iLID were both diluted with a 1:2 ratio with 2 μ L of M2B pH 6.1 (80 mM K-PIPES pH 6.1, 1 mM EGTA, 2 mM MgCl₂). Microtubules were diluted with a 1:7 ratio with 7 μ L of DTT M2B pH 6.1 (45 μ L of M2B pH 6.1 with 1 μ L 250 mM DTT and 333.4 mg/ μ L glucose). The reaction mix was then aged in the flowcell for 120 - 180 minutes before light activation and data acquisition.

Tracer Beads Preparation. To visualize the fluid dynamics of our system, we used 1 μ m polystyrene beads as tracer particles. The particles were incubated overnight in M2B pH 6.8 buffer with 50 mg/ml pluronic acid. The beads were then washed and palletted at 1000xg for 2 minutes and resuspended in M2B pH 6.8 before adding them into the reaction mix.

Fluorescent Beads Preparation. Fluorescent particles were used to demonstrate mixing and transport capability of our system. We used 0.5 μ m polystyrene beads that are dyed with highly hydrophobic dyes. The particles were incubated in M2B pH6.8 buffer iwth 50 mg/mL pluronic acid. The beads were then washed and palletted at 1000xg for 2 minutes and resuspended in M2B pH 6.8 buffer before adding them into the reaction mix.

Cell Culture The cells used in the transport study (Jurkat cells; ATCC TIB-1522 and Raji cells; ATCC CCL-86) were cultured in medium composed of high-glucose RPMI 1640 (Life Technologies, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS; Qualified, Life Technologies, Carlsbad, CA, USA). Jurkat cells were cultured to maintain cell density between 1×10^5 and 3×10^6 cells/mL. Before loading the cells in the aster mix, the cells were thoroughly washed with M2B buffer 6.8 (previously described in Materials and Methods). Cells culture were first centrifuged at 300x at 5 minutes to remove the culture media. Then washed twice with M2B 6.8 at 300x at 5 minutes to remove any remaining culture media and salts. Subsequently, cells suspended in M2B 6.8 were introduced into the microtubule buffer to attain the desired cell density. As an example, for a 5mL culture with a density of 3×10^6 cells/mL, the typical protocol would involve suspending the cells in 1 mL of M2B 6.8, of which 10 μ L would be utilized in every 45 μ L of the microtubule buffers. Raji cells are only used in Fig. 3.7 because they can form larger clusters.

Cell Live/Dead Staining. Calcein AM (Catalog number C1430 Life Technologies, Carlsbad, CA, USA) and Ethidium Homodimer-1 (EthD-1) (Catalog number E1169 Life Technologies, Carlsbad, CA, USA) solutions were prepared using DMSO/H₂O

1:4 (v/v) for a stock concentration of 1 mM. 2 μ M Live/dead stain working concentration were prepared in DPBS (Life Technologies, Carlsbad, CA, USA) by adding 20 μ L of each stain to 10 mL of DPBS. Cells culture were first centrifuged at 300x at 5 minutes to remove the culture media. The 2 μ M live/dead stain were added to the cell pallet. Cells were sufficiently stained after 1 hr. Cell culture were centrifuged at 300 $\times$ g at 5 minutes again to remove staining solution. Subsequently, cells were suspended in M2B 6.8 then added to the active matter mix.

Vesicle Preparation. Lipid vesicles were prepared according to a modified method in Ref. (V. Noireaux and A. Libchaber, 2004). In a 15 mL glass vial, 0.5 mL of chloroform was combined with 15.2 μ L of 25 mg/mL 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) (850457; Avanti Polar Lipids) and 0.65 μ L of 1 mg/mL dioleoyl-phosphoethanolamine-lissamine rhodamine B (rhodamine PE) (810158; Avanti Polar Lipids). The chloroform was then evaporated in a fume hood, following all safety guidelines for handling chloroform. The resulting lipid dry film was mixed with 1 mL of mineral oil (M8410; Sigma-Aldrich) to achieve a final concentration of 500 μ M in oil solution. This mixture was heated to 50 °C and dissolved by pulse vortexing and sonication for 20 minutes. The lipid-oil mixture was stored at room temperature under dark conditions and used within 2 days.

Inner and outer solutions of the lipid vesicles were prepared to match the osmolarity of the active matter mix. Due to the incompatibility of our active matter system with high levels of salts or sugars, and the scarcity of the mixture itself, we increased the inner and outer solution osmolarity by adding KCl to M2B 6.8 to 1820 mOsm, which is lower than the 2034 mOsm of the active matter mix but does not disrupt protein functions. To form the vesicles, 300 μ L of outer solution was placed in a 1.5 mL tube, and 300 μ L of lipid-oil mixture was gently layered onto the outer solution. This was incubated on ice for 60 min to allow for assembly of a lipid monolayer at the oil/outer-solution interface.

Just before use, 200 μ L of lipid-oil mixture was put into a 1.5 mL sample tube and cooled on ice for >15 min. Then, 20 μ L of inner-solution was added to the lipid-oil mixture and immediately emulsified by first pipetting 20 times and then vortexing (power max; Vortex-Genie 2; Scientific Industries, Bohemia, NY) for 10 s. The emulsion was incubated on ice for 5 min to stabilize by spontaneous alignment of lipid molecules at the inner-buffer/oil interface. Subsequently, 200 μ L of the emulsion was carefully placed on the lipid/oil mixture and the outer solution, then incubated for 5 minutes on ice. The 1.5 mL tube was then centrifuged (2,000 $\times$ g, 10

min, 4 °C) to push the emulsion droplets through the interface. After centrifugation, the oil layer and 500 μL of buffer solution were gently drawn off from the top of the tube.

For the final mixture, 1 μL of resuspended vesicles was added to a combination of 8 μL energy mix, 2 μL of diluted iLid motor, 2 μL of diluted micro motor, and 1 μL of 3x diluted microtubules.

Design and Implementation of Different Bar Patterns. We custom fitted an epi-illuminated pattern projector onto our microscope. The size of the projection field is 800 x 1280 pixels. Matrices containing coordinates of bars were first computed in Python and then converted to greyscale and eventually saved into tiff formats. Tiff image sequences were then processed by custom micro-manager script. The scripts can be found at <https://github.com/fy26/ActiveMatter>.

Data Acquisition and Projection of Patterns. All experiments were performed with an automated widefield epifluorescence microscope with custom epi-illuminated projector and LED gated transmitted light discussed in our previous work (T. D. Ross et al., 2019). All samples were imaged at 10X magnification. Image sequences were captured using a Nikon TI2 controlled with micro-manager. Images of the fluorescent microtubules (cy5) and tracer particles (brightfield) were also acquired every 8 seconds. Bar patterns are projected onto the image plane every 8 seconds with a brief 200 milliseconds flash of a 2.4 mW/mm² activation light from a 470 nm LED.

Duration of the activation light. The duration of the light was empirically determined through an iterative process: 1. We started with a short activation time, such as 50ms, and gradually increased it in 50 ms increments. 2. After each increment, we observed the sample for aster formation. 3. We continued steps 1 and 2 until we observed the formation of a stable aster, defining this as the optimal activation time. 4. Longer pulse duration resulted in contractile activity outside of the intended light pattern, helping establish an upper limit for the activation duration.

Particle Image Velocimetry (PIV). PIV was performed on the images of tracer beads using PIVlab (Thielicke and Stamhuis, 2014; Thielicke and Sonntag, 2021) to extract the solvent flow fields.

Derivation of the general principle for linear superposition. The generalized Stokes equation for the solvent flow is $\mathbf{f} + \mu \nabla^2 \mathbf{u} - \nabla p = 0$, where $\mathbf{f}$ is the body force applied by external fields or sources, for example, in our system the body force is from the active force generated by the crosslinked microtubules and motors. We

now construct a general principle for linear superposition of flows induced by n different force-generating sources. We denote the force applied on the fluid from the source i , in the absence of other sources, as $\mathbf{f}_i$, and the resultant flow and pressure field as $\mathbf{u}_i$ and p_i , respectively. Similarly, the body force, flow field and pressure field in the presence of all the n sources are denoted by $\mathbf{f}_t$, $\mathbf{u}_t$ and p_t , respectively. To establish a linear regime of fluid flows driven by different sources, we require $\mathbf{u}_t = \sum_i \mathbf{u}_i$ and $p_t = \sum_i p_i$, which can be substituted into the Stokes equation and yield $\mathbf{f}_t = \sum_i \mathbf{f}_i$. This result directly comes from the linearity of the Stokes equation in $\mathbf{u}$ and p , however, it is not trivial because $\mathbf{f}_i$ can be dependent on the flow velocity $\mathbf{u}$ and the formula $\mathbf{f}_t = \sum_i \mathbf{f}_i$ does not always hold. In our system, the force-generating sources are the microtubule networks and the force induced by a single network i , in the absence of all other networks, can be expressed as $\mathbf{f}_i = \gamma c_i (\mathbf{v}_i - \mathbf{u}_i)$, where γ is the drag coefficient of the microtubule and the solvent, c and $\mathbf{v}$ are the density and velocity of the microtubule network, respectively. Therefore, in the presence of n networks, the general principle $\mathbf{f}_t = \sum_i \mathbf{f}_i$ together with the additional linear relationships, $c_t = \sum_i c_i$ and $\mathbf{v}_t = \sum_i \mathbf{v}_i$, requires that $\sum_i c_i \cdot \sum_j \mathbf{v}_j = \sum_i c_i \mathbf{v}_i$ and $\sum_i c_i \cdot \sum_j \mathbf{u}_j = \sum_i c_i \mathbf{u}_i$. The former requires that $c_i \mathbf{v}_j = \mathbf{0}$ when $i \neq j$, which is automatically satisfied as long as there are no networks overlapping in the system. The latter requires that $c_i \mathbf{u}_j = \mathbf{0}$ for any $i \neq j$, which is the rule of linear superposition in a multiple-active-agent system.

Numerical Simulation. Finite difference method was used in numerical simulations with the central differencing scheme in space and the method of lines in time. The codes are written in Python and available at <https://github.com/fy26/ActiveMatter>.

Calculation of shear modulus G . The steady-state value of D_f depends on the Capillary number $Ca = \mu \frac{\partial u_x}{\partial x} r / G$ (Lac et al., 2004; Hsu and Leal, 2009; de Loubens et al., 2014), where r is the radius of the aster, via a linear relationship $D_f = ACa$. The value of coefficient A is calculated to be 25/6 for elastic capsules (Lac et al., 2004; de Loubens et al., 2014), and measured to be around 20 for viscoelastic drops (Hsu and Leal, 2009). Here we choose $A = 10$ to estimate the shear modulus G of the microtubule aster. Additionally using measurements $\mu = 0.02 \text{ Pa}\cdot\text{s}$ (Gagnon et al., 2020), $\partial u_x / \partial x = 0.0015 \text{ s}^{-1}$, $r = 100 \text{ }\mu\text{m}$, $L = 120 \text{ }\mu\text{m}$ and $B = 70 \text{ }\mu\text{m}$, the shear modulus of the aster G is calculated to be $1 \times 10^{-7} \text{ Pa}$.

Calculation of detachment force on cells in an extensional flow. The flow-induced friction $\mathbf{f}_p$ on a spherical particle translating in an unbounded fluid with velocity $\mathbf{v}$ is $\mathbf{f}_p = -6\pi\mu a \mathbf{v}$, which is used to approximate the force on detaching cells. We

denote the two attached cells by a and b , and the unperturbed flow velocity at the two cell centers by $\mathbf{u}_a$ and $\mathbf{u}_b$, respectively. Then the cell pair moves at the same velocity $(\mathbf{u}_a + \mathbf{u}_b)/2$. The magnitude of the flow-induced force on each cell is $f_p = 3\pi\mu a|\mathbf{u}_a - \mathbf{u}_b| = 3\pi\mu a|\partial\mathbf{u}/\partial\mathbf{x}|\Delta l$. The detachment force on each cell has the same magnitude as f_p .

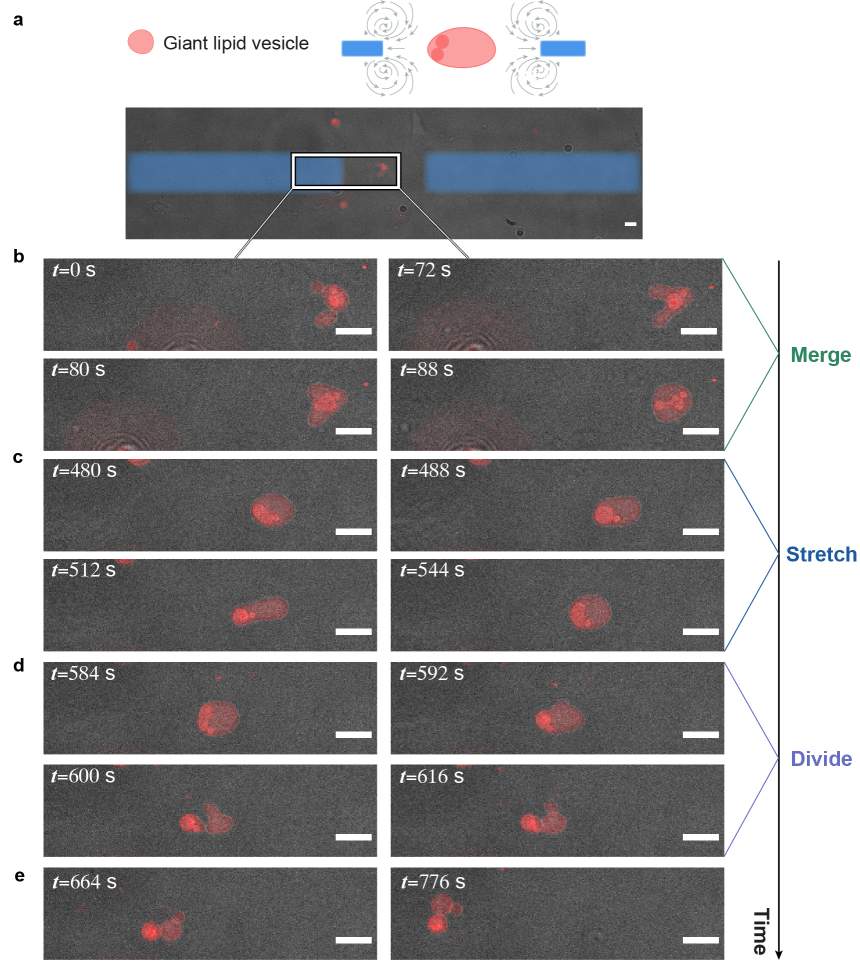


Figure 3.6: **The stretching module can stretch and divide a giant lipid vesicle (GV) into daughter vesicles using the stretching module.** **a**, Sketch of the experimental setup. The GVs are dyed red. **b**, 3 GVs merge into a single GV. **c**, The fused GV is stretched along the horizontal direction. **d**, The fused GV is divided into 3 daughter vesicles. **e**, The daughter vesicles are stable. Scale bars, $20\ \mu\text{m}$.

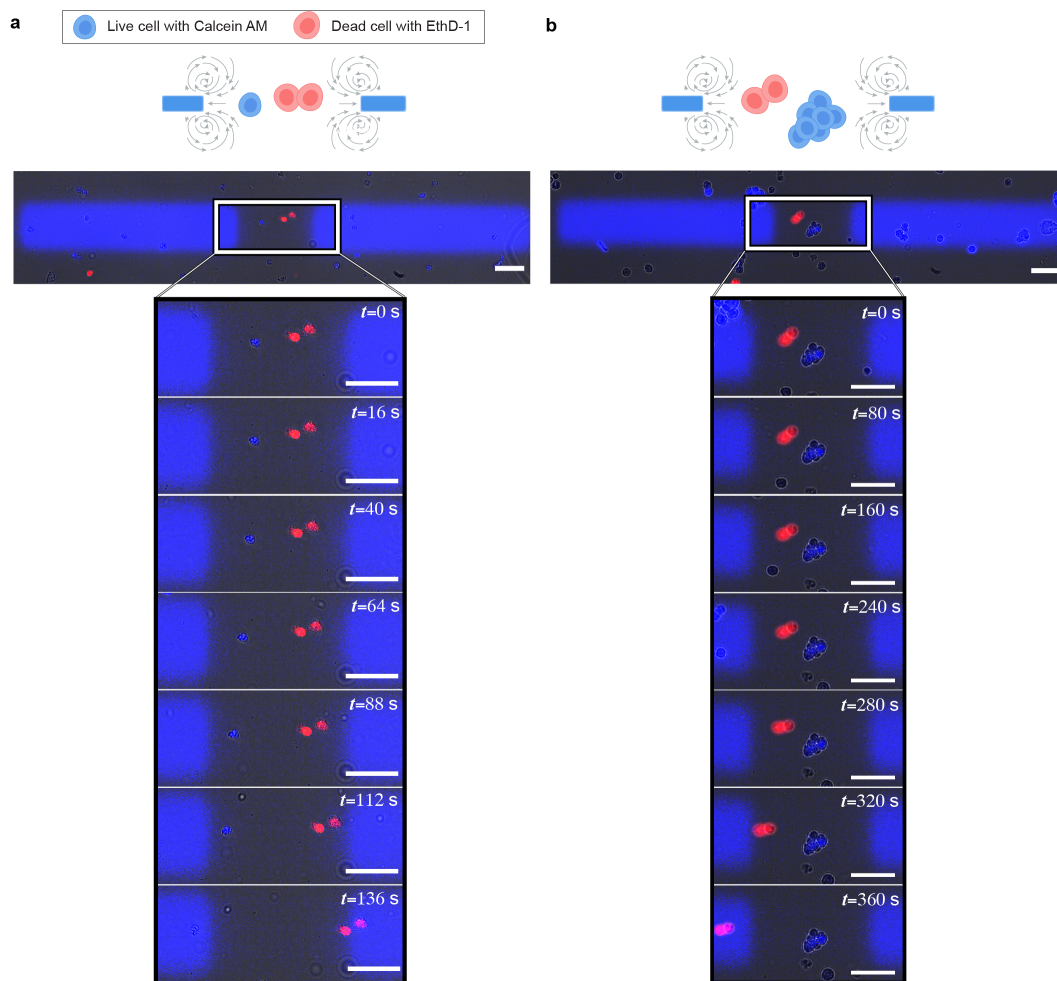


Figure 3.7: **The stretching module can isolate dead cells (red) from live cells (blue).** **a**, Time-lapse images of isolating one live cell from a pair of dead cells. **b**, Time-lapse images of isolating a dead-cell pair from a live-cell cluster, without disturbing the latter. Raji cells (Materials and Methods) are used in this figure because they can form large clusters.

*Chapter 4***LACTATE-SENSING SYNTHETIC CELLS RELEASE T-CELL ENGAGERS TO KILL TUMOR CELLS****Abstract**

Nonliving synthetic cells could deliver therapeutics locally at tumor sites without replication or persistence, but most existing systems require experimenter-supplied chemical triggers or fields, or use constitutive expression rather than environmental gating. We built giant unilamellar vesicle synthetic cells that sense lactate, a tumor-microenvironment metabolite, and autonomously express a blinatumomab-format T-cell engager for local release in a form that living T cells can use. In this architecture, alpha-hemolysin admits extracellular lactate, an engineered LldR/ALPaGA circuit gates cell-free expression, and listeriolysin O releases protein cargo through conditional output pores. When activated by tumor-conditioned medium, the synthetic cells released the T-cell engager and drove T-cell-dependent killing of CD19-positive Raji cells, including in direct co-culture where tumor cells served as the sole lactate source.

Many effective therapeutics fail because their activity is delivered systemically rather than generated locally. T-cell engagers such as blinatumomab activate cytotoxicity at picomolar concentrations yet cause dose-limiting toxicity—cytokine release syndrome and neurotoxicity—because they engage T cells throughout the body (R. Bargou et al., 2008; U.S. Food and Drug Administration, 2025). Live-cell therapies home to tumors and respond to local cues but introduce persistence, expansion and manufacturing burdens. We therefore asked whether a nonliving synthetic cell could reproduce a few therapeutic cell functions—sensing a local metabolic cue, manufacturing a protein effector and releasing it—inside a programmable and non-replicating chassis.

Synthetic cells built from lipid vesicles encapsulating cell-free transcription-translation (TX-TL) machinery have produced therapeutic proteins inside tumors (Krinsky et al., 2018), released neurotrophic factors that drive neural differentiation (Topalak et al., 2020), communicated with engineered mammalian cells (Monck, Elani, and Ceroni, 2024), and sensed model inducers through membrane pores (Vincent Noireaux and Albert Libchaber, 2004; Lentini et al., 2014). However, most systems

reported to date have relied on experimenter-added chemical triggers or fields, or on constitutive expression without environmental gating. We wanted to build a synthetic cell that detects a disease-relevant metabolite and responds with therapeutic output. We used lactate as the input because it is elevated to 10–40 mM in many tumor microenvironments through the Warburg effect (Pérez-Tomás and Pérez-Guillén, 2020; Cruz-López et al., 2019), compared to 1–2 mM in normal tissue. The goal was to build a nonliving system that senses tumor-derived lactate, produces a T-cell engager and releases it locally at a tumor site in a form that living T cells can use.

The synthetic cell was organized as a two-pore input/output system. Alpha-hemolysin (α HL) from *Staphylococcus aureus* forms heptameric pores of approximately 1.4 nm inner diameter that admit small molecules while retaining proteins (Song et al., 1996; Aksimentiev and Schulten, 2005). Purified α HL was inserted into the vesicle membrane after GUV formation, providing always-open pores through which small molecules equilibrate within minutes (Supplementary Fig. 2). Inside the vesicle, a lactate-responsive genetic circuit based on the LldR transcription factor and the engineered ALPaGA promoter (Zúñiga et al., 2021) linked lactate sensing to gene expression. ALPaGA was specifically engineered to function in glucose-rich and hypoxic environments that silence the wild-type *E. coli* LldPRD promoter, making it suitable for the tumor microenvironment. The circuit controlled co-expression of GFP (or a T-cell engager) and listeriolysin O (LLO), a cholesterol-dependent cytolysin that oligomerizes into large membrane pores of 20–30 nm diameter (Czajkowsky et al., 2004). This design separates small-molecule entry from protein release: α HL pores admit lactate but cannot release folded proteins; LLO pores release proteins but form only when the genetic circuit fires.

GUVs were prepared by inverted emulsion from POPC and cholesterol (55:45 mol ratio) supplemented with 1 mol% biotinylated PEG-lipid for surface immobilization and 0.2 mol% rhodamine-PE for membrane visualization. The 45 mol% cholesterol exceeds the threshold required for LLO binding and oligomerization (Bavdek et al., 2007). The vesicles encapsulated *E. coli* lysate-based TX-TL (myTXTL, Arbor Biosciences) and plasmid DNA encoding the lactate-responsive circuit.

We first tested the architecture with GFP as a fluorescent reporter, because intravesicular fluorescence reports both expression and release in real time (Fig. 4.1). We tested five configurations, each omitting one component. Constitutive GFP expres-

sion (P70a promoter) produced an increase to a plateau at approximately 7 hours and set the upper bound of vesicle-confined TX-TL expression. The complete lactate-responsive architecture remained near baseline without lactate, confirming tight LldR repression with approximately 13% basal leak hypothesized to result from delayed LldR accumulation from zero at reaction onset. Lactate (10 mM) did not activate expression when α HL was omitted, demonstrating that the charged lactate anion (>99% ionized at physiological pH) cannot cross the intact POPC/cholesterol bilayer and that α HL pores are required for sensing. When lactate and α HL were present but LLO was omitted, GFP accumulated steadily to approximately 90% of the constitutive level and remained trapped—confirming functional expression without release. In the complete system, internal GFP rose to a peak at approximately 3 hours and then declined steeply as LLO pores opened, returning to near-baseline by 5 hours. This rise-and-fall trajectory is the kinetic signature of coupled expression and release: GFP accumulates while LLO remains below the cooperative pore-formation threshold, then escapes rapidly once LLO oligomerizes. Peak fluorescence confirmed that both the no-LLO and full-system conditions expressed GFP. Release index, defined as (peak – final)/peak, was 0.58 ± 0.14 in the full system and near zero in all other conditions, including the no-LLO condition where expression was high but no pores formed. Lactate, α HL and LLO were therefore each required for the complete expression-and-release behavior. A lactate dose-response of the full system yielded a release EC₅₀ of approximately 2.5 mM, higher than the expression EC₅₀ alone (~1.8 mM; Supplementary Fig. 1), reflecting the additional cooperative threshold of LLO pore assembly.

We next replaced GFP with a blinatumomab-format CD19/CD3 T-cell engager and tested whether the released BiTE could kill target cells (Fig. 4.2). Blinatumomab is a bispecific CD19-directed CD3 T-cell engager whose clinical activity depends on physical recruitment of T cells to CD19-positive target cells (R. Bargou et al., 2008; U.S. Food and Drug Administration, 2025). In a staged donor-receiver assay, immobilized synthetic cells containing BiTE and LLO plasmids under ALPaGA control were incubated for six hours with either fresh non-lactate medium (OFF) or high-lactate, cell-free Raji-conditioned medium (ON). Raji cells are a CD19-positive Burkitt lymphoma line routinely used as targets in blinatumomab killing assays (ATCC, 2026). Conditioned medium contained 10.0 ± 0.98 mM lactate after 24 hours (Supplementary Fig. 1), well above the release EC₅₀. Donor supernatants were collected and transferred at 20% v/v to receiver wells containing calcein-AM-labeled Raji cells with activated primary human T cells at a 10:1 effector-to-target

ratio.

Raji-conditioned synthetic-cell supernatants killed Raji targets. Baseline Raji plus T cells showed $4.2 \pm 2.8\%$ killing, while commercial blinatumomab (1 nM) produced $83.5 \pm 5.8\%$ killing. Supernatant from synthetic cells incubated in fresh non-lactate medium (OFF) produced $18.5 \pm 8.2\%$ killing, whereas supernatant from synthetic cells incubated in high-lactate Raji-conditioned medium (ON) produced $62.4 \pm 12.5\%$ killing ($P < 0.01$, OFF versus ON). The residual killing in the OFF condition is hypothesized to result from non-specific membrane disruption from LLO and α HL present in the supernatant, rather than BiTE activity. Direct co-culture of synthetic cells with live Raji cells and T cells—without any preconditioning step or supernatant transfer—produced $59.1 \pm 14.8\%$ killing, demonstrating that live Raji cells can provide the activating lactate input in the same culture environment.

Together, these data show that nonliving synthetic cells can sense a tumor metabolite, produce a therapeutic protein, and release it in a form that drives CD19-positive target-cell killing. The system does not attempt to reproduce proliferation, migration or immune-synapse formation. Instead, it performs a narrower task: local metabolic sensing coupled to therapeutic protein production and release. Lactate alone is not a tumor-specific input—the ALPaGA EC_{50} (~ 1.8 mM) sits near normal tissue levels (1–2 mM), and conditioned medium from normal fibroblasts (HFF; 1.3 mM) produced partial sensor activation (Supplementary Fig. 1). Future versions will require threshold tuning through engineered LldR variants with higher EC_{50} , or combinatorial sensing of hypoxia, pH, or surface antigens. The two-pore design is flexible: changing the DNA template changes the therapeutic output, and replacing the LldR/ALPaGA sensor with other transcription factor–promoter pairs would enable sensing of different metabolites. This work shows that a single nonliving vesicle can combine metabolite sensing, protein production, and conditional release to drive immune-mediated killing.

Online Methods

Plasmids and genetic constructs. The lactate-responsive plasmid ALPaGA (Addgene 175272) (Zúñiga et al., 2021) encodes LldR under the constitutive P9 promoter and sfGFP under the ALPaGA promoter on the pSB4K5 backbone (kanamycin resistance, low copy, pSC101 origin; 5603 bp). This plasmid was used without modification for GFP-reporter experiments.

For the therapeutic construct (Plasmid A'), the sfGFP open reading frame (posi-

tions 1216–1932) was replaced with a blinatumomab-format anti-CD19/CD3 BiTE coding sequence by Gibson Assembly (NEB E2611). The ALPaGA backbone was amplified by inverse PCR using Q5 polymerase (NEB M0491), excluding sfGFP. The BiTE CDS was amplified with primers containing 20–25 bp overlaps complementary to the backbone ends. PCR products were treated with DpnI (NEB R0176) for 1 hour at 37°C, gel-extracted (Qiagen QIAquick), and assembled at 50°C for 1 hour. Assemblies were transformed into DH5 α and selected on kanamycin (50 μ g/mL). The P9-LldR module, ALPaGA promoter, B0034 strong ribosome binding site, B0015 terminator and pSB4K5 backbone were retained from the parent plasmid.

For the release construct (Plasmid B), sfGFP was replaced with the listeriolysin O (LLO) coding sequence using the same inverse-PCR/Gibson strategy. The weak ribosome binding site B0033 (TCACACAGGAAAG) was encoded in the forward primer for the LLO fragment, positioned between the ALPaGA promoter and the LLO start codon with a TACTAG spacer. An alternative Plasmid B encoding perfringolysin O (PFO) from DT034A (Addgene 159395) was constructed identically, excluding the C-terminal pdt degradation tag.

DT034A (Addgene 159395), encoding LuxR under a constitutive promoter and PFO under the pLux promoter on pSB1A3, was used without modification as a positive control for pore-mediated release.

A constitutive P70a-deGFP construct (provided with the myTXTL kit) was used as a TX-TL expression reference. All constructs were sequence-verified by Sanger sequencing (Genewiz) using iGEM standard primers VF2 (5'-TGCCACCTGACGTCTAAGAA-3') and VR (5'-ATTACCGCCTTTGAGTGAGC-3') with additional internal primers for full-insert coverage. Plasmid DNA was prepared using Qiagen Plasmid Midi kits (12145), eluted in nuclease-free water, quantified by NanoDrop 2000c, diluted to 200 nM working stocks, and stored at –20°C.

Alpha-hemolysin purification. Alpha-hemolysin was expressed from plasmid JF001A (Addgene 53116) (Lentini et al., 2014) encoding *Staphylococcus aureus* alpha-hemolysin with a C-terminal His₆ tag in pET21b. BL21(DE3) cells were grown in LB with ampicillin (100 μ g/mL) at 37°C to OD₆₀₀ ~0.6, induced with 1 mM IPTG, and expressed at 25°C for 5 hours. Cells were harvested (6000 $\times$ g, 10 min, 4°C), resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF), and lysed by sonication (30 s on, 30 s off, 6 cycles, on ice). Clarified lysate (20,000 $\times$ g, 30 min, 4°C) was loaded onto Ni-NTA resin (Qiagen 30210), washed with 10 column volumes of 20 mM imidazole in lysis

buffer, and eluted with 250 mM imidazole. Protein was dialyzed overnight against storage buffer (20 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol) using 10 kDa MWCO SnakeSkin dialysis tubing (Thermo Fisher Scientific). Purity was >95% by SDS-PAGE (single band at ~33 kDa). Aliquots were snap-frozen and stored at -80°C .

Cell-free transcription-translation. All reactions used myTXTL Sigma 70 Master Mix (Arbor Biosciences) with the provided energy buffer and amino acids. Plasmid DNA was added at 10–20 nM. For BiTE expression, reactions included the disulfide bond enhancement kit (Arbor Biosciences). Bulk reactions were assembled on ice and incubated at 29°C in black clear-bottom 96-well plates (Costar 3603) covered with 25 μL mineral oil. sfGFP fluorescence (excitation 485 nm, emission 528 nm) was monitored at 15-min intervals on a Tecan Infinite M200 plate reader. For dose-response experiments, sodium L-lactate (Sigma L7022) was added at 0–20 mM. Dose-response data were fit to a Hill equation: $f(x) = \text{baseline} + V_{\text{max}} \cdot x^n / (EC_{50}^n + x^n)$ using GraphPad Prism (version 10).

GUV preparation. Lipids were mixed from chloroform stocks at 55 mol% POPC (Avanti 850457), 45 mol% cholesterol (Avanti 700000), 1 mol% DSPE-PEG(2000)-biotin (Avanti 880129), and 0.2 mol% Lissamine Rhodamine PE (Avanti 810150) using Hamilton syringes in clean glass vials. The mixture was dried under nitrogen, placed under vacuum for >4 hours, and dissolved in sterile mineral oil (Sigma BioXtra 5310) at 380 μM total lipid by vortexing, heating to 80°C , and sonication ($50\text{--}60^{\circ}\text{C}$, 30–60 min). The osmolality of the inner solution (assembled myTXTL reaction, typically 290–310 mOsm/kg) was measured by freezing-point depression and matched to the outer solution (DPBS without $\text{Mg}^{2+}/\text{Ca}^{2+}$) within ± 25 mOsm/kg (Garamella et al., 2016).

GUVs were formed by inverted emulsion (Toparlak et al., 2020; Garamella et al., 2016). Lipid-oil solution (500 μL) was overlaid onto outer solution (200 μL) and equilibrated for 30–60 min. Inner solution (10 μL) containing assembled TX-TL reaction, plasmid DNA and supplements was emulsified in 500 μL lipid-oil by agitation (drawing the tube over a rack 10–15 times), overlaid onto the interface, and centrifuged at $5000\times g$ for 5 min. The oil layer was removed by aspiration and the vesicle pellet washed twice with DPBS ($5000\times g$, 1 min each).

Alpha-hemolysin insertion. Purified αHL was added to washed GUVs at the optimized concentration in DPBS. After 30 min at room temperature, unincorporated protein was removed by centrifugation ($5000\times g$, 1 min). The working concentration

was determined by titration (0–100 nM), selecting the lowest concentration that permitted fluorescein (376 Da) equilibration within 30 min while retaining FITC-dextran (10 kDa) (Supplementary Fig. 2).

GUV immobilization and imaging. Glass-bottom 96-well plates (Greiner 655090) were coated with 50 $\mu\text{g/mL}$ biotinylated BSA (Sigma A8549) in T50 buffer (10 mM Tris pH 8.0, 50 mM NaCl) for 30 min, followed by 10 $\mu\text{g/mL}$ streptavidin (Sigma S4762) in T50 for 30 min (Dolder, Müller, and Ballmoos, 2022). Wells were washed with imaging buffer (DPBS + 100 mM NaCl). GUVs were added and allowed to settle for 1 hour.

Confocal z-stacks were acquired on a Zeiss LSM 880 laser scanning confocal microscope with a 40 $\times$ objective. The red lipid-bilayer channel (Rhodamine PE; excitation 560 nm, emission 583 nm) was used to identify GUV membranes, and the green channel (sfGFP; excitation 485 nm, emission 528 nm) was used to quantify GFP signal. Display scaling was applied only for visualization; all measurements were performed on raw pixel intensities.

GUV detection. GUV candidates were detected automatically from the red channel using a circular Hough-transform workflow applied independently to every z plane. Each red-channel plane was background-subtracted with a Gaussian high-pass filter and lightly smoothed before circle detection over radii of 12–56 pixels. Candidate circles were filtered using red membrane-ring metrics and merged across z planes by non-maximum suppression to remove duplicate detections.

Each candidate was re-scored across the full z-stack using the red membrane signal. The focus score incorporated membrane-ring amplitude, radial full width at half maximum, angular support around the circumference, and radial peak offset from the candidate radius. A candidate was considered in focus if at least one z plane passed the red-ring focus filter. The measurement plane was the passing z plane with the highest red focus score.

For the strict automatic analysis, accepted GUVs were required to be non-partial, not edge-clipped, have an in-focus z plane, have radius ≥ 20 pixels, and have red focus score ≥ 10 . This detector identified 25 in-focus GUVs in the no-lactate condition and 22 in-focus GUVs in the lactate condition (Supplementary Figs. 3 and 4).

GFP quantification and classification. GFP signal was quantified from the selected best-focus z plane. To avoid including membrane-associated dye, the GUV interior was measured using a circular mask 5 pixels smaller than the detected red membrane

radius. For each GUV, mean green intensity, median green intensity, and integrated green intensity were calculated. Mean green intensity was used as the primary GFP-expression metric because it is normalized by GUV interior area.

GFP-positive calls were made relative to the no-lactate condition, which served as the autofluorescence/baseline control. GFP-positive GUVs were defined as accepted GUVs with mean green intensity greater than the no-lactate mean plus 3 standard deviations and median green intensity greater than the maximum no-lactate median. This yielded a mean green cutoff of 9.52 and a median green cutoff of 5.0. GUVs above both thresholds were classified as GFP-positive; all others were classified as GFP-negative/baseline.

For time-course experiments, immobilized GUVs were imaged at 5-min intervals for up to 10 hours. Mean intravesicular GFP was tracked for individual vesicles across time points. Data were normalized to the constitutive P70a-deGFP maximum within each experiment. Release index was calculated as (peak – final)/peak.

Cell culture. Raji cells (ATCC CCL-86) were cultured in RPMI 1640 (Gibco) with 10% FBS, penicillin-streptomycin (100 μ g/mL) and 2 mM L-glutamine at 37°C, 5% CO₂. For conditioned medium, Raji cells were seeded at 2×10^6 cells/mL in 10 mL fresh complete RPMI and cultured for 24 hours. Medium was collected and clarified by centrifugation (300 $\times$ g, 5 min). Lactate concentration was measured using the L-Lactate Assay Kit (Sigma MAK329) according to the manufacturer's instructions.

Human foreskin fibroblasts (HFF) were cultured in DMEM with 10% FBS under standard conditions. Conditioned medium was collected identically to Raji.

Human T cells were isolated from PBMCs by negative selection (EasySep Human T Cell Isolation Kit, STEMCELL Technologies) and activated with CD3/CD28 Dynabeads (Thermo Fisher 11131D) at 1:1 bead-to-cell ratio in RPMI + 10% FBS + 30 U/mL IL-2 for 4 days. Beads were removed magnetically the day before the assay.

Donor activation and BiTE release. Synthetic cells containing Plasmid A' (BiTE, 20 nM), Plasmid B (LLO, 4 nM), disulfide bond enhancement kit, and α HL were incubated for 6 hours at 29°C with either fresh non-lactate complete RPMI (OFF) or high-lactate, cell-free Raji-conditioned medium (ON). Samples were centrifuged (5000 $\times$ g, 5 min) and supernatants collected.

Cytotoxicity assay. Raji cells were labeled with 2 μ M calcein-AM (Thermo Fisher

C1430) for 30 min at 37°C, washed three times (300×g, 5 min), and resuspended in fresh RPMI. Labeled Raji (50,000 per well) and activated T cells (500,000 per well; 10:1 E:T) were seeded in 96-well round-bottom plates. Donor supernatant was added at 20% v/v (total 200 μ L). Commercial blinatumomab was added at 1 nM and 0.1 nM as positive controls. For direct co-culture, synthetic cells were added to Raji + T cells in fresh RPMI without supernatant transfer. After 16 hours at 37°C, plates were centrifuged (300×g, 5 min), 100 μ L supernatant was transferred to black clear-bottom plates, and calcein fluorescence (excitation 485 nm, emission 528 nm) was measured by plate reader. Specific lysis was calculated as:

$$\% \text{ Killing} = 100 \times \frac{F_{\text{sample}} - F_{\text{spontaneous}}}{F_{\text{maximum}} - F_{\text{spontaneous}}}$$

where F_{maximum} was determined by lysis with 0.5% Triton X-100 and $F_{\text{spontaneous}}$ by Raji cells in fresh medium without T cells or synthetic cells.

Statistics. Data are presented as mean $\pm$ SEM. For vesicle experiments, n represents independent vesicle preparations; individual vesicles were treated as technical observations nested within preparation. For killing assays, technical triplicates were averaged within each T-cell donor before testing; $n = 3$ independent T-cell donors ($n = 9$ total observations). Comparisons were by unpaired two-tailed Student's t -test or one-way ANOVA with Dunnett's post hoc test. $P < 0.05$ was considered significant. Dose-response curves were fit by nonlinear regression (Hill equation, variable slope). All analyses used GraphPad Prism version 10.

Author contributions

S.L., P.Z. and M.T. conceived the study. S.L. performed experiments and analysed data. S.L., P.Z. and M.T. interpreted results and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Data availability

Source data for Figs. 4.1 and 4.2 and supplementary figures will be provided with the final manuscript. Plasmids will be available from Addgene.

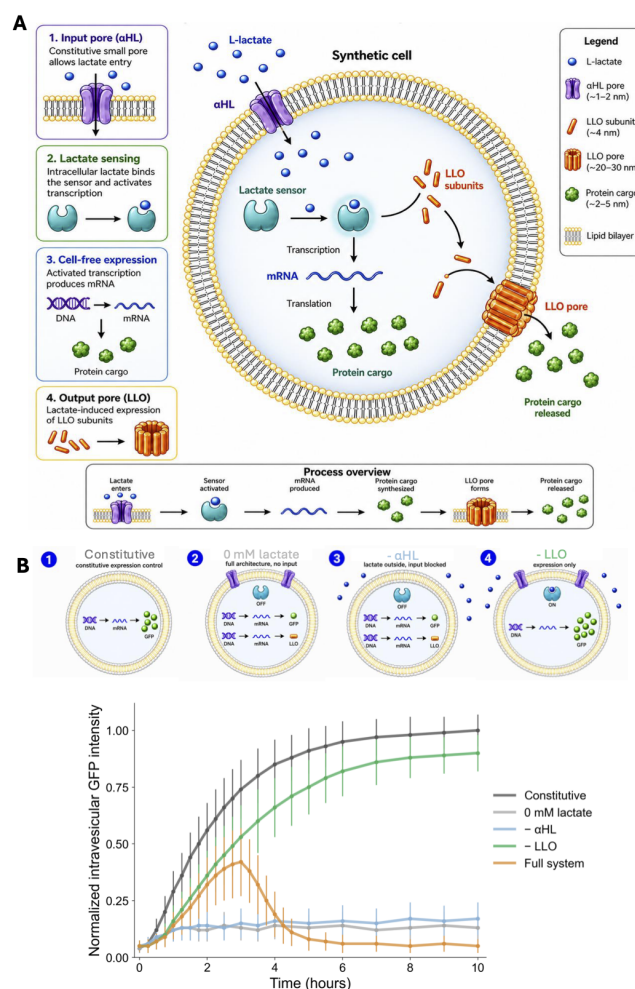


Figure 4.1: Lactate gates expression and release in synthetic cells. **a**, Design of the synthetic cell. Centre: a GUV encapsulating cell-free TX-TL machinery and plasmid DNA. L-lactate enters through alpha-hemolysin (α HL) input pores (~ 1 – 2 nm), binds the LldR-based lactate sensor, and activates transcription. Expressed protein cargo accumulates inside the vesicle. LLO monomers oligomerize into large output pores (~ 20 – 30 nm) that release cargo. Left panels: the four functional steps. Bottom: process overview from lactate entry to protein release. **b**, Four control configurations isolating each system component, alongside the full system shown in (a). (1) Constitutive GFP (P70a, no circuit). (2) Full architecture, 0 mM lactate (OFF). (3) 10 mM lactate, no α HL (input blocked). (4) 10 mM lactate + α HL, no LLO (expression only). Bottom: intravesicular GFP fluorescence over 10 hours by time-lapse microscopy (15-min intervals). Constitutive (dark grey) and no-LLO (green) conditions rise and plateau. OFF (light grey) and no- α HL (blue) remain near baseline ($\sim 13\%$ of max). The full system (orange) peaks at ~ 3 hours then drops as LLO pores open and GFP escapes. Data normalized to constitutive maximum. Mean $\pm$ SEM, $n = 5$ independent vesicle preparations.

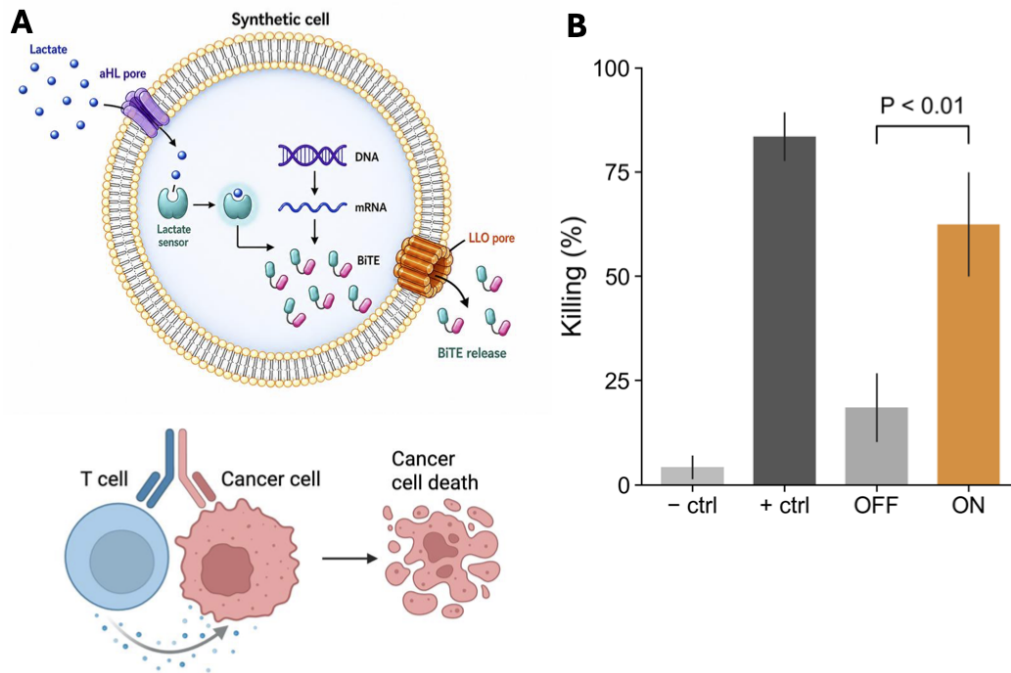


Figure 4.2: **Autonomous synthetic cells kill tumor cells through released BiTE.** **a**, Mechanism of action. Top: synthetic cell activated by tumor-derived lactate produces and releases a blinatumomab-format BiTE through LLO output pores. Bottom: released BiTE bridges CD3 on T cells to CD19 on Raji cells, redirecting T-cell cytotoxicity and inducing cancer cell death. **b**, Cytotoxicity assay. Synthetic cells were incubated with fresh non-lactate medium (OFF) or high-lactate Raji-conditioned medium (ON) for 6 hours. Donor supernatant (20% v/v) was transferred to calcein-AM-labeled Raji cells with activated human T cells (10:1 E:T). - ctrl: Raji + T cells in fresh medium. + ctrl: 1 nM soluble blinatumomab. ON versus OFF: $P < 0.01$, unpaired t -test. The residual killing in the OFF condition (~18%) is hypothesized to result from non-specific pore-protein leakage. Mean $\pm$ SEM, $n = 9$ (3 T-cell donors $\times$ 3 technical replicates).

Chapter 5

CONCLUDING REMARKS

PRC1 transformed a motorized microtubule network into a mechanical information-processing architecture. Kinesin converted ATP hydrolysis into sliding force, but PRC1 determined where that force traveled. At low PRC1 concentration, the network dissipated motor activity near the activation region. At intermediate PRC1 concentration, the network coordinated motion over micrometre length scales but failed to move beads efficiently or produce substantial work. At high PRC1 concentration, the network preserved the imposed light geometry, propagated force over hundreds of micrometres, and displaced external loads. The semiflexible-network model explained this sequence by separating connectivity from rigidity: crosslinks first produced a connected network and only later produced a load-bearing material. Connectivity organized motor activity; rigidity converted organized activity into work. Motors supplied the engine, but PRC1 built the transmission (Subramanian et al., 2010; Wijeratne and Subramanian, 2018; Alfieri, Gaska, and Forth, 2021; Head, Levine, and F. C. MacKintosh, 2003; Broedersz and Fred C. MacKintosh, 2014).

Light-patterned active matter carried the same logic from force propagation into spatial task execution. We used optical patterns to specify where motors acted, and the microtubule–motor material converted that spatial instruction into hydrodynamic output. Active stresses recruited material, shaped flows, transported particles, mixed fluids, separated objects, and generated extensional deformation. The system therefore processed spatial information through mechanics rather than through symbolic computation. Earlier microtubule–motor reconstitutions showed that purified biomolecules can form asters, extensile bundles, active fluids, and spontaneous flows; patterned activation turned those dynamics into programmable operations. In this project, geometry became the input, ATP-fueled active stress became the processing layer, and transport became the output (Nédélec et al., 1997; Sanchez, Daniel T. N. Chen, et al., 2012; Tyler D. Ross et al., 2019b; Zijie Qu et al., 2021; Yang et al., 2025).

Lactate-gated synthetic cells pushed the same information-processing architecture into a biochemical and therapeutic regime. The membrane defined what the reaction

could sense and what it could release. Alpha-hemolysin admitted lactate into the vesicle. ALPaGA/LldR converted lactate into a transcriptional state. Cell-free TX–TL consumed biochemical resources to synthesize protein output. LLO created a macromolecular release route. In the therapeutic implementation, the vesicle produced and released a blinatumomab-format T-cell engager that recruited living T cells to kill CD19-positive Raji cells. The GUV did not imitate a T cell. It built an acellular upstream controller: it sensed a metabolic context, manufactured an immune-facing molecule, and delegated cytotoxic execution to living immune cells (Vincent Noireaux and Albert Libchaber, 2004; Zúñiga et al., 2021; Boyd et al., 2023; Monck, Elani, and Ceroni, 2024; Heili et al., 2024).

Across the three projects, information processing occurs through matter rather than above it. PRC1 occupancy, overlap density, connectivity, and rigidity encode mechanical state. Light-defined activation geometry and active stress fields encode spatial state of active fluids. Membrane permeability, promoter occupancy, TX–TL reaction progress, and pore formation encode biochemical state. Each system then spent energy to make that state consequential. ATP hydrolysis drove force propagation and transport in the active materials; TX–TL drove de novo protein production and release in the synthetic cells. In this thesis, cellular intelligence therefore means controlling and determining how information, whether physical or chemical, is processed and routed through cells, or in our case, active materials (Bray, 1995; Barkai and Leibler, 1997; Hartwell et al., 1999; Daniel Needleman and Zvonimir Dogic, 2017).

Top-down biology will discover the next generation of modules for these architectures. Living cells already contain solutions to the problems that bottom-up systems struggle to solve: various crosslinkers that tune force transmission, receptors that classify environmental context, pores and transporters that gate exchange, regulators that store chemical state, trafficking pathways that localize secretion, and immune mechanisms that convert molecular recognition into killing. Top-down cell biology, perturbation screens, living-cell synthetic biology, and computational models can identify these modules and reveal the principles that make them robust in their native context. Bottom-up reconstitution can then remove those modules from the living chassis and test sufficiency under defined physical conditions. PRC1, microtubules, kinesins, ALPaGA/LldR, alpha-hemolysin, LLO, and T-cell engagers already show this logic in miniature: each came from a real biological system, but each became engineerable only after it was isolated, simplified, and rebuilt in a new architecture

(Dixit et al., 2016; Adamson et al., 2016; Morsut et al., 2016; Roybal et al., 2016; Replogle et al., 2022).

I envision that future work will meet both top-down and bottom-up approaches in the middle. For therapeutics, that convergence can produce acellular systems that borrow cellular logic without inheriting replication, long-term persistence, or genomic instability: compartments that sense disease context, manufacture effectors locally, and recruit living cells only where needed. For fundamental science, the same strategy creates constructive tests of mechanism. A top-down perturbation can identify a crosslinker, pore, receptor, regulator, or secretion module as necessary in cells; bottom-up reconstitution can ask whether that module is sufficient, what couplings it requires, and why it fails when isolated. The next step will not come from adding modules side by side. It will come from closing loops between modules: sensing to state, state to energy conversion, energy conversion to transport, and transport to output. Reconstituting cellular intelligence will therefore mean mining living systems for portable information-processing modules then rebuilding them in nonliving biomolecular matter.

I want to end with a broader speculation. We build almost everything today by shaping inert materials—plastic, metal, silicon—into precise forms. The entire discipline of engineering is essentially geometry: you design a shape, and you manufacture it. But none of the components in this thesis work that way. Alpha-hemolysin was not designed; it was selected because it carries a rule: admit small molecules, retain proteins. LLO carries a different rule: oligomerize above a cholesterol threshold. ALPaGA carries another: express when lactate is present. When you assemble rule-carrying components, you get an active machine—something that senses, decides, and acts. I think the future of manufacturing includes not just shaping materials but granting them operational rules, and that the line between a machine and a living thing will get harder to draw.

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Appendix A

SUPPLEMENTAL INFORMATION FOR FORCE PROPAGATION IN ACTIVE CYTOSKELETAL NETWORKS

A.1 PIV and Optical Flow Methods

Optical Flow

Optical flow is a method used to estimate the motion of objects between consecutive frames of a video sequence by analyzing the apparent motion of brightness patterns in the images. The Farneback method is a specific optical flow algorithm that uses polynomial expansions to estimate the velocity field.

Code implementation in `optical_flow.ipynb`

Optical Flow Assumptions

The basic assumption of optical flow is the brightness constancy constraint, which states that the intensity of a pixel remains constant between consecutive frames. Mathematically, this can be expressed as:

$$I_1(\mathbf{x}) = I_2(\mathbf{x} + \mathbf{d}(\mathbf{x})), \quad (\text{A.1})$$

where I_1 and I_2 are the intensities of the pixel at position $\mathbf{x}$ in the two frames, and $\mathbf{d}(\mathbf{x})$ is the velocity vector of the pixel.

Farneback Method

The Farneback method approximates the velocity field using polynomial expansions. It represents the local neighborhood of each pixel by a polynomial, which can be expressed as:

$$I(\mathbf{x}) \approx \mathbf{a}_0 + \mathbf{a}_1x + \mathbf{a}_2y + \mathbf{a}_3x^2 + \mathbf{a}_4xy + \mathbf{a}_5y^2, \quad (\text{A.2})$$

where $\mathbf{a}_i$ are the coefficients of the polynomial.

To estimate the velocity field, the method constructs an over-determined system of linear equations by sampling the polynomial coefficients in a neighborhood around each pixel. This system is then solved using least squares minimization to find the velocity vector $\mathbf{d}(\mathbf{x})$ that best fits the brightness constancy constraint.

Velocity Field Calculation

Once the velocity field $\mathbf{d}(\mathbf{x})$ is obtained, the velocity field $\mathbf{v}(\mathbf{x})$ is calculated by dividing the displacement by the time interval Δt between frames:

$$\mathbf{v}(\mathbf{x}) = \frac{\mathbf{d}(\mathbf{x})}{\Delta t}. \quad (\text{A.3})$$

Particle Image Velocimetry (PIV)

Particle Image Velocimetry (PIV) is a technique for measuring fluid flow velocity fields by tracking the displacement of tracer particles between sequential image frames.

PIV Processing Parameters

In our analysis, PIV was conducted using a three-pass algorithm to enhance the accuracy and resolution of the velocity field. Each pass refines the previous estimate by using smaller interrogation windows and step sizes, effectively increasing spatial resolution. The parameters used for each pass are:

- First pass: Interrogation window of 64 pixels, step size of 32 pixels.
- Second pass: Interrogation window of 32 pixels, step size of 16 pixels.
- Third pass: Interrogation window of 16 pixels, step size of 8 pixels.

These parameters are chosen to balance the trade-off between spatial resolution and robustness against noise.

Sub-Pixel Estimation and Correlation

For sub-pixel accuracy in the displacement measurement, we employed the Gauss 2x3-point estimator. This method fits a Gaussian function to the cross-correlation peak to estimate the displacement vector with sub-pixel precision. The displacement $\mathbf{d}(\mathbf{x})$ can be expressed as:

$$\mathbf{d}(\mathbf{x}) = \frac{\partial \Phi}{\partial \mathbf{x}} \bigg/ \frac{\partial^2 \Phi}{\partial \mathbf{x}^2}, \quad (\text{A.4})$$

where Φ represents the cross-correlation function. The peak of this function is iteratively refined to achieve higher precision.

Post-Processing and Validation

To ensure the reliability of the PIV results, post-processing steps were applied. Vector validation routines, including outlier detection and replacement, were performed using a threshold of 8 times the standard deviation. Additionally, a local median threshold of 3 was applied to detect and correct erroneous vectors. This validation step is crucial for eliminating spurious vectors and improving the overall quality of the velocity field.

PIV Velocity Field Calculation

The final velocity field $\mathbf{v}(\mathbf{x})$ is calculated by dividing the measured displacement $\mathbf{d}(\mathbf{x})$ by the time interval Δt between frames:

$$\mathbf{v}(\mathbf{x}) = \frac{\mathbf{d}(\mathbf{x})}{\Delta t}. \quad (\text{A.5})$$

This velocity field provides a detailed representation of the flow dynamics at each interrogation window's center.

Grid Averaging and Method Comparison

For comparative analysis between PIV and optical flow, both velocity fields need to be averaged over a uniform grid to facilitate direct comparison.

Grid Averaging Process

The grid averaging process involves interpolating the PIV and optical flow velocity data onto a common grid. Given a grid size G , the flow field is averaged over each grid cell. The velocity components u and v are computed as:

$$u_{avg}(i, j) = \frac{1}{n} \sum_{k=1}^n u_k, \quad (\text{A.6})$$

$$v_{avg}(i, j) = \frac{1}{n} \sum_{k=1}^n v_k, \quad (\text{A.7})$$

where u_k and v_k are the velocity components within the grid cell (i, j) , and n is the number of vectors within the cell. This averaging smooths the velocity field, reducing noise and allowing for a clearer comparison of the flow structures between methods.

Comparative Analysis

To compare the performance of the PIV and optical flow methods, we calculate three key metrics: relative speed errors, relative orientation errors, and cross-correlation between the methods and ground truth.

Relative Speed Error The relative speed error quantifies the discrepancy in the magnitudes of the velocity vectors between the method under evaluation and the ground truth. It is defined as:

$$\text{Relative Speed Error} = \frac{|V_{\text{method}} - V_{\text{gt}}|}{V_{\text{gt}}}, \quad (\text{A.8})$$

where V_{method} and V_{gt} are the magnitudes of the velocity vectors from the method under consideration (either PIV or optical flow) and the ground truth, respectively. This metric highlights the magnitude difference, providing insight into how accurately each method estimates the speed of the flow.

Relative Orientation Error The relative orientation error assesses the angular deviation between the estimated velocity vector from the method and the ground truth vector. It is calculated as follows:

$$\text{Relative Orientation Error} = \cos^{-1} \left(\frac{\mathbf{v}_{\text{method}} \cdot \mathbf{v}_{\text{gt}}}{|\mathbf{v}_{\text{method}}| |\mathbf{v}_{\text{gt}}|} \right), \quad (\text{A.9})$$

where $\mathbf{v}_{\text{method}}$ and $\mathbf{v}_{\text{gt}}$ are the velocity vectors obtained from the method and the ground truth, respectively. This metric measures the angular difference between the vectors, indicating how well each method captures the direction of the flow.

Cross-Correlation Analysis To evaluate the temporal consistency and overall agreement of the methods with the ground truth, we compute the cross-correlation of the mean speed across frames. The cross-correlation between the method's mean speed and the ground truth's mean speed is defined as:

$$\text{Cross-Correlation} = \frac{\sum (V_{\text{method}} - \bar{V}_{\text{method}})(V_{\text{gt}} - \bar{V}_{\text{gt}})}{\sqrt{\sum (V_{\text{method}} - \bar{V}_{\text{method}})^2 \sum (V_{\text{gt}} - \bar{V}_{\text{gt}})^2}}, \quad (\text{A.10})$$

where V_{method} and V_{gt} are the mean speeds of the method and ground truth for a given frame, and $\bar{V}_{\text{method}}$ and $\bar{V}_{\text{gt}}$ are the overall mean speeds across all frames. This

metric assesses how well the trends in the method's velocity estimates correlate with those of the ground truth over time.

Benchmarking

We benchmarked our custom optical flow algorithm with openPIV package from python. We used experimental data from the our previous work (Yang et al., 2022). The dataset consist of densely labeled Alexa-647 MTs and $1\ \mu\text{m}$ tracer beads were sequentially imaged every 8 seconds using fluorescence filter or brightfield microscopy. We performed PIV on the tracer beads data as the ground truth and performed PIV and image flow on the densely labeled data. We noticed that our image flow algorithm performed better in calculating both the magnitude of the velocity field and the orientation of the velocity vectors as shown in Figure A.1.

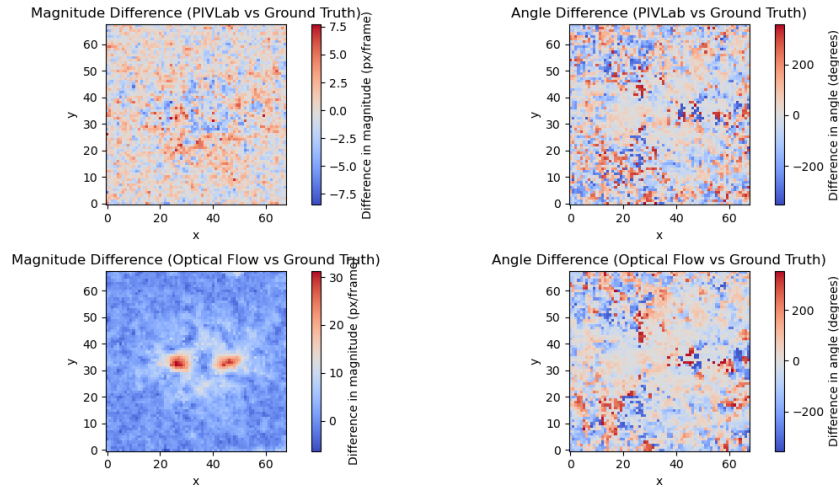


Figure A.1: **Comparison of Magnitude and Angle Differences between PIV/Optical Flow and Ground Truth for Frame 20.** The top row shows the **PIVLab** method compared to the ground truth: (left) magnitude difference and (right) angle difference. The bottom row shows the **Optical Flow** method compared to the ground truth: (left) magnitude difference and (right) angle difference. The magnitude difference is measured in pixels per frame, and the angle difference is in degrees. The PIVLab results exhibit less pronounced differences in magnitude and a scattered pattern of angle differences. In contrast, the optical flow method shows localized areas with significant magnitude differences but more uniform angle discrepancies.

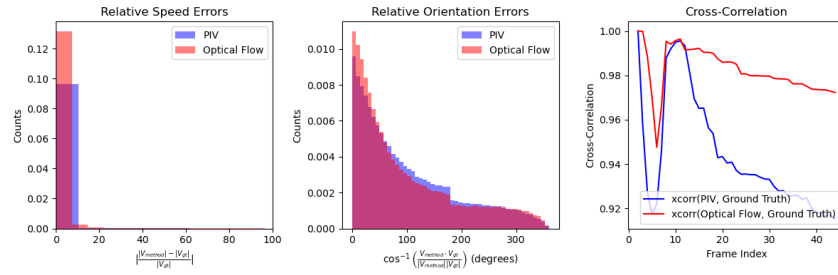


Figure A.2: **Histograms of Relative Speed and Orientation Errors, and Cross-Correlation for PIV and Optical Flow Methods.** From left to right: (1) The **relative speed errors** histogram shows the distribution of speed discrepancies normalized by the ground truth velocity. (2) The **relative orientation errors** histogram shows the distribution of angular discrepancies between the estimated and ground truth vectors. (3) The **cross-correlation** plot between the methods' mean speed and the ground truth's mean speed over 45 frames. Higher cross-correlation values indicate better temporal consistency with the ground truth. The optical flow method (red) maintains a high and stable correlation, while the PIV method (blue) shows a decline in correlation over time.

A.2 Microtubule Concentration Calibration

To establish a quantitative relationship between microtubule concentration and image intensity, we developed a calibration curve using a controlled experimental setup. This calibration allows us to convert observed fluorescence intensities in our main experiments to microtubule densities.

Code implementation in `figure_1 _density.ipynb`

Experimental Procedure

We prepared microtubule solutions at eight different concentrations. Each concentration was loaded into a separate flow cell. For each concentration, we captured 10 fluorescence microscopy images at different locations within the flow cell.

Data Analysis

For each image, we calculated the average pixel intensity. We then computed the mean intensity and standard deviation across the 10 images for each concentration. The known microtubule concentrations were converted to densities ($\text{MT}/\mu\text{m}^2$) based on the flow cell dimensions.

Calibration Curve

We plotted the average pixel intensity against the microtubule density and performed a linear regression. The resulting calibration equation is:

$$I = 143.05\rho - 39.76 \quad (\text{A.11})$$

where:

- I is the average pixel intensity
- ρ is the microtubule density in $\text{MT}/\mu\text{m}^2$

A.3 Velocity Correlation Function and Correlation Length Calculation

To quantify the spatial coherence of the velocity field in our active microtubule network, we computed the two-point velocity correlation function and derived the correlation length. This analysis provides insights into the length scale over which the system exhibits coordinated motion.

Code implementation in `figure_1 _correlation.ipynb`

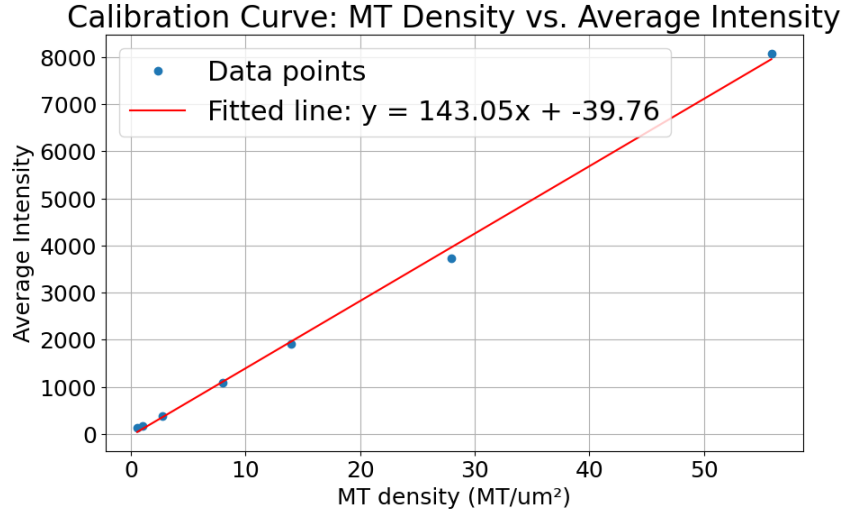


Figure A.3: **Microtubule density calibration curve**

Velocity Correlation Function

The two-point velocity correlation function $C(r)$ is defined as:

$$C(r) = \frac{\langle \mathbf{v}(\mathbf{r}_0) \cdot \mathbf{v}(\mathbf{r}_0 + \mathbf{r}) \rangle}{\langle |\mathbf{v}(\mathbf{r}_0)|^2 \rangle} \quad (\text{A.12})$$

where $\mathbf{v}(\mathbf{r})$ is the velocity vector at position $\mathbf{r}$, and the angle brackets denote an ensemble average over all positions $\mathbf{r}_0$ and time. Velocity vectors were computed based on Supplemental Information 1.1 In practice, we compute this correlation function as follows:

We select a region of interest in the center of the image, spanning ± 100 pixels in the y-direction. We compute the mean velocity field by averaging over frames 90 to 110. For each frame, we calculate the velocity fluctuations: $\delta \mathbf{v}(\mathbf{r}) = \mathbf{v}(\mathbf{r}) - \langle \mathbf{v}(\mathbf{r}) \rangle$. We then compute the correlation between a reference point (rightmost column of the ROI) and all other points to its left:

$$C(r) = \frac{\langle \delta \mathbf{v}(\mathbf{r}_{\text{ref}}) \cdot \delta \mathbf{v}(\mathbf{r}_{\text{ref}} + \mathbf{r}) \rangle}{\sqrt{\langle |\delta \mathbf{v}(\mathbf{r}_{\text{ref}})|^2 \rangle \langle |\delta \mathbf{v}(\mathbf{r}_{\text{ref}} + \mathbf{r})|^2 \rangle}} \quad (\text{A.13})$$

We average this correlation over all frames to obtain the final correlation function.

Correlation Length Calculation

The correlation length L_c is a measure of the distance over which velocities remain correlated. We use two methods to calculate L_c , depending on the behavior of the correlation function:

For short incubation times (local phase): We fit the correlation function to an exponential decay:

$$C(r) = Ae^{-r/L_c} \quad (\text{A.14})$$

where A is the amplitude and L_c is the correlation length. We use non-linear least squares fitting to determine L_c .

For long incubation times (global phase): The correlation function exhibits more complex behavior, often becoming negative at larger distances. In this case, we define L_c as the distance at which the correlation function first drops to $1/e$ of its initial value:

$$C(L_c) = \frac{1}{e}C(0) \quad (\text{A.15})$$

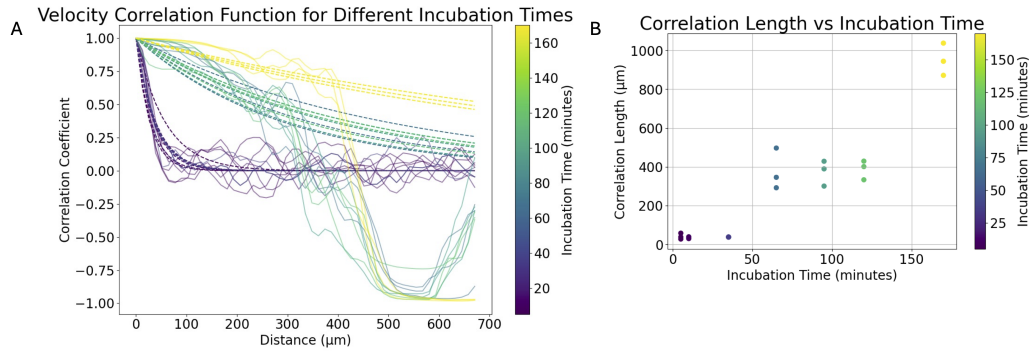


Figure A.4: **Exponential decay fitted to the two-point correlation function, and the corresponding correlation length l**

A.4 Flux Analysis

Code implementation in figure_1_flux.ipynb

Elliptical Region of Interest (ROI) Definition

We define an elliptical ROI using the equation:

$$\left(\frac{x - x_c}{a}\right)^2 + \left(\frac{y - y_c}{b}\right)^2 \leq 1 \quad (\text{A.16})$$

where (x_c, y_c) is the center of the ellipse, and a and b are the semi-major and semi-minor axes, respectively.

Boundary Point Selection

We use a k-d tree algorithm to efficiently find the nearest points on the ROI boundary to a set of evenly distributed points on a perfect ellipse. This ensures uniform sampling around the ROI while adhering to the pixelated nature of the image.

Flux Calculation

For each boundary point i at time t , we calculate the flux $F_i(t)$ as:

$$F_i(t) = \pm v_x(i, t) \cdot \rho(i, t) \cdot \Delta x \quad (\text{A.17})$$

where $v_x(i, t)$ is the x-component of the velocity, $\rho(i, t)$ is the microtubule density, and Δx is the pixel size. The sign is positive for points on the left half of the ellipse and negative for points on the right half.

Density Calibration

We convert intensity to microtubule density using the linear relationship using the equation obtained in section 2.

Data Smoothing

We apply Gaussian smoothing to the flux data:

$$F_{\text{smooth}}(x) = \int F(t) \cdot G(x - t) dt \quad (\text{A.18})$$

where $G(x)$ is a Gaussian function with standard deviation $\sigma = 2$.

Cumulative and Total Flux

Cumulative flux for each point i : $C_i = \sum_t F_i(t)$ Total flux at time t : $T(t) = \sum_i F_i(t)$

Velocity Field Interpolation

We interpolate the velocity field to the ROI boundary points using a grid-based approach:

$$v(x, y) \approx v(\lfloor x/g \rfloor \cdot g, \lfloor y/g \rfloor \cdot g) \quad (\text{A.19})$$

where g is the grid size (30 pixels in this case).

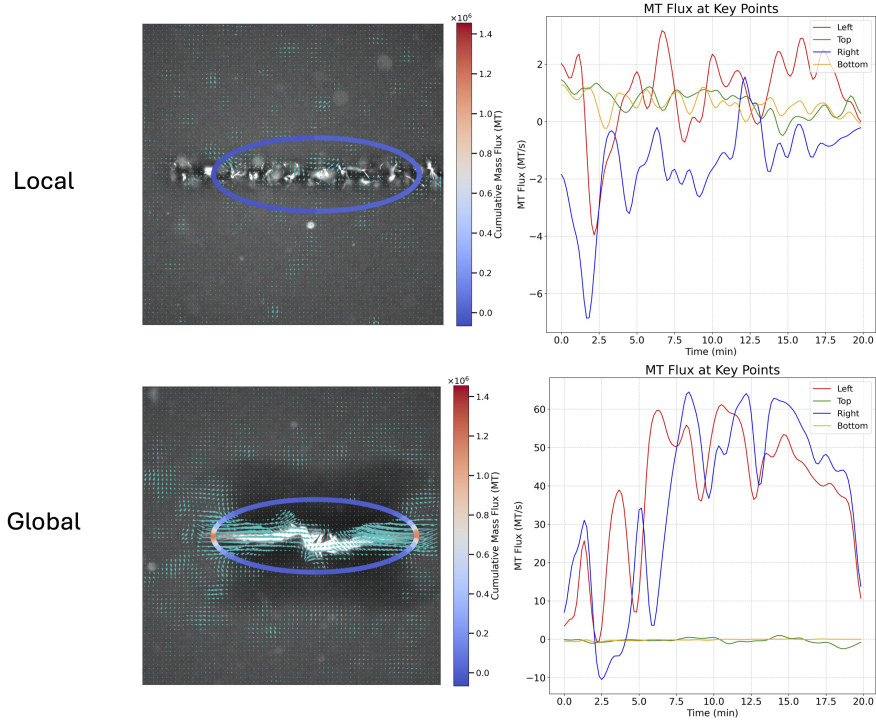


Figure A.5: **Microtubule flux at vertices and co-vertices of the ROI in representative global and local phase.**

A.5 Fluorescence Recovery After Photobleaching (FRAP)

Fluorescence Recovery After Photobleaching (FRAP) is a technique used to study the dynamics of molecular diffusion and interactions within cellular environments. In a FRAP experiment, a region of interest (ROI) within a fluorescently labeled sample is photobleached using a high-intensity laser, and the subsequent recovery of fluorescence within the bleached area is monitored over time. The rate of fluorescence recovery provides insights into the mobility and binding characteristics of the molecules in the ROI. Since we cannot directly image and measure the microtubule structures in the experiments, we employed FRAP to measure for the microtubule structure size. Code implementation in figure_2_FRAP.ipynb

Data Normalization

Normalization of the fluorescence intensity values is performed to account for variability in initial fluorescence levels and photobleaching efficiency. The normalization is defined as:

$$I_{\text{normalized}}(t) = \frac{I(t) - I_{\min}}{I_{\max} - I_{\min}}, \quad (\text{A.20})$$

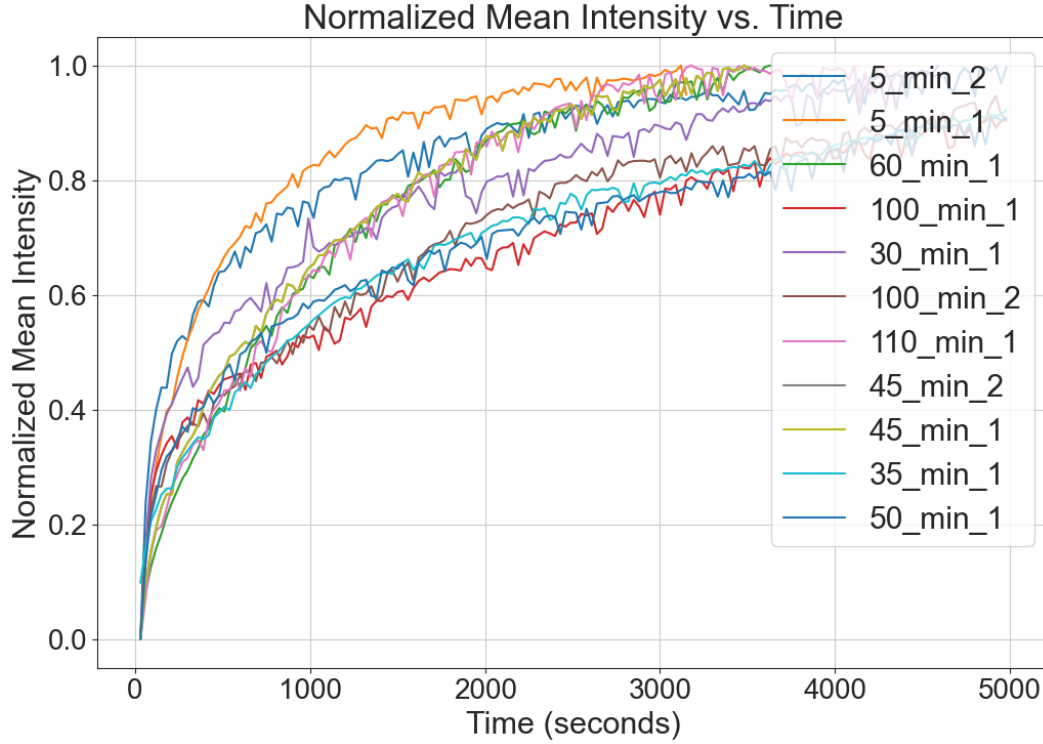


Figure A.6: **Fluorescence Intensity During Recovery**

where $I(t)$ is the intensity at time t , $I_{\min}$ is the minimum intensity, and $I_{\max}$ is the maximum intensity observed in the recovery phase. This normalization ensures that the fluorescence intensity values range between 0 and 1.

Half-time Recovery Calculation

The half-time recovery ($\tau_{1/2}$) is a critical parameter in FRAP analysis, representing the time required for the fluorescence intensity to recover to half of its final value. Mathematically, it is determined as:

$$\tau_{1/2} = \min \{t \mid I_{\text{normalized}}(t) \geq 0.5\}. \quad (\text{A.21})$$

This value is identified by finding the time point at which the normalized intensity reaches 50

Diffusion Coefficient Calculation

The diffusion coefficient D quantifies the rate at which molecules diffuse within the bleached area. It is derived from the half-time recovery using the following equation

from Axelrod's paper:

$$D = \frac{w^2}{4\tau_{1/2}} \gamma_D, \quad (\text{A.22})$$

where w is the radius of the laser beam used for photobleaching, and $\gamma_D = 0.88$ is a geometric correction factor for circular beams. The radius w is calculated as:

$$w = \text{resolution} \times \text{pixels}, \quad (\text{A.23})$$

with the resolution of the optical setup being $0.294 \mu\text{m}/\text{pixel}$ and the number of pixels being 390, giving:

$$w = 0.294 \mu\text{m}/\text{pixel} \times 390 \text{ pixels} = 114.66 \mu\text{m}. \quad (\text{A.24})$$

Thus, the diffusion coefficient is:

$$D = \frac{(114.66 \mu\text{m})^2}{4\tau_{1/2}} \times 0.88. \quad (\text{A.25})$$

A.6 Stokes Force Calculation

To calculate the forces exerted by the fluid on tracer particles, we employed Stokes' law. This method involved tracking the motion of tracer beads within the fluid, determining their velocities, and then calculating the forces based on the fluid's viscosity and the particles' radii.

Particle Tracking

We utilized the same particle tracking code as described in the previous section, but with $10 \mu\text{m}$ tracer beads introduced into the system. The positions of these beads, $\mathbf{x}_i(t)$, were tracked over time t , and their velocities, $\mathbf{v}_i(t)$, were computed by differentiating their trajectories with respect to time:

$$\mathbf{v}_i(t) = \frac{d\mathbf{x}_i(t)}{dt}. \quad (\text{A.26})$$

Velocity Calculation

The velocities of the tracer beads were initially calculated in micrometers per second ($\mu\text{m}/\text{s}$). These velocities were then converted to meters per second (m/s) for use in the Stokes' law calculation:

$$v_{i,\text{m/s}} = v_{i,\mu\text{m/s}} \times 10^{-6}. \quad (\text{A.27})$$

Stokes' Law

Stokes' law relates the force F exerted by a viscous fluid on a spherical particle moving through the fluid to the particle's radius r , the fluid's viscosity η , and the particle's velocity v . The equation is given by:

$$F = 6\pi\eta r v. \quad (\text{A.28})$$

For our system, we used the following parameters:

- Radius of the tracer particles: $r = 6.5 \times 10^{-6}$ meters.
- Viscosity of the fluid: $\eta = 2$ Pa.s.

The calculated forces for each particle were then averaged to obtain a representative value of the force in the system. The results, including velocities in both $\mu\text{m/s}$ and m/s , and the corresponding forces in Newtons (N), were compiled into a DataFrame for further analysis and plotting.

Example Calculation

For clarity, an example calculation is provided below. Given a tracer bead velocity of $10 \mu\text{m/s}$, the force exerted on the particle is calculated as follows:

$$\begin{aligned} v_{i,\text{m/s}} &= 10 \times 10^{-6} \text{ m/s}, \\ F_i &= 6\pi \times 2 \times 6.5 \times 10^{-6} \times 10 \times 10^{-6}, \\ F_i &= 2.44 \times 10^{-9} \text{ N}. \end{aligned}$$

A.7 Cytosim and Percolation Simulation Summary

The Cytosim and percolation analyses from the previous manuscript version are included here as supplemental material. These simulations use microtubule length distributions as an architectural control and compare Cytosim aster formation with a graph-based percolation model. Detailed percolation and Cytosim methods are provided in the following sections.

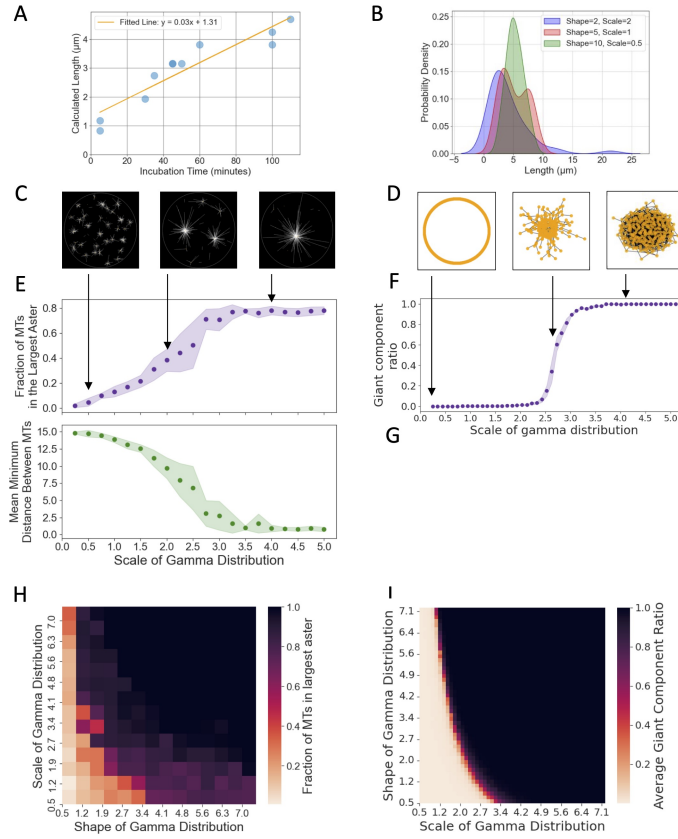


Figure A.7: Cytosim and percolation simulations of length-dependent active-network connectivity. **A**, Incubation time increases the effective microtubule bundle length, measured by fluorescence recovery after photobleaching (FRAP). **B**, Kernel density estimates of microtubule length distributions drawn from modified Gamma distributions, where each segment length is constrained to $1\text{--}25\ \mu\text{m}$ and the total length does not exceed $200\ \mu\text{m}$. Three parameter sets, $(k, \theta) = (2, 2)$, $(5, 1)$ and $(10, 0.5)$, illustrate how these constraints alter the probability density profiles relative to a standard Gamma distribution. **C**, Representative Cytosim simulations for different microtubule length distributions. **D**, Representative percolation simulations for different microtubule length distributions. **E**, Fraction of microtubules in the largest aster and the mean minimum distance between pairs of microtubules as a function of the Gamma scale parameter, with shape parameter fixed at $k = 1$. **F**, Giant-component ratio as a function of the Gamma scale parameter for the percolation simulations, with shape parameter fixed at $k = 1$. **G**, Representative Gamma distribution for simulated microtubule length. **H**, Heat map showing the relationship between the Gamma shape and scale parameters and the fraction of microtubules in the largest aster. **I**, Heat map showing the relationship between the Gamma shape and scale parameters and the giant-component ratio.

A.8 Percolation Analysis

Mapping active-gel connectivity onto ER percolation

In the main text we coarse-grain the microtubule–kinesin network into a *length-controlled* random graph: each microtubule bundle is a node; an *undirected* edge is drawn whenever any two bundles overlap or cross-link within a chemically short range. Code implementation of the simulation is provided in `percolation.ipynb`; a simpler version is available on Colab. If we assume (i) bundles are well mixed in 2-D chambers and (ii) the probability of a contact scales only with their mean length $\langle L \rangle$, then the adjacency matrix becomes *dense* and *uniform*. Under this assumption the ensemble is analytically equivalent to the classical Erdős–Rényi (ER) model

$$\mathcal{G}(n, q), \quad q = \frac{c}{n}, \quad c = 1 + \delta,$$

where n is the number of bundles, q the edge probability and c the mean degree. The control parameter is the reduced distance to criticality $\delta = c - 1$.

Order parameter and susceptibility

For every generated graph we label connected components with `scipy.sparse.csgraph.connected_components`. Let N_{GC} be the size of the largest cluster (the *giant component*). We monitor two standard observables:

$$S = N_{\text{GC}}/n, \tag{A.29}$$

$$\chi = \frac{1}{n} \sum_{\alpha \neq \text{GC}} N_{\alpha}^2, \tag{A.30}$$

where the sum runs over all clusters except the giant one. S plays the rôle of a magnetisation-like order parameter, while χ is the cluster-size susceptibility.

Finite-size scaling protocol

System sizes. We simulate three graph sizes,

$$n \in \{6\,400, 12\,800, 25\,600\},$$

chosen so that $n^{1/3}$ is an integer power of two, simplifying the asymptotic window selection.

Asymptotic window. Mean-field theory predicts $\delta_{\text{min}} \sim n^{-1/3}$ and we restrict all power-law fits to

$$|\delta| \in [n^{-1/3}, 0.04].$$

The upper bound 0.04 is well inside the critical regime yet far from finite-size saturation.

Replicas and averaging. For each (n, δ) pair we generate $N_{\text{rep}} = 60$ independent graphs. Sample means $\bar{S}$ and $\bar{\chi}$ are then used in the fits.

Exponent extraction. In the ER universality class

$$S \sim \delta^\beta, \quad \chi \sim |\delta|^{-\gamma}, \quad \beta = \gamma = 1,$$

for $\delta > 0$ and $\delta < 0$ respectively. We estimate slopes with a simple log-log linear regression; zeros and infinities are masked.

Data collapse. Setting $\nu = \frac{1}{2}$ (exact mean-field value) we rescale

$$x = \delta n^{1/3}, \quad y = S n^{1/3},$$

and verify that all sizes fall on a single master curve (Supplementary Figure A.8).

Numerical result. Table A.1 summarises the fitted exponents. The statistical uncertainty (standard error from the linear fit) is $\lesssim 0.05$ for all n .

Table A.1: Numerical exponents for S and χ .

n	β	γ
6 400	1.04	0.96
12 800	1.01	1.02
25 600	0.98	1.01

Gamma-length phase diagram

To emulate the experimentally observed bundle-length distribution we draw filament lengths ℓ from a *clamped* Gamma law,

$$\ell \sim \Gamma(k, \theta) [0, \ell_{\text{max}} = 25 \mu\text{m}],$$

parameterised by the shape k and scale θ of the underlying (unclamped) Gamma density. For every (k, θ) pair we compute the *tail probability*

$$q(k, \theta) = 1 - F_{\Gamma}(\ell_{\text{thr}} = 9 \mu\text{m}; k, \theta),$$

where F_Γ is the cumulative distribution function of the *unclamped* Gamma. The threshold $\ell_{\text{thr}}=9\,\mu\text{m}$ corresponds to the minimum overlap length at which two bundles are experimentally observed to cross-link with high probability.

We then feed the edge probability q directly into an Erdős–Rényi graph $\mathcal{G}(n, q)$ with $n = 25\,600$. Instead of Monte-Carlo sampling we exploit the $n \rightarrow \infty$ closed-form solution

$$S = 1 + \frac{W(-c e^{-c})}{c}, \quad c = n q,$$

where W is the Lambert W function. This expression is exact for ER graphs and makes the 120×120 parameter scan complete in under one second on a laptop.

Panel I of Supplementary Figure A.7 plots the resulting surface $S(k, \theta)$ as a heat-map. The color jump delineates a thin percolation front: increasing either the shape or the scale parameter by $\Delta k \lesssim 0.2$ or $\Delta \theta \lesssim 0.2$ is enough to nucleate a system-spanning (giant) component.

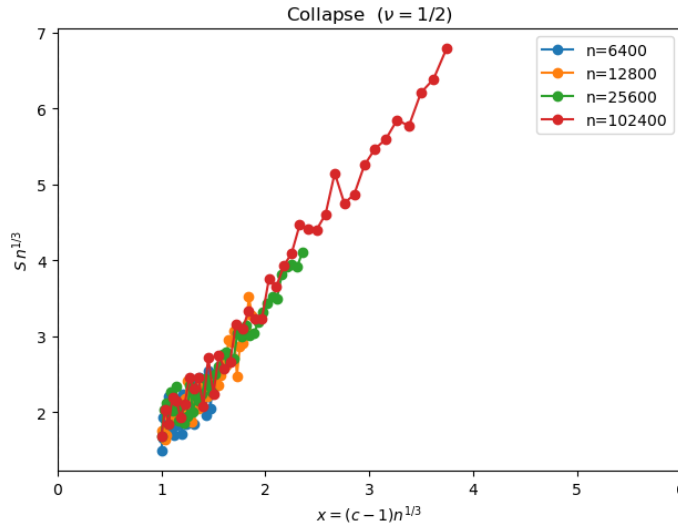


Figure A.8: Finite-size scaling collapse of the giant-component order parameter. Symbols denote Erdős–Rényi graphs with $n = 6,400$ (blue), $12,800$ (orange), $25,600$ (green) and $102,400$ (red). Only data inside the asymptotic window $n^{-1/3} \leq |\delta| \leq 0.08$ (see Supplementary Note S3) are plotted, so the rescaled axis begins at $x_{\min} \approx 1$. After the mean-field rescaling $x = (c - 1) n^{1/3}$ and $y = S n^{1/3}$ all sizes fall on a single master curve, confirming the expected exponents $\nu = \frac{1}{2}$ and $\beta = 1$.

Reproducibility notes

- All simulations were run with Python 3.10, SciPy 1.11, NumPy 1.26, scikit-learn 1.4, and Matplotlib 3.8.

- A fully annotated Jupyter notebook (`percolation.ipynb`) accompanies this file. A lighter Colab version is linked in the main text.
- Random streams are seeded with `np.random.default_rng(seed=2024)` for bit-wise reproducibility.
- Runtime on a laptop (Apple M1, 8 cores) is ~ 30 s for the scaling analysis and ~ 20 s for the phase diagram.

A.9 Cytosim Simulation

Mathematical Background and Cytosim Framework

Cytosim is a powerful software package designed to simulate the collective dynamics of cytoskeletal fibers, such as microtubules and actin filaments (Nedelec and Foethke, 2007). It uses a coarse-grained representation of fibers and includes various biophysical processes, such as fiber flexibility, polymerization, depolymerization, and motor protein interactions. The mathematical framework underlying Cytosim is based on Langevin dynamics, which describes the motion of fibers and motors in a viscous medium subject to thermal fluctuations. In Cytosim, fibers are modeled as inextensible elastic rods, which are discretized into segments. The dynamics of each segment i is governed by the Langevin equation:

$$\gamma \frac{d\mathbf{r}_i}{dt} = \mathbf{F}_i^{\text{elastic}} + \mathbf{F}_i^{\text{motor}} + \mathbf{F}_i^{\text{thermal}} + \mathbf{F}_i^{\text{constraint}} \quad (\text{A.31})$$

where γ is the drag coefficient, $\mathbf{r}_i$ is the position of segment i , and $\mathbf{F}_i$ represents the forces acting on the segment. The elastic forces $\mathbf{F}_i^{\text{elastic}}$ are derived from the bending and twisting energies of the fibers, while the motor forces $\mathbf{F}_i^{\text{motor}}$ are described by a stochastic binding and unbinding kinetics. The thermal forces $\mathbf{F}_i^{\text{thermal}}$ are modeled as Gaussian white noise, and the constraint forces $\mathbf{F}_i^{\text{constraint}}$ ensure the inextensibility of the fibers and the connectivity between segments. The Langevin equation is solved numerically using a semi-implicit integration scheme:

$$\mathbf{r}_i(t + \Delta t) = \mathbf{r}_i(t) + \frac{\Delta t}{\gamma} \left(\mathbf{F}_i^{\text{elastic}} + \mathbf{F}_i^{\text{motor}} + \mathbf{F}_i^{\text{thermal}} + \mathbf{F}_i^{\text{constraint}} \right) \quad (\text{A.32})$$

where Δt is the time step. This scheme ensures numerical stability and allows for the efficient simulation of large systems with many fibers and motors.

Generating Cytosim Configuration Files

To run simulations in Cytosim, users need to provide configuration files that specify the properties and initial conditions of the cytoskeletal components. In this study,

we focus on the role of microtubule length distribution in the collective dynamics of microtubule-kinesin systems. We generate Cytosim configuration files with different microtubule length distributions based on the gamma distribution, which is characterized by two parameters: shape (k) and scale (θ).

We define a function `generate_microtubule_lengths(shape, scale, total_length)` that generates a set of microtubule lengths following a gamma distribution with the specified shape and scale parameters. The function also takes a `total_length` argument, which determines the target sum of all generated microtubule lengths. The generated lengths are rounded to one decimal place and constrained to be between 1 and 25 units, which is a biologically relevant range for microtubule lengths.

The generated microtubule lengths are then used to create microtubule objects in the Cytosim configuration files. For each unique length value in the generated lengths list, a microtubule object is created with the corresponding length and the number of microtubules with that length. For example:

```
new 5 microtubule
{
length = 1.5
}
new 3 microtubule
{
length = 2.3
}
```

In this example, there are 5 microtubules with a length of 1.5 and 3 microtubules with a length of 2.3.

To explore the effect of the gamma distribution parameters on the microtubule lengths and the resulting collective dynamics, we generate config files for two different scenarios: a 1D parameter sweep and a 2D parameter sweep. In the 1D sweep, we fix the shape parameter and vary the scale parameter, while in the 2D sweep, we vary both parameters simultaneously. Code implementation can be found in `1d-phase-diagram.ipynb` and `2d-phase-diagram.ipynb`, and `manual-config.ipynb`

A.10 Semiflexible Fiber Network Model and Image-Derived Mechanics

Semiflexible fiber network model

To model the mechanics of crosslinked MT networks, we developed a 2D semiflexible fiber intersection network. The model places $N_f = B/\lambda$ straight fibers in an $L \times L$ periodic box, where B is a fixed material budget and λ is the mean fiber length. Each fiber has a random center position (uniform in $[0, L]^2$), random orientation (uniform in $[0, \pi]$), and a length drawn from a Gamma distribution with mean λ and shape parameter k : $\ell_i \sim \text{Gamma}(k, \lambda/k)$. At fixed B , increasing λ produces fewer but longer fibers, controlling the network connectivity.

The network graph is constructed from geometric intersections. All pairwise fiber–fiber crossings are computed (accounting for periodic images), and each intersection becomes a graph node. A fraction p_{xl} of intersections are retained as crosslinks, representing the probability that a MT–MT contact is stabilized by PRC1. Consecutive crosslinks along the same fiber define the graph edges, with rest lengths set to the initial intersection spacing. This construction naturally produces a sub-isostatic network: most nodes lie in the interior of fibers and have coordination $z = 2$ (one neighbor on each side along the fiber), with only branch points at shared intersections contributing $z > 2$.

The total elastic energy has two contributions:

$$E = \frac{k_s}{2} \sum_{\langle i,j \rangle} (\ell_{ij} - \ell_{ij}^0)^2 + \frac{\kappa_b}{2} \sum_{\langle a,b,c \rangle} \frac{|\mathbf{d}_{abc}|^2}{L_1 + L_2}. \quad (\text{A.33})$$

The first term is a harmonic stretching energy over all edges, where ℓ_{ij} is the current edge length and ℓ_{ij}^0 is the rest length ($k_s = 1$). The second term is a bending energy over all consecutive triples (a, b, c) along the same fiber. Here $\mathbf{d}_{abc}$ is the positional deviation of node b from the line connecting a and c , computed as

$$\mathbf{d} = -(w_2 \mathbf{d}_{ba} + w_1 \mathbf{d}_{bc}), \quad (\text{A.34})$$

with weights $w_1 = L_1/(L_1 + L_2)$ and $w_2 = L_2/(L_1 + L_2)$, where $L_1 = |ab|$ and $L_2 = |bc|$ are the rest-state segment lengths. This formulation is equivalent to penalizing changes in angle along the fiber backbone, but is numerically stable (no trigonometric functions or $1/r$ singularities). The parameter κ_b denotes the bending stiffness of the modeled fiber segments and should be distinguished from geometric curvature measured in microscopy images.

To measure the shear modulus, we apply a simple shear strain $\gamma = 0.01$ using Lees–Edwards periodic boundary conditions, which impose shear without fixed walls.

The affine displacement $\Delta x_i = \gamma (y_i - L/2)$ is applied to all nodes, and the network is then relaxed to mechanical equilibrium using the FIRE (Fast Inertial Relaxation Engine) algorithm (Bitzek et al., 2006). The shear modulus is computed from the elastic energy of the relaxed configuration:

$$G = \frac{E(+\gamma) + E(-\gamma)}{\gamma^2 L^2}. \quad (\text{A.35})$$

The control parameter λ drives two sequential transitions as it increases. First, a connectivity transition: the giant component fraction S rises from near zero to $S \approx 1$ as longer fibers create enough intersections to span the system. Second, a rigidity transition: the shear modulus G rises from zero at a higher value of λ , separated from the connectivity threshold by a gap where the network is topologically connected but mechanically floppy. The width of this connectivity–rigidity gap depends on the crosslink fraction p_{xl} and the fiber length heterogeneity (controlled by the Gamma shape parameter k). The rigidity transition is entirely bending-mediated: setting $\kappa_b = 0$ yields $G = 0$ for all λ , even when the giant component is fully formed. This confirms that the sub-isostatic fiber networks in this model cannot resist shear through stretching alone—rigidity requires bending stiffness to transmit forces along the fiber backbone between crosslinks.

Application to image-derived networks

To test these predictions on real MT/PRC1 networks, we extracted skeleton networks from fluorescence microscopy images at four PRC1 concentrations (185, 195, 200, and 225 nM; $n = 20, 19, 20$, and 20 image windows) and loaded them directly into the same mechanical framework, bypassing the synthetic fiber placement and using the experimentally determined node positions, edges, and bending triples. We computed the shear modulus at four values of κ_b (0, 0.1, 1.0, 10.0). Setting $\kappa_b = 0$ yields $G = 0$ for all 79 networks, confirming that the image-derived networks are sub-isostatic and require fiber bending stiffness for rigidity. At $\kappa_b = 1$, a clear mechanical transition emerges: networks at 185–195 nM PRC1 show $G/G_{\text{max}} \approx 0.2$, while networks at 200–225 nM jump to $G/G_{\text{max}} \approx 0.5$ –0.6, coinciding with a roughly threefold increase in crosslink density. These results demonstrate that PRC1 crosslinking drives a bending-rigidity percolation transition in real MT networks: PRC1 creates the connectivity, but it is the intrinsic bending stiffness of the MT backbone that makes that connectivity load-bearing.

Image-derived fiber detection and network reconstruction

Fiber networks were reconstructed from fluorescence micrographs using a conservative ridge-detection and skeletonization pipeline. Images were converted to grayscale, background-subtracted, contrast-enhanced, filtered with multiscale vesselness filters, thresholded, morphologically cleaned, and skeletonized to obtain one-pixel-wide centerlines. Analysis windows were selected by ranking candidate crops for ridge signal, contrast, sharpness, and skeleton occupancy, while penalizing saturation, broad bright blobs, and edge-dominated windows.

To improve recovery of dim filaments, each selected window was also analyzed after intensity inversion. The inverted images were contrast-enhanced and filtered for dark ridge-like structure using a combination of Frangi, Meijering, and local line-contrast responses. Inverted-pass skeleton fragments were not accepted automatically; each fragment was retained only if it satisfied criteria based on length, linearity, proximity to the original skeleton, branchpoint count, and local contrast. Rejected fragments were preserved as an audit layer but excluded from the final mechanics networks.

The final image-derived skeleton was defined as the union of the original skeleton and the retained inverted-pass candidate fragments. Broad saturated or blurry regions were marked as ambiguous because they may contain unresolved overlapping fibers that cannot be assigned unambiguously from a single image. Thus, the reconstruction was designed to capture the visually resolved network backbone rather than every possible fluorescent filament.

For mechanics simulations, each skeleton pixel was treated as a network node and each 8-neighbor skeleton adjacency was treated as a spring edge. Coordinates were rescaled to a square model domain with side length $L = 10$. Bending interactions were assigned only along degree-2 centerline pixels, without imposing fiber continuity through junctions or crossings.

Because dim, saturated, and out-of-focus structures may be missed, these networks should be interpreted as conservative image-derived backbones rather than complete ground-truth segmentations. Mechanical stiffness computed from these networks is therefore a lower-bound-like estimate of the stiffness supported by the resolved load-bearing structure.

A.11 Microtubule Aster Tracking

Tracking microtubule asters involves detecting and tracking their positions over time in image sequences. To improve detection accuracy, variable thresholding is

applied, which adjusts the thresholding percentile based on the image characteristics, ensuring consistent detection across frames with varying intensity distributions.

"figure_4_aster_traj.ipynb"

Preprocessing with Variable Thresholding

Variable thresholding adapts the threshold value dynamically by calculating the percentile of pixel intensities in each frame. This method helps in distinguishing microtubules from the background more effectively. The preprocessing steps are:

1. Calculate the threshold value as a specified percentile of pixel intensities.
2. Create a binary mask where pixels above the threshold are set to 255 (white) and others to 0 (black).
3. Apply binary dilation to enhance the visibility of microtubule structures.

For different segments of the image sequence, different percentiles are used:

- The first 20 frames use a 99.4 percentile threshold.
- Frames 21 to 30 use a 99.5 percentile threshold.
- Frames 31 and beyond use a 99.97 percentile threshold.

Aster Tracking

After preprocessing, the TrackPy library is used to detect and track microtubule asters. The tracking steps are:

1. Detect particles in each frame using a bandpass filter.
2. Link particles across frames to form trajectories based on proximity and predicted motion.
3. Calculate velocities by differentiating trajectories with respect to time.

This method allows for accurate tracking of microtubule asters, providing valuable data on their dynamics under different experimental conditions.

Aster Area Calculating

Aster area can be computed by calculating the area above the intensity threshold. Implemented in "figure_4_aster_size.ipynb"

A.12 Cell and Bead Tracking and Velocity Calculation

We tracked cells and beads in brightfield images to analyze their movements and calculate associated physical quantities such as velocity, force, work, and power. The tracking was performed using a similar approach to particle tracking. Code implementation in "figure_4_taj.ipynb"

Cargo Detection and Tracking

Cargo were imaged using brightfield microscopy. The detection and tracking process involved the following steps:

1. **Detection:** Cargo was identified in each frame based on intensity and morphological criteria.
2. **Linking:** Detected cargo was linked across frames to form trajectories, ensuring that each cell/bead's movement was accurately captured over time.

The position $\mathbf{x}_i(t)$ of cargo i at time t was recorded, allowing for the calculation of displacement and velocity.

Velocity Calculation

The velocity $\mathbf{v}_i(t)$ of cargo i was calculated by differentiating its trajectory with respect to time.

$$\text{velocity} = \text{velocity} \times 0.43 \mu\text{m/pixel} \quad (\text{A.36})$$

Tracer Bead Force Calculation

The force exerted by or on the cargo was calculated using Stokes' law, previously described in section 5. In short, we calculate the force as the force experienced by a spherical particle moving through a viscous fluid to its velocity:

$$F = 6\pi\eta r v, \quad (\text{A.37})$$

where:

- F is the force,
- η is the viscosity of the fluid,
- r is the radius of the cell,
- v is the velocity of the cell.

In this case, we used a radius $r = 6.5 \times 10^{-6}$ meters ($6.5 \mu\text{m}$) and fluid viscosity $\eta = 2 \times 10^{-3}$ Pa.s. The velocity v was converted from $\mu\text{m/s}$ to m/s using the pixel-to-meter conversion factor.

Work and Power Calculation

The work done by the moving the cargo was calculated by integrating the force over the displacement for each time step:

$$\text{work_done} = F \cdot \text{displacement}, \quad (\text{A.38})$$

where the displacement was converted from pixels to meters.

The total work done over the entire observation period was obtained by summing the work done at each time step.

$$\text{total_work_done} = \sum \text{work_done} \quad (\text{A.39})$$

The power, which is the rate of doing work, was calculated by dividing the work done at each time step by the corresponding time interval:

$$\text{power} = \frac{\text{work_done}}{\Delta t}, \quad (\text{A.40})$$

where Δt is the time between frames (1/8 seconds).

The total power at each time step was then summed to obtain the overall power profile as a function of time, which was plotted to visualize the energy expenditure of the cells.

$$\text{total_power} = \sum \text{power} \quad (\text{A.41})$$

The power was plotted against time to show how the energy expenditure varied over the course of the experiment.

A.13 Experimental Setup

Light-induced kinesin expression and purification

We constructed two chimeras of *D. melanogaster* kinesin K401: K401-iLiD and K401-micro as previously described (Addgene 122484 and 122485) (Tyler D Ross et al., 2019a). In short, for the K401-iLiD plasmid we inserted iLiD with a His tag after the C-terminus of K401. For the K401-micro plasmid, we inserted K401 between the His-MBP and the micro. The MBP domain is needed to ensure the microdomain remains fully functional during expression (Guntas et al., 2015b). After the expression, the MBP domain can be cleaved off by TEV protease.

For protein expression, we transformed the plasmids to BL21(DE3)pLysS cells. The cells were grown in LB and induced with 1mM IPTG at 18°C for 16 hours after reaching OD 0.6. The cells were then pelleted at 4000G and resuspended in lysis buffer (50 mM sodium phosphate, 4 mM MgCl₂, 250 mM NaCl, 25 mM imidazole, 0.05mM MgATP, 5 mM BME, 1 mg/ml lysozyme and 1 tablet/50 mL of Complete Protease Inhibitor). After a 1-hour incubation with stirring, the lysate was passed through a 30kPSI cell disruptor. The lysate was clarified at 30,000 G for 1 hour. The supernatant was then incubated with Ni-NTA agarose resin for 1 hour. The lysate/Ni-NTA mixture was loaded into a chromatography column and washed three times with wash buffer (Lysis buffer with no lysozyme nor complete EDTA tablet), and eluted with 500mM imidazole. Protein elutions were dialyzed overnight using 30 kDa MWCO membrane against 50 mM sodium phosphate, 4 mM MgCl₂, 250 mM NaCl, 0.05 mM MgATP, and 1 mM BME. For the K401-micro elution, we added TEV protease at a 1:25 mass ratio to remove the MBP domain. Then, we used centrifugal filters to exchange to pH 6.7 protein storage buffer (50 mM imidazole, HCl for pH balancing, 4 mM MgCl₂, 2 mM DTT, 50 μ M MgATP, and 36% sucrose). Proteins were then aliquoted and flash frozen in LN₂ and stored under -80°C.

Microtubule Polymerization and Length Distribution

Fluorescent microtubule polymerization was previously described (Tyler D Ross et al., 2019a). In short, we used a protocol based on one found on the Dogic lab homepage. The procedure began by preloading and starting a 37°C water bath. GMP-cpp, reagents, and tubes were cooled on ice. A 20 mM DTT solution was prepared using Pierce no-weigh format, and a GMP mixture consisting of M2B, DTT, and GMP-cpp was made and stored on ice. The ultracentrifuge and rotor were pre-cooled to 4°C. Tubulin (20 mg/mL) and labeled tubulin (20 mg/mL) were

thawed in the water bath until mostly thawed and then cooled on ice. In a cold room, labeled tubulin was added to the stock vial of unlabeled tubulin and mixed gently. The GMP mixture was then added to the tubulin mixture and stirred gently. This combined mixture was pipetted into ultracentrifuge tubes and incubated on ice for 5 minutes before being centrifuged at 90,000 rpm, 4°C for 8 minutes. The supernatant was carefully collected without disturbing the pellet and transferred to an Eppendorf tube, mixed, and stored on ice. The mixture was incubated in a 37°C water bath for 1 hour, protected from light. Aliquots were then dispensed into PCR strip tubes, which were spun to collect the fluid at the bottom. Finally, the PCR strips were flash frozen in liquid nitrogen and stored in a -80°C freezer.

To measure the length distribution of microtubules, we imaged fluorescently labeled microtubules immobilized onto the cover glass surface of a flow cell. The cover glass was treated with a 0.01% solution of poly-L-lysine (Sigma P4707) to promote microtubule binding. The lengths of microtubules were determined by image segmentation. Each microtubule image was normalized and underwent local and global thresholding to correct for non-uniform backgrounds and obtain thresholded images of putative microtubules. Morphological operations were applied to reconnect small breaks in filaments. Objects near the image boundary were removed, and small or circular objects were filtered out based on size and eccentricity thresholds. Potential microtubule crossovers were identified and eliminated by analyzing the angles of lines within the image.

Sample Chambers for Experiments

Glass slides and coverslips were first cleaned using a series of washes. Slides and coverslips were placed in respective containers, and 2% Hellmanex solution was prepared by mixing 6 mL Hellmanex with 300 mL DI water, heated, and poured into the containers. The containers were sonicated for 10 minutes, followed by three DI water rinses and an ethanol rinse. Ethanol was added to the containers and sonicated again for 10 minutes, followed by another ethanol rinse and three DI water rinses. Next, 0.1 M KOH was added to the containers, sonicated for 10 minutes, and rinsed three times with DI water. The slides were then etched overnight with 5% HCl and rinsed three times with DI water. Clean slides and coverslips were stored in DI water.

For silane coupling, a 2% acrylamide solution was prepared using 40% acrylamide stock solution, and degassed under vacuum. In a chemical hood, 98.5% ethanol,

1% acetic acid, and 0.5% silane agent were mixed to prepare the silane-coupling solution, which was immediately poured into the containers with the slides and coverslips and incubated at room temperature for 20-30 minutes. The slides were then rinsed once with ethanol, three times with DI water, and baked at 110°C for 30 minutes or 50°C overnight.

For acrylamide polymerization, the degassed 300 mL of 2% acrylamide solution was moved to a stir plate, and 105 μ L TEMED and 210 mg ammonium persulfate were added. The solution was immediately poured over the silane-coupled slides and coverslips and left to polymerize overnight at 4°C. Before use, the slides and coverslips were rinsed with DI water and air-dried.

Reaction Mixture

For the self-organization experiments, K401-micro, K401-iLID, and microtubules were combined into a reaction mixture to achieve final concentrations of approximately 0.1 μ M for each motor type and 1.5-2.5 μ M for tubulin, referring to protein monomers for K401-micro and K401-iLID constructs, and protein dimers for tubulin. The sample preparation was conducted under dark-room conditions to minimize unintended light activation, using room light filtered to block wavelengths below 580 nm (Kodak Wratten Filter No. 25). The base reaction mixture included a buffer, MgATP as an energy source, glycerol as a crowding agent, pluronic F-127 for surface passivation, and components for oxygen scavenging (pyranose oxidase, glucose, catalase, Trolox, DTT), along with ATP-recycling reagents (pyruvate kinase/lactic dehydrogenase, phosphoenolpyruvic acid). The reaction mixture consisted of 59.2 mM K-PIPES pH 6.8, 4.7 mM MgCl₂, 3.2 mM potassium chloride, 2.6 mM potassium phosphate, 0.74 mM EGTA, 1.4 mM MgATP, 10% glycerol, 0.50 mg/mL pluronic F-127, 2.9mg/mL pyranose oxidase, 3.2 mg/mL glucose, 0.086 mg/mL catalase, 5.4 mM DTT, 2.0 mM Trolox, 0.026 units/ μ L pyruvate kinase/lactic dehydrogenase, and 26.6 mM phosphoenolpyruvic acid.

We note that the sample is sensitive to the buffer pH and mixture incubation time. For our experimental conditions, the mixture pH is around 6.4 and we perform the experiments within 2 hours of constructing the mixture.

Microscope Setup

We conducted the experiments using an automated widefield epifluorescence microscope (Nikon Ti-2), custom-modified for two additional imaging modes: epilluminated pattern projection and LED-gated transmitted light. Light patterns from

a programmable DLP chip (EKB Technologies DLP LightCrafter™ E4500 MKII™ Fiber Couple) were projected onto the sample via a user-modified epi-illumination attachment (Nikon T-FL). The DLP chip was illuminated by a fiber-coupled 470 nm LED (ThorLabs M470L3). The epi-illumination attachment featured two light-path entry ports: one for the projected pattern light path and the other for a standard wide-field epi-fluorescence light path. These light paths were combined using a dichroic mirror (Semrock BLP01-488R-25). The magnification of the epi-illumination system was calibrated to ensure the camera's imaging sensor (FliR BFLY-U3-23S6M-C) was fully illuminated when the entire DLP chip was activated. Micro-Manager software, running custom scripts, controlled the pattern projection and stage movement.

Tracer Particle Method for Measuring Fluid Velocity

To measure the fluid velocity, we employed 1 μm polystyrene beads (Polysciences 07310-15) as tracer particles. The hydrophobic surface of these beads was passivated by incubating them overnight in M2B buffer with 50 mg/ml of pluronic F-127. This incubation process helps to coat the beads with pluronic, which prevents nonspecific interactions with the surrounding fluid and ensures accurate velocity measurements.

Before each experiment, the pluronic-coated beads were thoroughly washed. This was achieved by pelleting the beads through centrifugation and subsequently resuspending them in M2B buffer containing 0.5 mg/ml pluronic. This concentration of pluronic was chosen to match that of the reaction mixture, thereby maintaining consistency in the experimental conditions.

The beads, once prepared, were introduced into the fluid of interest. Their motion was tracked using high-resolution imaging techniques, and their velocities were calculated by analyzing their trajectories over time. This method provides precise measurements of the fluid velocity by leveraging the well-characterized motion of the tracer particles.

Culturing Jurkat Cells Using RPMI Medium

Jurkat cells, a type of immortalized T lymphocyte cell line, were cultured using RPMI 1640 medium. The RPMI 1640 medium provides the necessary nutrients and environment for the optimal growth and maintenance of Jurkat cells.

The cells were maintained in RPMI 1640 medium supplemented with the following components to enhance cell viability and proliferation:

- 10% fetal bovine serum (FBS) to provide essential growth factors, hormones,

and proteins.

- 1% penicillin-streptomycin to prevent bacterial contamination.
- 2 mM L-glutamine to support protein and nucleotide synthesis.

The culturing process involved the following steps:

1. **Cell Seeding:** Jurkat cells were seeded into culture flasks at an initial density of 0.2×10^6 cells/ml.
2. **Incubation:** The flasks were incubated at 37°C in a humidified atmosphere containing 5% CO₂. This environment mimics the physiological conditions of the human body, promoting optimal cell growth.
3. **Medium Change:** The culture medium was replaced every 2-3 days to remove metabolic waste products and replenish nutrients. This was done by gently pelleting the cells through centrifugation, discarding the old medium, and resuspending the cells in fresh RPMI 1640 medium with supplements.
4. **Cell Passage:** When the cells reached a density of 1×10^6 cells/ml, they were subcultured to maintain optimal growth conditions. This involved diluting the cell suspension with fresh medium and seeding it into new culture flasks.

We also tested cell viability after prolonged incubation in active matter mix using Trypan Blue. Jurkat cells showed minimal cell death during the first 180 minutes of incubation in active matter mixture.

Active Matter in Oil Emulsion

To prepare the active matter in oil emulsion using the EURx MICELLULA DNA Emulsion kit, follow these steps:

1. Prepare the Oil Emulsion Mix	Component	Volume (μL)
	Component 1	22
	Component 2	2
	Component 3	6

2. Combine the Reaction Mix with the Oil Emulsion Mix

- Add 5 μL of the reaction mix to 30 μL of the prepared oil emulsion mix.

3. Create a Stable Emulsion

- Run the tube containing the mixture against a tube rack to create a stable emulsion.
- Vortex the mixture if necessary to ensure proper emulsification.

A.14 Code Availability

Code can be found at <https://github.com/shichenliu97/active-matters-programmed-phase-transition>.

Appendix B

SUPPLEMENTAL INFORMATION FOR DYNAMIC FLOW CONTROL THROUGH ACTIVE MATTER PROGRAMMING LANGUAGE

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B.1 Coarse-grained model

Overview

Active networks of microtubules and motors exhibit diverse, and sometimes seemingly contradictory, behaviors under different conditions (M. C. Marchetti et al., 2013; D. Needleman and Z. Dogic, 2017). For example, both contractile (P. J. Foster et al., 2015; T. D. Ross et al., 2019) and extensile (Sanchez, D. T. N. Chen, et al., 2012) microtubule gels have been observed. Classified by symmetry, microtubule networks can exist in isotropic (Z. Qu et al., 2021), polar (B. A. Dalton et al., 2022) or nematic (Sanchez, D. T. N. Chen, et al., 2012; DeCamp et al., 2015) states. The active contraction in our system is mainly driven by the number density difference of the linked motors and microtubules, where the isotropic contractile forces are the central driving force. Indeed, such forces have been found to induce the active contraction of microtubule stripes (P. J. Foster et al., 2015).

In the past two decades, various coarse-grained models for motor-microtubule systems have been derived based on thermodynamic principles, such as the active gel theory (Joanny et al., 2007; Prost, Jülicher, and Joanny, 2015; Jülicher, Grill, and Salbreux, 2018), or microscopic interactions (Ahmadi, M. C. Marchetti, and Liverpool, 2006; Fürthauer, Lemma, et al., 2019; Fürthauer, D. J. Needleman, and Shelley, 2021). However, successful comparison of models and experiments for both microtubules and the ambient fluid is still lacking. Past attempts at modeling fluid flows in motor-microtubule systems are mainly through the boundary force model (T. D. Ross et al., 2019; Z. Qu et al., 2021), by placing force singularities at the boundaries of the illuminated region. The singularities are assumed immobile and thus the dynamics of active microtubules is not captured with this approach. Here we construct a multiphase complex fluids model that can quantitatively predict the coupled dynamics of microtubules and solvent flows.

Our system consists of three phases, namely the crosslinked microtubules, the freely-moving microtubules and the solvent solution. Other chemical species in our model include ATP and motor proteins. Upon illumination, the unlinked homodimer motor proteins will link to form tetramer motors, which can be further classified depending on whether they are bound on microtubules or not. To avoid confusion, we term the homodimer and tetramer motors “unlinked” and “linked” motors, respectively. The chemical reactions in our system include the reversible linking of motors under light and the reversible crosslinking of microtubules with linked motors. These chemical reactions will enter our model through the transport equations in section B.1.

In terms of dynamics, our system is mainly driven by the active stresses generated in the crosslinked microtubules. The stress-strain response of the crosslinked microtubules is modeled as a viscoelastic gel. The free microtubules are treated as passive particles and the solvent solution a Stokes flow. The ATP and motor proteins are much smaller molecules and assumed to follow the solvent flow, with the exception of the linked motors bound on microtubules, which follow the crosslinked microtubules. Each of the three phases also exerts friction on the other two. The momentum equations will be given in section B.1

As discussed before, we find *a posteriori* that the isotropic active stresses dominate in our system and the polar and nematic effects can be neglected. For simplicity we will not model the polarity distribution and its dynamical impacts in this paper.

Continuity Equations

The chemical reactions are sketched in Fig. B.1. The motor proteins can reversibly link and the transport equations are

$$\frac{\partial m}{\partial t} + \nabla \cdot (m\mathbf{u}) = 2(p^m d_f - p^d m^2) + D_m \nabla^2 m \quad (\text{B.1})$$

where m is the unlinked motor concentration, t is time, d_f is the freely-moving linked motor concentration, $\mathbf{u}$ is the solvent flow velocity, D_m is the diffusivity, p^m and $p^d(x, y, t)$ are the monomerization and dimerization rates of iLID proteins, respectively. For a static rectangular light bar, $p^d(x, y, t)$ is assumed to be

$$p^d(x, y, t) = \frac{p_0^d}{\left(1 + \left(\frac{x-c_x}{r_x}\right)^8\right) \left(1 + \left(\frac{y-c_y}{r_y}\right)^8\right)}, \quad (\text{B.2})$$

which is approximately a rectangular function with its center at (c_x, c_y) , side lengths $2r_x$ and $2r_y$, and maximum value p_0^d . The exponent “8” is empirically chosen. In

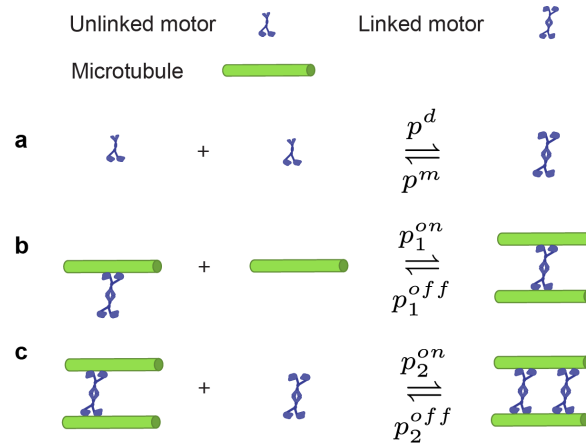


Figure B.1: **Illustrations of chemical reactions in the model.** **a**, Reversible linking of motors. **b**, Reversible crosslinking of a freely-moving microtubule i and another microtubule j . The latter can be either free or pre-crosslinked. **c**, Reversible binding of a linked motor on two pre-crosslinked microtubules.

general this exponent should be an even number and a larger exponent yields a sharper boundary of the rectangle. For a rotating light bar, one just needs to rotate the above expression in the $x - y$ plane at different times.

The transport equations for the crosslinked and freely-moving microtubules are

$$\frac{\partial c}{\partial t} + \nabla \cdot (c\mathbf{v}) = p_1^{on}c_f d_f - p_1^{off}c, \quad (\text{B.3a})$$

$$\frac{\partial c_f}{\partial t} + \nabla \cdot (c_f\mathbf{v}_f) = -p_1^{on}c_f d_f + p_1^{off}c + D_f \nabla^2 c_f. \quad (\text{B.3b})$$

where D_f is the diffusivity of the freely-moving microtubules, p_1^{on} and p_1^{off} are the crosslinking and un-crosslinking rates, respectively, see Fig. B.1b. The velocities of the crosslinked and freely-moving microtubules are denoted by $\mathbf{v}$ and $\mathbf{v}_f$, respectively.

The transport equations for the freely-moving and the bound linked motors are

$$\frac{\partial d_f}{\partial t} + \nabla \cdot (d_f\mathbf{u}) = -p^m d_f + p^d m^2 - p_1^{on}c_f d_f - p_2^{on}c d_f + p_2^{off}d_b + D_d \nabla^2 d_f, \quad (\text{B.4a})$$

$$\frac{\partial d_b}{\partial t} + \nabla \cdot (d_b\mathbf{v}) = p_1^{on}c_f d_f + p_2^{on}c d_f - p_2^{off}d_b, \quad (\text{B.4b})$$

where d_b is the bound linked motor concentration, p_2^{on} is the rate of the linked motors binding on the crosslinked microtubules, D_d is the diffusivity of the free linked motors, p_2^{off} is the unbinding rate of a linked motor without uncrosslinking the two microtubules, see Fig. B.1c. All freely-moving motors are assumed to follow the solvent flow and the bound motors follow the crosslinked gel.

The transport equation for the ATP is

$$\frac{\partial c_A}{\partial t} + \nabla \cdot (c_A \mathbf{u}) = -k_A d_b c_A + D_A \nabla^2 c_A, \quad (\text{B.5})$$

where k_A is the consumption rate of the ATP by the bound linked motors, and D_A is the ATP diffusivity. The continuity equation for the solvent flow is

$$\nabla \cdot \mathbf{u} = 0. \quad (\text{B.6})$$

Momentum Equations

We find it crucial to model the crosslinked and freely-moving microtubules as two separate phases. The former can be modeled as a self-contracting active gel and the latter passive particles. The solvent flow is assumed to be a Stokes flow. In this section, we list the momentum equations for each phase.

Crosslinked microtubules as an active gel

The crosslinked microtubules form an active gel, which is assumed to be viscoelastic. The momentum equation can be written as

$$\nabla \cdot (\boldsymbol{\sigma}_a + \boldsymbol{\sigma}_v + \boldsymbol{\sigma}_{el} + \boldsymbol{\sigma}_{st}) + \mathbf{f}_{fl} + \mathbf{f}_f = \mathbf{0}, \quad (\text{B.7})$$

where $\boldsymbol{\sigma}_a$, $\boldsymbol{\sigma}_v$, $\boldsymbol{\sigma}_{el}$ and $\boldsymbol{\sigma}_{st}$ are the active, viscous, elastic and steric stresses in the gel, $\mathbf{f}_{fl}$ and $\mathbf{f}_f$ are the frictions with the solvent and freely-moving microtubules, respectively. Their explicit expressions can be derived from microscopic interactions (Fürthauer, Lemma, et al., 2019; Fürthauer, D. J. Needleman, and Shelley, 2021). The active contractile stress is assumed to be

$$\boldsymbol{\sigma}_a = \alpha c_A d_b c \mathbf{I}, \quad (\text{B.8})$$

where c , c_A and d_b are the concentrations of the crosslinked microtubules, ATP and the bound linked motors, respectively, α is a constant activity coefficient and $\mathbf{I}$ is the identity tensor. This active stress is derived to be $\boldsymbol{\sigma}'_a \propto c^2 \mathbf{I}$, in Ref (Fürthauer, D. J. Needleman, and Shelley, 2021), where the bound motor concentration is implicitly assumed to be uniform and does not appear in active stresses. In our experiments, the concentration difference of the linked motors inside and outside of the illuminated regions is the key to form active gels. We can modify the theory in Ref (Fürthauer, D. J. Needleman, and Shelley, 2021) by adding a prefactor d_b/c in the active stresses, which is the number of bound motors per microtubule. In another word, the expression (B.8) can be derived using the same methods in Ref

(Fürthauer, D. J. Needleman, and Shelley, 2021) by taking the motor concentration into account and assuming the activity is proportional to the ATP concentration.

The viscous stress is

$$\boldsymbol{\sigma}_v = \eta d_{bc} \left(\nabla \mathbf{v} + \nabla \mathbf{v}^T \right), \quad (\text{B.9})$$

where $\mathbf{v}$ is the velocity of the crosslinked microtubules and η is the viscosity coefficient. This viscous stress in Ref (Fürthauer, D. J. Needleman, and Shelley, 2021) is $\boldsymbol{\sigma}'_v \propto c^2 (\nabla \mathbf{v} + \nabla \mathbf{v}^T)$. In our model the coefficient is changed from c^2 to d_{bc} to incorporate the effects of the motor concentrations.

The elastic stress $\boldsymbol{\sigma}_{el}$ is assumed to follow the Oldroyd-Maxwell model with a long relaxation time (Alves, Oliveira, and Pinho, 2021),

$$\frac{D\boldsymbol{\sigma}_{el}}{Dt} - [\boldsymbol{\sigma}_{el} \cdot \nabla \mathbf{v} + \nabla \mathbf{v}^T \cdot \boldsymbol{\sigma}_{el}] = \eta_{el} d_{bc} \left(\nabla \mathbf{v} + \nabla \mathbf{v}^T \right) - p_1^{off} \boldsymbol{\sigma}_{el}, \quad (\text{B.10})$$

where η_{el} is the elasticity coefficient and D/Dt is the material derivative. The steric stress is

$$\boldsymbol{\sigma}_{st} = -\xi c^2 \mathbf{I}, \quad (\text{B.11})$$

with a constant coefficient ξ .

The friction with freely-moving microtubules is

$$\mathbf{f}_f = \beta c c_f (\mathbf{v}_f - \mathbf{v}), \quad (\text{B.12})$$

where β is the friction coefficient. The friction with the solvent flow is

$$\mathbf{f}_{fl} = \gamma c (\mathbf{u} - \mathbf{v}) \quad (\text{B.13})$$

with the drag coefficient γ . In general the hydrodynamic drag coefficient depends on the fiber orientation (Leal, 2007). In this paper we neglect the polarity in both active stresses and hydrodynamic friction.

Freely-moving microtubules

The freely-moving microtubules are passive particles that only experience frictions from the gel and the ambient fluid. The force balance is $\beta c_f c (\mathbf{v} - \mathbf{v}_f) + \gamma c_f (\mathbf{u} - \mathbf{v}_f) = \mathbf{0}$, and their velocity is therefore

$$\mathbf{v}_f = \frac{\beta c \mathbf{v} + \gamma \mathbf{u}}{\beta c + \gamma}. \quad (\text{B.14})$$

Solvent flows

The flow cells used in the experiments are typical Hele-Shaw cells, the horizontal dimensions (x - and y -direction) of which greatly exceed their vertical dimension (z -direction). We now derive the averaged flow equation in the xy -plane. The three-dimensional momentum equation is

$$-\nabla\tilde{\Pi} + \mu\tilde{\nabla}^2\tilde{\mathbf{u}} + \gamma\tilde{c}(\tilde{\mathbf{v}} - \tilde{\mathbf{u}}) + \gamma\tilde{c}_f(\tilde{\mathbf{v}}_f - \tilde{\mathbf{u}}) = 0, \quad (\text{B.15})$$

where $\tilde{\Pi}$ and μ are the fluid pressure and viscosity, respectively. We use a tilde “ $\sim$ ” on top to indicate the variable is a function of (x, y, z) and if the variable is a vector, it is a three-dimensional vector, e.g., $\tilde{\Pi} = \tilde{\Pi}(x, y, z)$ and $\tilde{\mathbf{u}}(x, y, z) = (\tilde{u}_x(x, y, z), \tilde{u}_y(x, y, z), \tilde{u}_z(x, y, z))$. Since the vertical length scale is much smaller than horizontal length scales, the classical lubrication theory (Leal, 2007) shows that $\tilde{\Pi}(x, y, z) \approx \Pi(x, y)$, i.e., the fluid pressure is constant along z -direction, and $\tilde{u}_z \approx 0$. Substituting these two results into equation (B.15) yields $\tilde{v}_z \approx 0$ and $\tilde{v}_{f,z} \approx 0$. Furthermore, the x - and y -components of equation (B.15) can be approximated by

$$-\nabla\Pi + \mu\frac{\partial^2\tilde{\mathbf{u}}}{\partial z^2} + \gamma\tilde{c}(\tilde{\mathbf{v}} - \tilde{\mathbf{u}}) + \gamma\tilde{c}_f(\tilde{\mathbf{v}}_f - \tilde{\mathbf{u}}) = 0, \quad (\text{B.16})$$

where all vectors only have x - and y -components, i.e., $\tilde{\mathbf{u}} = (\tilde{u}_x(x, y, z), \tilde{u}_y(x, y, z))$, $\tilde{\mathbf{v}} = (\tilde{v}_x(x, y, z), \tilde{v}_y(x, y, z))$ and $\tilde{\mathbf{v}}_f = (\tilde{v}_{f,x}(x, y, z), \tilde{v}_{f,y}(x, y, z))$. Therefore, the analytical solution of $\tilde{\mathbf{u}}$ requires knowledge of the three-dimensional distribution of $\tilde{c}$, $\tilde{\mathbf{v}}$ and $\tilde{\mathbf{v}}_f$. To derive a two-dimensional model, we further simplify equation (B.16) by replacing the frictions with their z -directional average, i.e.,

$$-\nabla\Pi + \mu\frac{\partial^2\tilde{\mathbf{u}}}{\partial z^2} + \gamma c(\mathbf{v} - \mathbf{u}) + \gamma c_f(\mathbf{v}_f - \mathbf{u}) = 0, \quad (\text{B.17})$$

where $c(x, y) = h^{-1} \int_0^h \tilde{c} dz$, $\mathbf{v}(x, y) = h^{-1} \int_0^h \tilde{\mathbf{v}} dz$, $\mathbf{v}_f(x, y) = h^{-1} \int_0^h \tilde{\mathbf{v}}_f dz$, and $\mathbf{u}(x, y) = h^{-1} \int_0^h \tilde{\mathbf{u}} dz$ with the top and bottom walls of channel at $z = 0$ and $z = h$, respectively. These notations are consistent with the rest of the paper. The solution of equation (B.17) is

$$\tilde{\mathbf{u}} = \frac{1}{2\mu} [\nabla\Pi - \gamma c(\mathbf{v} - \mathbf{u}) - \gamma c_f(\mathbf{v}_f - \mathbf{u})] z(z - h). \quad (\text{B.18})$$

Combining $\mathbf{u} = h^{-1} \int_0^h \tilde{\mathbf{u}} dz$ and equations (B.14) and (B.18), we have the average two-dimensional flow velocity

$$\mathbf{u} = -\frac{h^2}{12\mu} [\nabla\Pi - (\gamma c + \gamma_f c_f)(\mathbf{v} - \mathbf{u})], \quad (\text{B.19})$$

with $\gamma_f = \gamma\beta c/(\beta c + \gamma)$. The above result is a modified Darcy's law in a Hele-Shaw cell (Leal, 2007) by incorporating the microtubule friction.

Non-dimensionalization

Using the initial microtubule concentration c_0 , initial unlinked motor concentration m_0 , initial ATP concentration c_{A0} , and the typical length scale of the illuminated region l , we can non-dimensionalize the governing equations by

$$\begin{aligned} \bar{c} &= \frac{c}{c_0}, \quad \bar{\nabla} = l_0 \nabla, \quad \bar{m} = \frac{m}{m_0}, \quad \bar{d}_f = \frac{d_f}{m_0}, \quad \bar{d}_b = \frac{d_b}{m_0}, \quad \bar{c}_A = \frac{c_A}{c_{A0}} \\ \bar{t} &= \frac{t}{t_0}, \quad \bar{v} = \frac{v}{v_0}, \quad \bar{v}_f = \frac{v_f}{v_0}, \quad \bar{u} = \frac{u}{v_0}, \quad \bar{\sigma}_{el} = \frac{\sigma_{el}}{\sigma_0}, \quad \bar{\Pi} = \frac{\Pi}{\Pi_0}, \end{aligned} \quad (\text{B.20})$$

where we use overlines to denote dimensionless variables, t_0 , v_0 , σ_0 and Π_0 are typical scales of time, velocity, stress, and fluid pressure, respectively. By balancing the contractile stress (B.8) and the viscous stress (B.9) in the active gel, we have $v_0 = \alpha c_{A0} l / \eta$. The typical time scale follows as $t_0 = l_0 / v_0 = \eta / \alpha c_{A0}$. The typical stress scale can be obtained from equation (B.8), which is $\sigma_0 = \alpha c_{A0} c_0 m_0$. From equation (B.19), we have $\Pi_0 = c_0 \gamma v_0 l_0 = \alpha \gamma c_0 c_{A0} l_0^2 / \eta$.

The dimensionless transport equations for the unlinked (B.1) and linked (B.4) motors are

$$\frac{\partial \bar{m}}{\partial \bar{t}} + \bar{\nabla} \cdot (\bar{m} \bar{u}) = 2(\bar{p}^m \bar{d}_f - \bar{p}^d \bar{m}^2) + \text{Pe}_m^{-1} \bar{\nabla}^2 \bar{m}, \quad (\text{B.21a})$$

$$\frac{\partial \bar{d}_f}{\partial \bar{t}} + \bar{\nabla} \cdot (\bar{d}_f \bar{u}) = -\bar{p}^m \bar{d}_f + \bar{p}^d \bar{m}^2 - \bar{p}_1^{on} \bar{c}_f \bar{d}_f - \bar{p}_2^{on} \bar{c} \bar{d}_f + \bar{p}_2^{off} \bar{d}_b + \text{Pe}_d^{-1} \bar{\nabla}^2 \bar{d}_f, \quad (\text{B.21b})$$

$$\frac{\partial \bar{d}_b}{\partial \bar{t}} + \bar{\nabla} \cdot (\bar{d}_b \bar{v}) = \bar{p}_1^{on} \bar{c}_f \bar{d}_f + \bar{p}_2^{on} \bar{c} \bar{d}_f - \bar{p}_2^{off} \bar{c}, \quad (\text{B.21c})$$

where the dimensionless reaction coefficients are $\bar{p}^m = p^m t_0$, $\bar{p}^d = p^m m_0 t_0$, $\bar{p}_1^{on} = p_1^{on} c_0 t_0$, $\bar{p}_2^{on} = p_2^{on} c_0 t_0$ and $\bar{p}_2^{off} = p_2^{off} t_0$. We use Pe to denote the Péclet number and $\text{Pe}_m = v_0 l / D_m$, $\text{Pe}_d = v_0 l / D_d$.

The dimensionless transport equations for the microtubules (B.3) are

$$\frac{\partial \bar{c}}{\partial \bar{t}} + \bar{\nabla} \cdot (\bar{c} \bar{v}) = \bar{p}_1^{on} \bar{c}_f \bar{d}_f - \bar{p}_1^{off} \bar{c}, \quad (\text{B.22a})$$

$$\frac{\partial \bar{c}_f}{\partial \bar{t}} + \bar{\nabla} \cdot (\bar{c}_f \bar{v}_f) = -\bar{p}_1^{on} \bar{c}_f \bar{d}_f + \bar{p}_1^{off} \bar{c} + \text{Pe}_f^{-1} \bar{\nabla}^2 \bar{c}_f \quad (\text{B.22b})$$

with $\bar{p}_1^{off} = p_1^{off} t_0$, $\text{Pe}_f = v_0 l_0 / D_f$. For ATP, equation (B.5) becomes

$$\frac{\partial \bar{c}_A}{\partial \bar{t}} + \bar{\nabla} \cdot (\bar{c}_A \bar{u}) = -\bar{k}_A \bar{d}_b \bar{c}_A + \text{Pe}_A^{-1} \bar{\nabla}^2 \bar{c}_A, \quad (\text{B.23})$$

where $\bar{k}_A = k_A m_0 t_0$ and $\text{Pe}_A = v_0 l_0 / D_A$.

The continuity equation for the solvent flow (B.6) is

$$\bar{\nabla} \cdot \bar{\mathbf{u}} = 0. \quad (\text{B.24})$$

Using equations (B.7-B.14), the dimensionless momentum equations for the active gel are

$$\bar{\nabla} \cdot \left[\left(\bar{c}_A \bar{d}_b \bar{c} - \bar{\xi} \bar{c}^2 \right) \mathbf{I} + \bar{\nabla} \bar{\mathbf{v}} + \bar{\nabla} \bar{\mathbf{v}}^T + \bar{\boldsymbol{\sigma}}_{el} \right] + \left(\bar{\gamma}_f \bar{c}_f + \bar{\gamma} \bar{c} \right) (\bar{\mathbf{u}} - \bar{\mathbf{v}}) = \mathbf{0} \quad (\text{B.25})$$

where $\bar{\xi} = \xi c_0 / \alpha c_{A0} m_0$, $\bar{\gamma} = \gamma l_0^2 / m_0 \eta$, $\bar{\gamma}_f = \gamma_f l_0^2 / m_0 \eta$ and

$$\frac{D \bar{\boldsymbol{\sigma}}_{el}}{D \bar{t}} - [\bar{\boldsymbol{\sigma}}_{el} \cdot \bar{\nabla} \bar{\mathbf{v}} + \bar{\nabla} \bar{\mathbf{v}}^T \cdot \bar{\boldsymbol{\sigma}}_{el}] = \bar{\eta}_{el} \bar{d}_b \bar{c} (\bar{\nabla} \bar{\mathbf{v}} + \bar{\nabla} \bar{\mathbf{v}}^T) - \bar{p}_1^{off} \bar{\boldsymbol{\sigma}}_{el}, \quad (\text{B.26})$$

with $\bar{\eta}_{el} = \eta_{el} / \alpha c_{A0}$.

The dimensionless velocity of the freely-moving microtubules (B.14) is

$$\bar{\mathbf{v}}_f = \frac{\bar{\beta} \bar{c} \bar{\mathbf{v}} + \bar{\gamma} \bar{\mathbf{u}}}{\bar{\beta} \bar{c} + \bar{\gamma}}, \quad (\text{B.27})$$

with $\bar{\beta} = \beta l_0^2 c_0 / m_0 \eta$. Note that only two of $\bar{\beta}$, $\bar{\gamma}$ and $\bar{\gamma}_f$ are independent, which are connected through $\bar{\gamma}_f = \bar{\gamma} \bar{\beta} \bar{c} / (\bar{\beta} \bar{c} + \bar{\gamma})$.

The dimensionless solvent flow (B.19) is

$$\bar{\mathbf{u}} = -\bar{\zeta} \left[\bar{\nabla} \bar{\Pi} - \left(\bar{c} + \frac{\bar{\gamma}_f}{\bar{\gamma}} \bar{c}_f \right) (\bar{\mathbf{v}} - \bar{\mathbf{u}}) \right], \quad (\text{B.28})$$

with $\bar{\zeta} = h^2 c_0 \gamma / 12 \mu$.

Simulations

Physical parameters

All experiments in the paper are controlled to be at similar conditions. Therefore, the same physical parameters are used in simulations throughout the paper, with one exception in Fig. 2c in the main text where $\bar{\zeta}$ is varied. The physical parameters are documented below:

$$\begin{aligned} l_0 &= 240 \mu\text{m}, \quad t_0 = 40 \text{ s}, \quad p_0^d = 15, \quad p^m = 12, \quad p_1^{on} = 15, \quad p_2^{on} = 0.75, \\ p_1^{off} &= 10, \quad p_2^{off} = 20, \quad \text{Pe}_f^{-1} = 10^{-3}, \quad \text{Pe}_m^{-1} = \text{Pe}_d^{-1} = \text{Pe}_A^{-1} = 10^{-2}, \\ \bar{k}_A &= 0.8, \quad \bar{\xi} = 0.05, \quad \bar{\eta}_{el} = 1.5, \quad \bar{\gamma} = 0.045, \quad \bar{\beta} = 0.45, \quad \bar{\zeta} = 20.5. \end{aligned} \quad (\text{B.29})$$

Additionally, the duration of each light pulse is 0.04 and the time interval between two light pulses is 0.2.

Aspect ratios of single bars

In the main text we show that the bar sizes do not affect the power law of flow decay. Another important geometric factor is the aspect ratio. A previous study on flows generated with different aspect ratios (AR) (Z. Qu et al., 2021) shows that they can change the flow directions outside the light bar: when $AR \geq 0.5$, the flow is directed away from the light region, as opposed to when $AR \leq 0.25$, the flow goes into the light bar.

To understand how the AR of a rectangular light bar affects scaling of flow decay, we fix the bar width and simulate single-bar flows with different aspect ratios. The center-line flow magnitudes are documented in Fig. B.2. Fig. B.2a shows that aspect ratios can change flow directions. When $AR \leq 0.25$, the center-line flow is always pointing towards the bar center. When $AR \geq 0.5$, the flow near the light bar edge is an outflow, pointing away from the bar. These findings are consistent with experiments (Z. Qu et al., 2021). Furthermore, our numerical results reveal that the flows outside the illuminated regions always decay in a power law, with the exponents ranging from -4 to -3.5, regardless of the aspect ratios (Fig. B.2b). To provide experimental evidence, we measure the center-line flow field induced by a single light bar with $AR = 0.3$ in Fig. B.2c. Both experimental and simulated data show that the flow decay follows the power law $(x/w)^{-3.5}$ outside the illumination region.

ATP consumption

ATP is the power source of our system and we calculate the time course of ATP in simulations in Fig. B.3. As the microtubule-kinesin aster forms a dense core, the consumption of ATP increases and results in a local depletion that cannot be sufficiently replenished by transport of ATP. More work will be done in future to characterize how ATP affects the flow intensity and stability.

B.2 Experimental system characterization

Stability of the solvent flow structure

How long does it take for our system to build up a structured solvent flow and how long can the flow persist? To address these questions, we measure the average flow speed around the light bar over time in Fig. B.4a. The time course of the average flow speed in the rectangular region in Fig. B.4a is plotted in Fig. B.4b. We find that the flow can be quickly initiated and reach its maximum value, around $6 \mu\text{m/s}$,

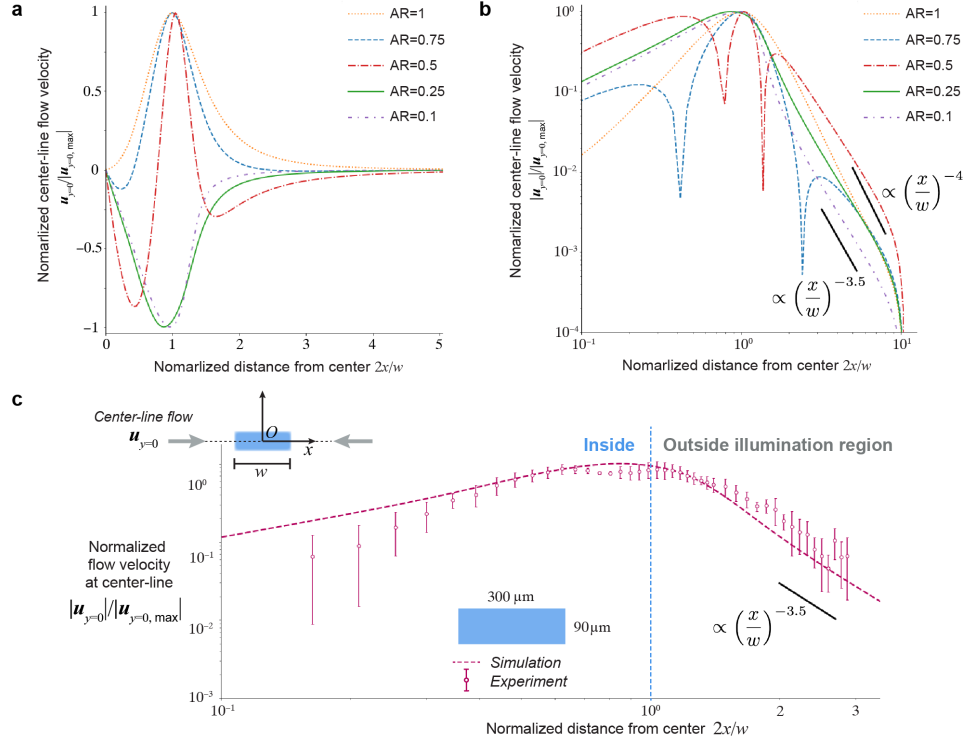


Figure B.2: **a**, Simulated center-line flows induced by single bars with different aspect ratios (AR). Positive (negative) values represent the flow is directed away from (into) the light bar. **b**, Absolute values of data in **a** in a log-log plot. **c**, Measured and simulated center-line flow field induced by a single bar with AR= 0.3. The flow fields are time-averaged over 240 s. Error bars show standard deviations of 5 experiments.

within 3 min. Then the active flow slowly decays over a time span of approximately 60 min. To quantify how stable the flow structure persists, we calculate the cross-correlation of the flow field over time in Fig. B.4c. The pointwise cross-correlation $Corr(x, y, t)$ is defined by $Corr(x, y, t) = \frac{\mathbf{u}(x, y, t) \cdot \mathbf{u}_0(x, y)}{|\mathbf{u}(x, y, t)| |\mathbf{u}_0(x, y)|}$, where we first compute the inner product of the experimental flow field $\mathbf{u}(x, y, t)$ and a benchmark flow field $\mathbf{u}_0(x, y)$, and then normalize by their norms. The benchmark flow field $\mathbf{u}_0(x, y)$ is chosen to be the time average of the flow field between $t = 1$ and 10 min. We calculate the spatial average of the cross-correlation $Corr(x, y, t)$ in the $500 \times 400 \mu\text{m}$ region shown in Fig. B.4a, which is denoted by $\overline{Corr}(t)$ and plotted in Fig. B.4a. The range of $\overline{Corr}(t)$ is $[-1, 1]$ where $\overline{Corr}(t) = 1$ represents perfect alignment of the experimental flow field with the benchmark flow. Both the average flow magnitude and cross-correlation decay monotonously after $t = 3$ min. The flow magnitude drops to its medium value $4 \mu\text{m/s}$ at around $t = 20$ min, when the cross-correlation $\overline{Corr}(t = 20 \text{ min})$ is 0.7. Additionally, Fig. B.4a shows that the flow field at $t = 20$

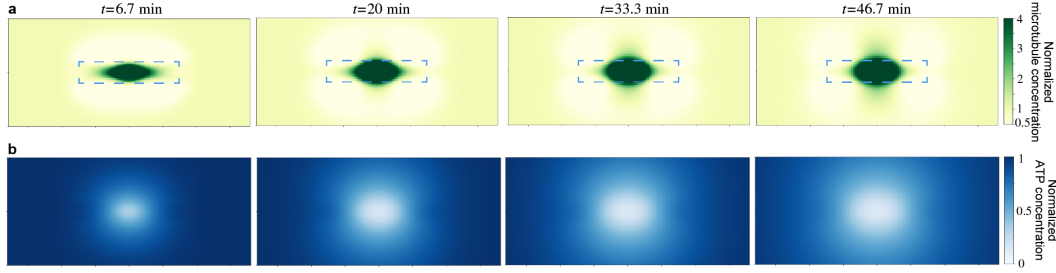


Figure B.3: **a**, Microtubule and **b**, ATP concentration field under a single light bar in simulations. The dashed line in **a** shows the light bar boundary. Both microtubule and ATP concentrations are normalized by their initial conditions.

min can clearly maintain its four-vortex structure. These results suggest that our active solvent flow can maintain its intensity and structure over a time span of 20 min, which is also the time scale for applications.

Microtubule concentration field

We also measure the time course of microtubule concentration generated by a single bar in Fig. B.5. The microtubule concentration is measured by the fluorescent intensity of Cy5 tagged on microtubules, where a linear relationship has been confirmed in our other paper (Liu et al., 2024). Fig. B.5a shows the spatial distribution of microtubules over time and Fig. B.5b shows the average microtubule concentration in the rectangular region in Fig. B.5a. The question of concern is how the accumulation of microtubules affects the flow field. The active solvent flow is driven by the microtubule fluxes going into the two short sides of the light bar. On the one hand the accumulation of microtubules could generate a higher active stress that might strengthen the flow, on the other hand the dense microtubule aster could increase flow resistance inside the channel and thus might decrease the flow. Fig. B.5b shows that average microtubule concentration in the aster grows linearly with time in experiments. As a consequence, the average mass flux of microtubules going into the aster is constant in time, which indicates that the accumulation of kinesin motors inside the microtubule network is not able to recruit more active matter influxes over time. As the flow resistance in the channel increases with the microtubule concentration, the flow strength decreases in time, as shown in Fig. B.4b. Fig. B.4b and Fig. B.5b demonstrate that the active matter system is more efficient in driving solvent flows when the aster is not dense. The exact mechanisms of the optimal microtubule network density and its values under different physical and chemical conditions remain an open question.

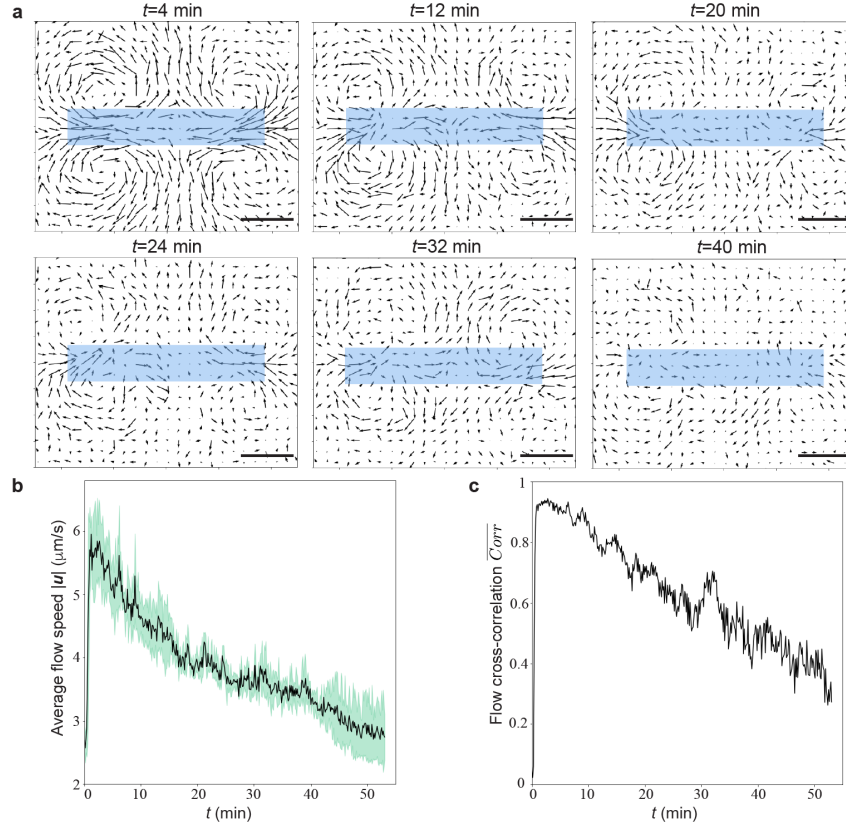


Figure B.4: Experimental flow characterization. **a**, Measured flow field over time. The flow fields are averaged over 5 experiments. Scale bars, $100\ \mu\text{m}$. **b**, Average flow magnitude over time. The flow speed is spatially averaged in the $500 \times 400\ \mu\text{m}$ rectangular region in **a**. The data are presented by the mean values $\pm$ s.d. of 5 experiments. **c**, Cross-correlation of the flow field over time. The flow field is averaged over 5 experiments.

The time course of average microtubule concentration in simulations is also shown in Fig. B.5b. Up to $t=15$ min, the simulated microtubule concentration also increases linearly. However, it plateaus at later times. The discrepancy between simulations and experiments may be due to neglecting higher-order active stresses in our model. Our model only considers the isotropic active stresses while the polar and nematic effects are neglected. At late times, such higher-order effects can be important when the microtubule aster is dense, which can result in dynamic and unstable shapes as shown in Fig. B.5a. These unstable shapes are not reproducible with our current model. Furthermore, our model assumes a simple isotropic steric stress that is quadratic in the microtubule concentration, which could be too strong such that the microtubule concentration plateaus at late times. Future efforts will be including these high-order effects in both active and steric stresses.

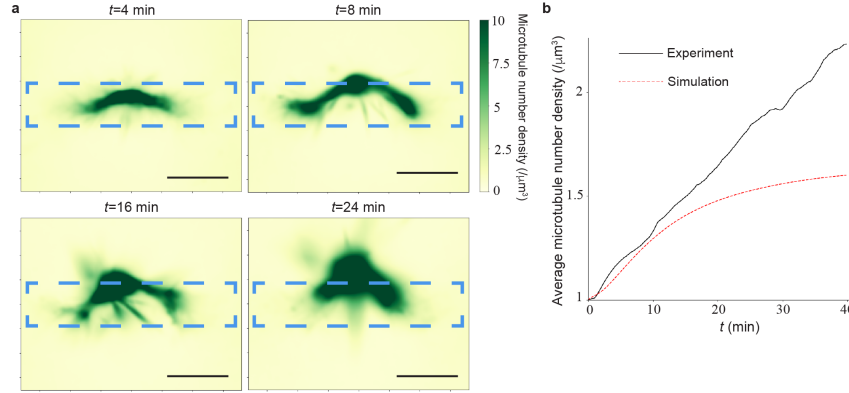


Figure B.5: Microtubule concentration characterization. **a**, Microtubule concentration field generated by a single light bar. The boundary of the light bar is labeled by a dashed line. Scale bars, $50\ \mu\text{m}$. **b**, Average microtubule concentration over time. The microtubule concentration is spatially averaged in the $180 \times 140\ \mu\text{m}$ rectangular region in **a**.

Microtubule length distribution

The microtubule length calculation begins with image normalization to account for background intensities across samples. We then apply a combination of local and global thresholding techniques to segment microtubules from the background, adapting to local intensity variations while maintaining consistent overall detection sensitivity. Morphological operations are used to refine the segmented structures, closing small gaps in filaments that may result from thresholding. To improve measurement accuracy, we implement several artifact removal steps: objects touching image borders are excluded to avoid partial measurements, small objects below a size threshold are removed to reduce noise, and circular objects are filtered out based on eccentricity to focus on linear microtubule structures. The data and codes been deposited at <https://github.com/fy26/ActiveMatter/tree/main/Microtubule%20length%20distribution>. The measured cumulative distribution function of microtubule lengths is plotted in Fig. B.6. The median length is $1.0\ \mu\text{m}$.

Comparison of flow magnitudes between superposition and experiments

To quantify the errors introduced by superposition, we compare the flow magnitudes between the superposed flow of 2 single-bar flows and experimental measurement of 2-bar flows (Fig. B.7a). The flow data are the same with Figs. 1b and 1c in the main text. We also calculate the experimental variations of flow magnitudes and compare their distribution with the superposition-induced error distribution, which is plotted in Fig. B.7b. The results show that the errors induced by linear superposition are

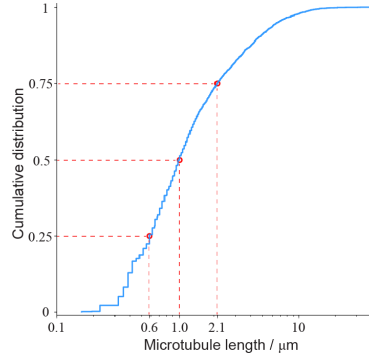


Figure B.6: Cumulative distribution of microtubule lengths.

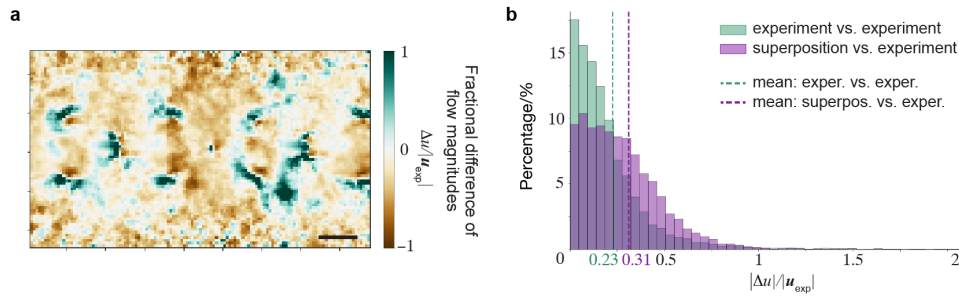


Figure B.7: **a**, Comparison of flow magnitudes between two-bar experiments and superposition of 2 single-bar flows. The heatmap represents the fractional difference between measured and superposed flow magnitudes $\Delta u/|u_{\text{exp}}|$, where $\Delta u = |u_{\text{sup}}| - |u_{\text{exp}}|$. **b**, Distributions of discrepancy in **a** and experimental variations. This demonstrates that the superposition-induced discrepancy is relatively small, only increasing the mean of errors from 0.23 to 0.31. All scale bars are $100 \mu\text{m}$ and all flow fields are time-averaged from $t = 160 \text{ s}$ to 400 s .

small, only increasing the mean of errors from 0.23 to 0.31.

Superposition of two pentagons

To further demonstrate that the principle of linear superposition is not limited to rectangular shapes, we experimentally show that superposition of two single-pentagon flows also matches with the flow field generated by two pentagons in Fig. B.8. The flow field induced by a single pentagonal light pattern has 10 counter-rotating vortices, as shown in Fig. B.8a. Such flow structures also persist when there are two pentagonal light patterns next to each other, as shown in Fig. B.8b. We linearly superpose two single-pentagon flows in Fig. B.8a to match the flow in Fig. B.8b. The superposed flow field and flow-direction discrepancy $\Delta\theta$ are shown in Fig. B.8c. We then quantify the distribution of $\Delta\theta$ in Fig. B.8d. The mean of $\Delta\theta$ is 0.58, comparable to the mean values of discrepancy found in two-bar and

nine-bar superposition, which are 0.3 and 0.6, respectively. Therefore, the principle of linear superposition is not limited to rectangular bars and can be generalized to other shapes.

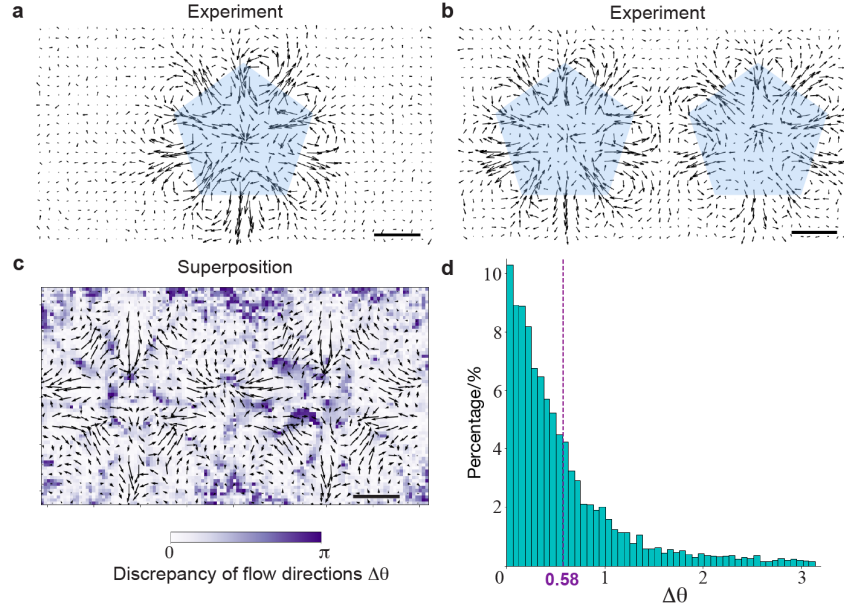


Figure B.8: Linear superposition can be applied to pentagons. **a**, Experimental flow field generated by a single pentagonal light pattern. **b**, Experimental flow field generated by two pentagons. **c**, The vector field is a superposition of two single-pentagon flows in **a** to match the flow field in **b**. The heat map shows the discrepancy of flow directions $\Delta\theta$ between superposed and measured flows. **d**, Distribution of the flow discrepancy $\Delta\theta$. The mean is 0.58. All flow fields are time-averaged over 240 s. Scale bars, 100 μm .

B.3 Optimization scheme for particle transport

In this section we show how to optimize particle transport along a straight path using linear transformation of single-bar flow data (Fig. B.9a). To ensure that the particle move along a straight path, the line segment AB should coincide with the axis of symmetry in the composition of light patterns (Fig. 3b, main text): when using only one bar, the particle should be placed at the center line perpendicular to the longer sides of the light bar to avoid being absorbed into the aster; when using two bars, the particle should move along their axis of symmetry, with a constraint that the two bars are separate above a critical spacing $\bar{w}_c$ to avoid merging; when using three bars, the optimal solution is just a sum of the solutions using one and two bars. Therefore we only need to solve for the optimal solution using two bars. This can be further simplified into finding the optimal location and orientation of

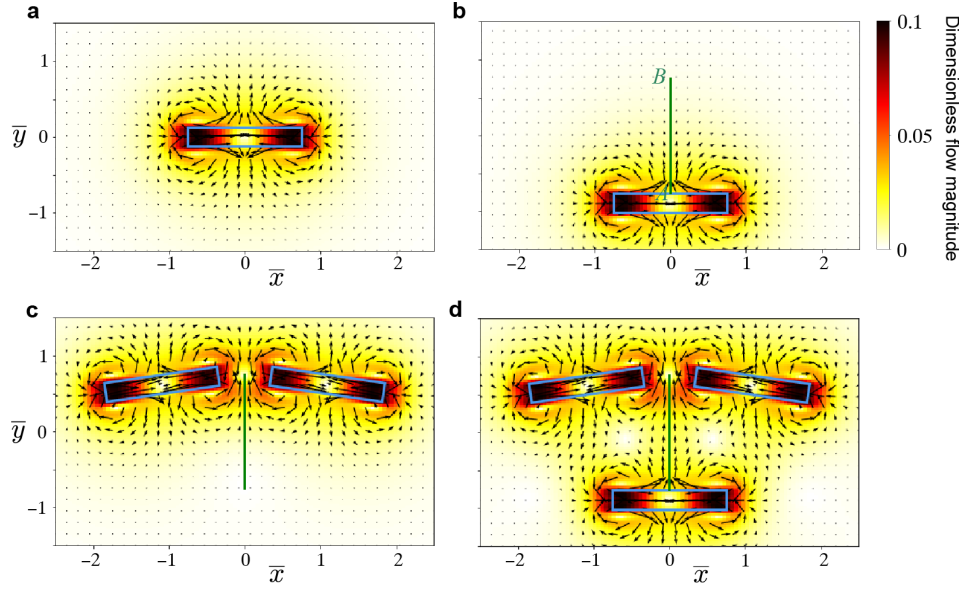


Figure B.9: **a**, Simulated single-bar flow field. Optimal solutions for particle transport using **b**, $n = 1$, **c**, $n = 2$ and **d**, $n = 3$ light bars, and their corresponding flow fields. The objective is to move a particle along the line segment AB (green). The outlines of light bars are plotted in blue.

a single bar to maximize f , and the second bar is just its reflection with respect to line AB. In this case, the objective function f only depends on three variables, the $\bar{x}$ - and $\bar{y}$ -coordinates of the bar center, and its orientation angle. The optimal solution can be found by directly searching the maximum value over the three-dimensional optimization landscape using simulated single-bar flow data. The region $\bar{x} < \bar{w}_c + \bar{w}_y/2$, where $\bar{w}_y$ is the width of the shorter side, is prohibited due to the constraint $\bar{w}_g < \bar{w}_c$ (Fig. 3c, main text).

Problem formulation

Objective: Move a particle along a line segment AB from $\bar{y} = -0.75$ to 0.75 along the $\bar{y}$ -axis and maximize its speed using n bars.

The objective function is defined as

$$f = \int_A^B \bar{u} \cdot d\bar{x}. \quad (\text{B.30})$$

The constraints are:

1. The particle does not move into the aster.

2. The spacing between bars should be above a critical length $\bar{w}_c$ to avoid interactions.

Solutions up to $n = 3$

To move the particle along a straight path, the axis of symmetry in the composition of light patterns should coincide with the $\bar{y}$ -axis.

$n = 1$

There are two symmetry axes for a single rectangular bar (Fig. 1a, main text), and the fluid flow goes into the bar lengthwise and out of the bar breadthwise. To satisfy constraint 1, the light bar should be placed at one end of AB with its long side perpendicular to the $\bar{y}$ -axis (Fig. B.9b)

$n = 2$

When there are two bars, the only way to construct a straight streamline, except for directly using the symmetry axes of single bars, is to make the two bars symmetric about $\bar{y}$ -axis. Therefore, the problem is reduced to finding the location and orientation of a single bar to maximize f , and the second bar is just its reflection with respect to $\bar{y}$ -axis.

We denote the single-bar flow field in Fig. B.9a as $\bar{\mathbf{u}}_s$. We now calculate the flow field $\bar{\mathbf{u}}$ induced by a single bar placed at $\bar{\mathbf{x}}_s$ with an orientation angle $\psi \in [0, \pi)$. The rotation matrix $\mathbf{R}$ associated with ψ is

$$\mathbf{R}(\psi) = \begin{pmatrix} \cos \psi & -\sin \psi \\ \sin \psi & \cos \psi \end{pmatrix}. \quad (\text{B.31})$$

Through simple linear transformation of coordinates, the flow field $\bar{\mathbf{u}}$ is just

$$\bar{\mathbf{u}}(\bar{\mathbf{x}}) = \mathbf{R}(\psi) \cdot \bar{\mathbf{u}}_s \left(\mathbf{R}(\psi)^T \cdot (\bar{\mathbf{x}} - \bar{\mathbf{x}}_s) \right). \quad (\text{B.32})$$

The constraint 2 can be formulated as

$$\bar{x} - \frac{\bar{w}}{2} |\cos \psi| - \frac{\bar{w}_y}{2} \sin \psi > \frac{\bar{w}_c}{2}, \quad (\text{B.33})$$

where $\bar{w}$ and $\bar{w}_y$ are the length and breadth of the light bar.

The optimal solution can be found directly by searching for the maximum value of the objective function (B.30) using (B.32) and the constraint (B.33), since it only

depends on 3 variables. Note that in plotting the optimization landscape Fig. 3c in the main text, at each location $(\bar{x}, \bar{y})$, we first calculate the maximum value of f with respect to the orientation angle ψ and also save the optimal $\psi^*(\bar{x}, \bar{y})$. In this way, the optimal solution $(\bar{x}^*, \bar{y}^*, \psi^*(\bar{x}^*, \bar{y}^*))$ can be determined by the maximum value of f in the optimization landscape. To show how the orientation angle ψ affects the objective function f , we plot the value of f over ψ at the optimized location $(\bar{x}^*, \bar{y}^*)$ in Fig. B.10.

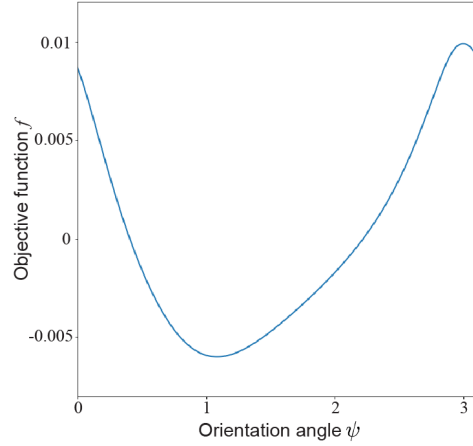


Figure B.10: Objective function f over orientation angle ψ .

Due to symmetry, there is one optimal solution in each quadrant. We can further confine the computation domain to be in Quadrant I and the solution is $\bar{x}^* = 1.09$, $\bar{y}^* = 0.62$ and $\psi^* = 3.0$ with $\bar{w}_c = 0.325$. The overall optimal two-bar configuration is shown in Fig. B.9c.

$n = 3$

Due to symmetry, the optimal solution for 3 bars can only be the linear superposition of the optimal solutions for $n = 1$ and $n = 2$, as shown in Fig. B.9d.

Appendix C

SUPPLEMENTARY INFORMATION FOR LACTATE-SENSING SYNTHETIC CELLS RELEASE T-CELL ENGAGERS TO KILL TUMOR CELLS

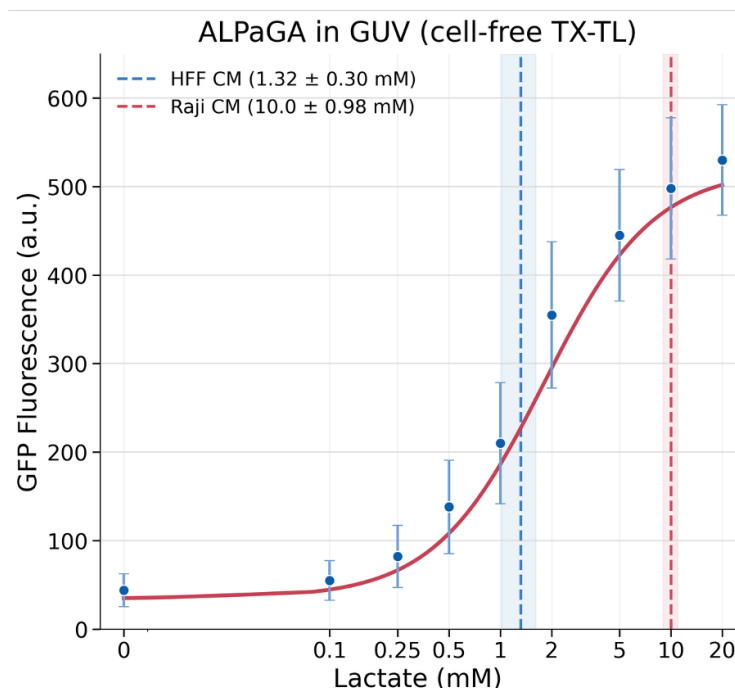


Figure C.1: ALPaGA lactate dose-response in cell-free TX-TL. GFP fluorescence at 6-hour endpoint from the ALPaGA plasmid (10 nM) in myTXTL reactions encapsulated in GUVs with pre-inserted α HL. Sodium L-lactate concentrations ranged from 0 to 20 mM. Data were fit to a Hill equation (solid line; $EC_{50} = 1.8$ mM, Hill coefficient $n = 1.35$). Dashed vertical lines indicate measured lactate concentrations in 24-hour conditioned medium from HFF cells (blue; 1.32 ± 0.30 mM, near the EC_{50}) and Raji cells (red; 10.0 ± 0.98 mM, saturating). Shaded bands represent ± 1 s.d. of the conditioned-medium lactate measurements. HFF-level lactate produces partial activation ($\sim 30\%$ of maximum), while Raji-level lactate drives near-maximal expression, providing a selectivity window between normal-cell and tumor-derived lactate. Mean $\pm$ SEM, $n = 5$ independent vesicle preparations.

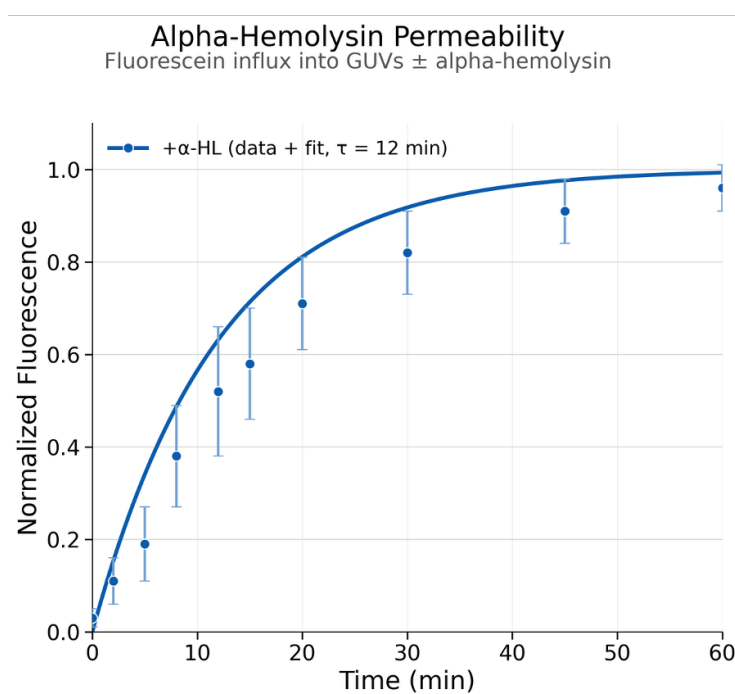


Figure C.2: Alpha-hemolysin enables small-molecule permeability across the GUV membrane. Fluorescein (376 Da) influx into GUVs after addition of purified α HL (10 nM) to the vesicle exterior. Intravesicular fluorescence was normalized to the external fluorescein concentration and monitored by time-lapse microscopy. Data were fit to an exponential approach to equilibrium (solid blue line; $c(t) = c_{\text{out}} \times (1 - e^{-t/\tau})$, $\tau = 12$ min). Half-equilibration occurs at ~ 8 minutes, with full equilibration by ~ 30 minutes. The rapid influx kinetics confirm that α HL pores permit small-molecule transit on a timescale much faster than cell-free gene expression (~ 1 – 2 hours), ensuring that lactate sensing is not rate-limiting in the complete system. Mean $\pm$ SEM, $n = 5$ independent vesicle preparations.

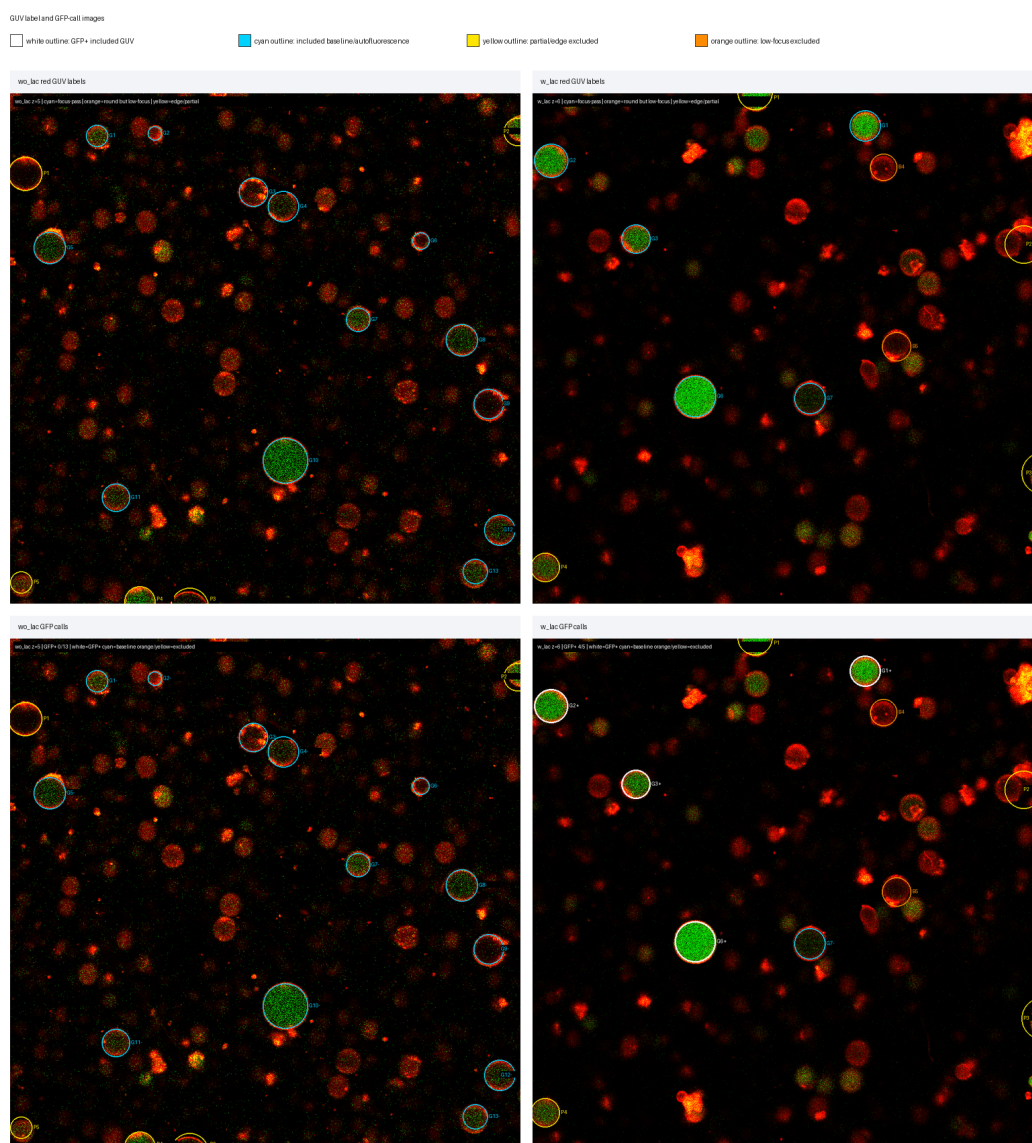


Figure C.3: Automated GU detection and GFP classification. Top row: red-channel (Rhodamine PE) GU detection overlays for the no-lactate (left) and lactate (right) conditions. Cyan outlines indicate in-focus GUs passing all acceptance criteria. Orange outlines indicate low-focus candidates excluded from analysis. Yellow outlines indicate partial or edge-clipped GUs excluded from analysis. Bottom row: GFP-call overlays for the same fields. White outlines indicate GFP-positive GUs (mean green intensity above the no-lactate baseline + 3 s.d. and median green intensity above the maximum no-lactate median). Cyan outlines indicate accepted GUs classified as GFP-negative/baseline. The strict automatic detector identified 25 accepted GUs without lactate (0 GFP-positive) and 22 accepted GUs with lactate (9 GFP-positive, 40.9%).

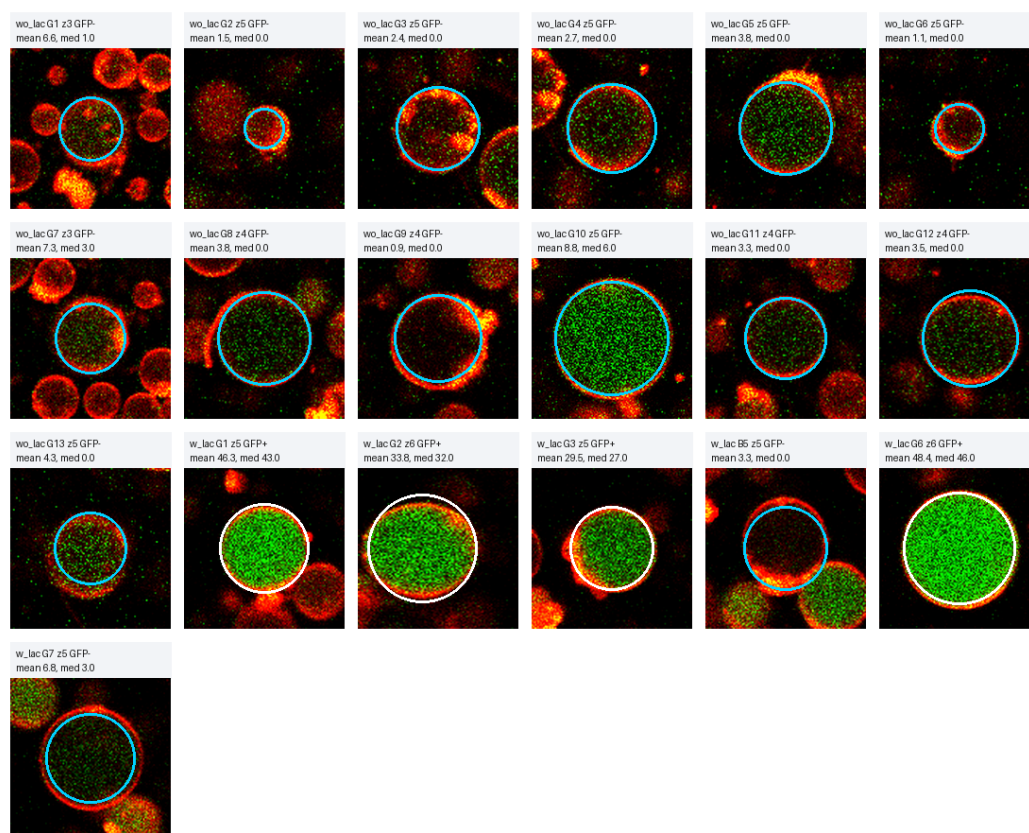


Figure C.4: Individual GU thumbnails showing GFP expression ± lactate. Cropped confocal images of a conservative best-focus GU subset from the z-scan analysis. Red: Rhodamine PE membrane. Green: GFP. Top two rows: no-lactate condition (wo_lac). All GUs are GFP-negative with low mean green intensities (0.9–7.3; median 0.0). Bottom rows: lactate condition (w_lac). GFP-positive GUs (white outlines) show elevated mean green intensities (29.5–48.4; median 27.0–46.0), while a subset remain GFP-negative (mean 3.3–6.8), consistent with stochastic variation in DNA encapsulation efficiency. The interior measurement mask (cyan circle) was set 5 pixels smaller than the detected membrane radius to exclude membrane-associated fluorescence. Mean and median green intensities are displayed per GU.