

# Gene-driven self-morphing microtubule-based active matter

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## Table of Contents

|                                                                                                 |    |
|-------------------------------------------------------------------------------------------------|----|
| 1. Data Analysis .....                                                                          | 3  |
| 1.1 Particle image velocimetry (PIV).....                                                       | 3  |
| 1.2 Wavelength analysis.....                                                                    | 3  |
| 1.2a Image preprocessing and blister bending detection .....                                    | 3  |
| 1.2b Definitions of blister geometry.....                                                       | 3  |
| 1.2c Tracking across frames .....                                                               | 4  |
| 1.2d Track management .....                                                                     | 4  |
| 1.2e Post-processing filters.....                                                               | 4  |
| 1.2f Manual track curation.....                                                                 | 4  |
| 1.2g Characteristic blister width analysis .....                                                | 4  |
| 1.2h Characteristic wavelength analysis.....                                                    | 4  |
| 1.2i Blister width analysis .....                                                               | 4  |
| 1.3 Active mixing does not enhance TXTL expression .....                                        | 4  |
| 1.4 Quantification of kinesin expression from GFP fluorescence .....                            | 5  |
| 1.4a Conversion of fluorescence to concentration.....                                           | 5  |
| 2. Mechanochemical Model Overview .....                                                         | 5  |
| 2.1 Common transcription-translation assumptions.....                                           | 6  |
| 2.2 Modeling framework and biological context .....                                             | 6  |
| 2.3 Numerical integration and mechanical readout.....                                           | 7  |
| Circuit 1: Constitutive expression of kinesin under the T7 promoter .....                       | 7  |
| Circuit 2: Constitutive kinesin expression with PRC1-mediated mechanical loading.....           | 7  |
| Circuit 3: Activator-repressor gene circuit: kinesin- $\sigma$ 28 feedback with CI repression + |    |
| ClpXP degradation .....                                                                         | 8  |
| 2.4 Flow concentration model .....                                                              | 9  |
| 2.5 Addition of PRC1 protein .....                                                              | 9  |
| 3. Tables .....                                                                                 | 11 |
| Table S1: Plasmids and DNA constructs used in this study. ....                                  | 11 |
| Table S2 : Model parameters used in the gene circuit simulations .....                          | 12 |
| Table S3: Parameter values used for numerical simulations in figures .....                      | 12 |
| 4. References .....                                                                             | 13 |
| 5. Supplementary Figures .....                                                                  | 14 |
| Figure S1: Experimental platform for cell-free microtubule-associated protein synthesis.....    | 14 |
| Figure S2: GFP protein expression in the presence of a microtubule sheet does not generate      |    |
| flow.....                                                                                       | 15 |
| Figure S3: Active microtubule mixing does not alter cell-free gene expression. ....             | 16 |
| Figure S4: Kinetics of kinesin production as a function of DNA concentration in a cell-free     |    |
| system.....                                                                                     | 17 |
| Figure S5: Time-lapse imaging of kinesin-driven active microtubule (MT) flows. ....             | 18 |
| Figure S6: Time-lapse imaging of GFP co-expression with kinesin (no fusion).....                | 19 |
| Figure S7: Dynamics of bending instability growth. ....                                         | 20 |

|                                                                                                                                              |    |
|----------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure S8: Planar dynamics of a single blister.....                                                                                          | 21 |
| Figure S9: Estimation of MT sheet thickness. ....                                                                                            | 22 |
| Figure S10: Scaling of bending instability width with DNA concentration. ....                                                                | 23 |
| Figure S11: Mean velocity as a function of time during co-expression of kinesin and PRC1,<br>complementary to the data shown in Fig. 3c..... | 24 |
| Figure S12: Oscillations in kinesin gene circuit activity with reporter expression. Three .....                                              | 25 |
| Figure S13: Velocity magnitude as a function of time for the activator-repressor gene circuit<br>(data shown in Fig. 3e).....                | 26 |
| Figure S14. Complementary version of Extended Data Fig. 3 showing the full dynamic range<br>of the predicted gene expression levels.....     | 27 |
| 6. Video Captions.....                                                                                                                       | 28 |
| Video 1 .....                                                                                                                                | 28 |
| Video 2 .....                                                                                                                                | 28 |

## 1. Data Analysis

**1.1 Particle image velocimetry (PIV):** Mean velocity magnitudes were calculated from velocity fields obtained via PIV analysis of MT fluorescence image sequences using PIVlab 3.01 (MATLAB R2023b). PIV analysis parameters, including interrogation window size and number of passes, were selected using the program-recommended settings based on the segment of each video exhibiting the fastest flows to ensure adequate capture of the full velocity range. For each time step, velocity fields were computed from consecutive image frames. The focal plane was set at the top of the initial MT sheet to minimize any effects of surface adhesion to the well bottom. Velocity vectors from low-contrast regions were excluded from mean velocity magnitude calculations.

**1.2 Wavelength analysis:** The focal plane for instability wavelength analysis was determined from the intensity profile of the undeformed MT sheet. At the first acquisition time point, the mean pixel intensity was calculated for each z-slice and plotted as a function of z-index. The z-plane at or near the peak intensity was selected for all subsequent analyses performed in MATLAB. This methodology was used for the data in Figs. 1, 4.

**1.2a Image preprocessing and blister bending detection:** Raw grayscale x-y images were converted to double precision and inverted so that dark regions appeared bright. Noise was reduced using 5 iterations of anisotropic diffusion filtering to preserve edges while smoothing homogeneous regions. Edges were detected using a Sobel operator with an adaptive threshold scaled by a user-defined factor (fudgeFactor), adjusted for imaging conditions (objective, modality, and exposure) in MATLAB. The binary edge map was refined by sequential dilation (linear and disk structuring elements), followed by hole filling and morphological closing to improve boundary continuity and remove small artifacts. Objects below a minimum area threshold (set by magnification) and those touching image borders were excluded. Connected components were then labeled, and region properties (centroid, area, orientation, perimeter, and pixel indices) were extracted using the built-in function regionprops.

### 1.2b Definitions of blister geometry:

**Area:** The area of each blister was defined as the number of pixels within each filled binary region.

**Length:** Blister masks were skeletonized (bwskel, MATLAB), and the longest geodesic path was identified and extended to the object boundary. Length was calculated as the cumulative Euclidean distance along this path.

**Width:** Blister width was computed as the mean perpendicular distance between boundaries along a smoothed (Savitzky-Golay, MATLAB) skeleton. Measurements were taken at up to 20 points along the original skeleton; if fewer valid points were available, the perpendicular distances at all valid points were averaged.

**1.2c Tracking across frames:** Each blister was tracked over time using its centroid position, geometric properties (width, length, orientation), object mask, and a Kalman filter for motion prediction. A constant-velocity Kalman filter was initialized upon the first detection of each blister. Object associations between consecutive frames were performed by matching detected blisters to existing tracks using a cost-minimization approach. Track positions were first predicted using the Kalman filter, and a cost matrix was computed between predicted and detected objects using several parameters: distance between centroids, changes in area and aspect ratio, orientation difference, and spatial overlap (after dilation). Assignments were determined using the Hungarian algorithm with a maximum cost threshold. To ensure physically meaningful matches, associations were accepted only if the overlap with the previous object (after dilation) exceeded 20%.

**1.2d Track management:** Matched tracks were updated using new measurements and Kalman filter correction. Unmatched tracks were terminated after three consecutive missed frames, and new tracks were initialized for unmatched detections. In merging events, only one track was continued, while the others were terminated. Splitting events were flagged and terminated, marking the end of a blister.

**1.2e Post-processing filters:** Tracks shorter than three consecutive frames were discarded. Tracks exhibiting abrupt area reductions greater than 40% in successive frames were truncated at the point of change. Final geometric measurements were converted from pixel units to physical units using calibration parameters.

**1.2f Manual track curation:** Tracked blisters were overlaid on the original MT fluorescence images and visually inspected. Results were manually corrected where necessary to ensure consistency with the underlying data.

**1.2g Characteristic blister width analysis:** Tracked blisters were analyzed over time, and for each blister, the maximum width observed across its lifetime was determined. The characteristic width  $w$  at each  $DNA_k$  was then defined as the mean of these maximum widths across all tracked blisters.

**1.2h Characteristic wavelength analysis:** For each blister, the nearest-neighbor centroid-to-centroid distance was measured across its lifetime. The steady-state characteristic wavelength,  $\lambda$ , defined as the mean of these distances computed from frames in which  $\geq 85\%$  of the maximum number of blisters in a sample was detected, was calculated at each  $DNA_k$ .

**1.2i Blister width analysis:** For each  $DNA_k$  concentration, 20 blisters from a single sample were selected, and their widths were measured manually at a representative point along each blister. The mean width at each relevant DNA concentration was then calculated from these values. This methodology was used for the data in Fig. 2c due to the lower resolution of the data set.

**1.3 Active mixing does not enhance TXTL expression:** To test whether active microtubule flows feedback on protein synthesis, we decoupled mechanical activity from gene expression by externally adding purified streptavidin-clustered kinesin motors to the TXTL reaction. Motor

clusters were assembled from purified biotinylated kinesin and tetrameric streptavidin following the standard active-matter protocol described previously<sup>1</sup>. A GFP reporter protein was expressed from a 5nM plasmid in the presence of either no motors, 20 nM kinesin clusters, or 100 nM kinesin clusters. Active flow persisted throughout the experiment in the motor-containing samples, whereas GFP expression was monitored for 10 h. Across three independent repeats, neither the GFP production kinetics nor the final GFP expression level differed between conditions (Fig. S3), indicating that active mechanical mixing does not measurably enhance cell-free gene expression under the reaction conditions used here and abundant ATP availability for sustained flow.

**1.4 Quantification of kinesin expression from GFP fluorescence:** To estimate kinesin production, GFP expression was compared with and without kinesin co-expression. A constant concentration of GFP coding plasmid (0.4 nM, T7 promoter) was co-expressed with varying concentrations of kinesin-coding plasmid (0.1 – 9 nM, T7 promoter), enforcing competition for shared transcriptional and translational resources (T7 RNA polymerase, ribosomes, Amino acids, nucleic acids). Fluorescence was measured over time and converted to GFP-equivalent protein concentration using a calibration curve. Kinesin concentration,  $[kinesin]$ , at every time point was inferred from the reduction in GFP expression upon co-expression,  $\Delta[GFP]$ , reflecting resource diversion to kinesin synthesis. This GFP-equivalent signal was converted to kinesin concentration using a molecular weight-based scaling factor:

$$[kinesin] = \frac{\Delta[GFP]}{a}; a = \frac{MW_{kinesin}}{MW_{GFP}}$$

Using  $MW_{GFP} \sim 27 \text{ kDa}$  and  $MW_{kinesin} \sim 55 \text{ kDa}$ , we estimated  $a \sim 2$ . Based on three independent repeats, a plasmid concentration of 3 nM was found to correspond to the production of  $\sim 3 \mu\text{M}$  kinesin monomers after 6 hours of expression. This corresponds to  $\sim 1.5 \mu\text{M}$  dimers and 750 nM tetrameric motor clusters (Fig. S4).

**1.4a Conversion of fluorescence to concentration:** To quantify kinesin expression in the CFE system, purified GFP fluorescence was used as a calibrated fluorescence unit for molar protein concentration. A calibration curve was generated using purified GFP at known concentrations (0 – 5  $\mu\text{M}$ ), imaged under known microscope settings in the expression experiment above (objective, illumination intensity, binning, exposure time, and camera parameters). After background subtraction, fluorescence intensity was converted to molar concentration via linear regression. Each calibration point represents the mean of three independent repeats.

## 2. Mechanochemical Model Overview

We model three cell-free gene circuits that regulate kinesin motor production and translate motor concentrations into predicted MT flow speed. Each circuit is described by a system of ordinary differential equations (ODEs) governing mRNA and protein concentrations.

1. **Circuit 1 (constitutive kinesin expression):** Kinesin is expressed constitutively from a T7 promoter, with mRNA turnover and translation without protein degradation.
2. **Circuit 2 (kinesin with PRC1-mediated mechanical loading):** Kinesin (expressed under a T7 promoter) and the passive crosslinker PRC1 (expressed under a p<sub>70</sub> promoter) are expressed independently. Kinesin concentration determines the baseline active stress, while PRC1 modulates the resulting MT flow through an additive load-dependent stall factor that captures PRC1-induced mechanical friction.
3. **Circuit 3 (activator-repressor circuit with shared degradation capacity):** The activator protein  $A$  (a kinesin- $\sigma^{28}$  fusion) is expressed constitutively and transcriptionally repressed by a repressor  $R$  (CI)<sup>2</sup>. Repressor expression is activated by  $A$  via Michaelis-Menten kinetics. Both  $A$  and  $R$  are degraded by a shared protease pool (ClpXP), modeled through an effective concentration-dependent lifetime  $\tau_{XP}(A, R)$  that captures competition for finite degradation capacity.

All gene circuits components are summarized in table S1. All simulations are performed over 8 hours, with time in the ODEs expressed in minutes.

**2.1 Common transcription-translation assumptions:** All circuits are modeled using coarse-grained rate equations appropriate for CFE transcription-translation systems (Table S2). Transcription is assumed to scale linearly with DNA template concentration  $D$ , with promoter-specific effective transcription rates  $k_{TX}$ . All mRNA species undergo first-order decay characterized by a common lifetime  $\tau_m$ . Translation is proportional to mRNA abundance, with an effective translation rate  $k_{TL}$ .

Protein degradation is treated in a circuit-dependent manner. In Circuits 1 and 2, proteins are assumed to be stable on experimental timescales, and no explicit protein degradation is included. In Circuit 3, protein turnover is mediated by a shared ClpXP protease pool, modeled explicitly through a concentration-dependent effective lifetime (see below).

**2.2 Modeling framework and biological context:** The gene circuits are modeled in an *E. coli* transcription-translation CFE system using effective mass-action and Michaelis-Menten kinetics for transcriptional regulation, translation, and degradation. This approach follows established coarse-grained ODE models for gene networks in lysates, in which molecular complexity is compressed into experimentally measurable rate constants and regulatory thresholds<sup>2,3</sup>. All rates and concentrations used for the model are summarized in **Tables S2,S3**.

In CFE transcription-translation, untagged proteins are effectively stable, whereas SsrA-tagged proteins are selectively degraded by the ClpXP protease. mRNA lifetimes in this system are typically on the order of 10-20 minutes. Transcription from the  $\sigma^{70}$  promoter (p<sub>70</sub>) is driven by endogenous *E. coli* RNA polymerase and facilitated by the abundant  $\sigma^{70}$  present in the CFE reaction mixture. Transcription from T7 promoters is driven by supplemented T7 RNA polymerase.

**2.3 Numerical integration and mechanical readout:** All circuits are simulated over an 8-hour duration using numerical integration (ode15s). The resulting protein concentration dynamics are mapped to predicted microtubule flow speeds using a nonlinear Hill-type transfer function. Where applicable, this baseline motor-driven flow is further modulated by a phenomenological factor that captures PRC1-dependent mechanical loading.

**Circuit 1: Constitutive expression of kinesin under the T7 promoter (Extended Data Fig. 2a).**

The equations governing constitutive kinesin expression are:

$$\frac{dm_k}{dt} = k_{TX}^{T7} D_k - \frac{m_k}{\tau_m}, \quad (1)$$

$$\frac{dK}{dt} = k_{TL} m_k - \gamma_{k_{12}} K, \quad (2)$$

where  $m_k$  denotes the kinesin mRNA concentration,  $k_{TX}^{T7}$  is the effective transcription rate driven by T7 RNA polymerase, and  $D_k$  is the experimentally controlled concentration of the T7-kinesin DNA template, and  $\tau_m$  is the mRNA lifetime in the CFE transcription-translation reaction. The kinesin protein concentration is denoted by  $K$ , with translation occurring at a rate  $K_{TL}$ . The term  $\gamma_{K_{12}}$  represents a first-order kinesin loss or disassembly rate, which is set to zero in the present model, consistent with the stability of untagged kinesin in the extract.

**Circuit 2: Constitutive kinesin expression with PRC1-mediated mechanical loading.** Circuit 2 extends Circuit 1 by introducing constitutive expression of the passive crosslinker PRC1 alongside kinesin (Extended Data Fig. 2b). Kinesin is expressed from a T7 promoter, while PRC1 is expressed independently from a  $\sigma^{70}$ -dependent  $p_{70}$  promoter. The transcriptional dynamics of the two genes are uncoupled, and their interaction enters only through the mechanical readout of microtubule flow.

The circuit is described by the following equations:

$$\frac{dm_k}{dt} = k_{TX}^{T7} D_k - \frac{m_k}{\tau_m}, \quad (3)$$

$$\frac{dK}{dt} = k_{TL} m_k - \gamma_{k_{12}} K, \quad (4)$$

$$\frac{dm_p}{dt} = k_{TX}^{70} D_p - \frac{m_p}{\tau_m}, \quad (5)$$

$$\frac{dP}{dt} = k_{TL} m_p - \gamma_p p. \quad (6)$$

Here,  $m_k$  and  $m_p$  denote the mRNA concentrations of kinesin and PRC1, respectively, and  $K$  and  $P$  denote the corresponding protein concentrations. The parameters  $k_{TX}^{T7}$  and  $k_{TX}^{70}$  represent the effective transcription rates driven by T7 RNA polymerase and endogenous *E. coli* RNA

polymerase with  $\sigma^{70}$ , respectively.  $D_K$  and  $D_P$  are the experimentally controlled concentrations of the T7-kinesin and p<sub>70</sub>-PRC1 DNA templates. All mRNAs decay with a common lifetime  $\tau_m$ , and translation proceeds at a rate  $K_{TL}$ .

The parameters  $\gamma_{k12}$  and  $\gamma_p$  represent first-order protein loss rates for kinesin and PRC1, respectively. In the present model, both are set to zero, consistent with the stability of untagged proteins in the CFE.

**Circuit 3: Activator-repressor gene circuit: kinesin- $\sigma^{28}$  feedback with CI repression + ClpXP degradation.** Circuit 3 implements an activator-repressor feedback architecture in which a kinesin- $\sigma^{28}$  fusion protein acts as an activator that both drives mechanical output and regulates its own expression through a CI repressor (Extended Data Fig. 2c). The circuit dynamics are described by

$$\frac{dm_A}{dt} = k_{TX}^{70} D_A \cdot Rep(R) - \frac{m_A}{\tau_m}, \quad (7)$$

$$\frac{dA}{dt} = k_{TL} m_A - \frac{A}{\tau_{XP}(A, R)}, \quad (8)$$

$$\frac{dm_R}{dt} = k_{TX}^{28} D_R \cdot Act(A) - \frac{m_R}{\tau_m}, \quad (9)$$

$$\frac{dR}{dt} = k_{TL} m_R - \frac{R}{\tau_{XP}(A, R)}. \quad (10)$$

Here,  $m_A$  and  $m_R$  denote the mRNA concentrations of the activator and repressor, respectively, and  $A$  and  $R$  are the corresponding protein concentrations. The activator  $A$  is a kinesin- $\sigma^{28}$  fusion protein expressed from a p<sub>70</sub> promoter and transcriptionally repressed by the CI repressor  $R$ . The repressor  $R$  is expressed from a  $\sigma^{28}$ -dependent promoter p<sub>28</sub> and is activated by  $A$ .

Regulatory interactions are modeled as

$$Rep(R) = \frac{1}{1 + \left(\frac{R}{K_I}\right)^4},$$

$$Act(A) = \frac{A}{K_A + A},$$

where  $K_I$  is the effective repression threshold for CI binding and  $K_A$  is the activation threshold for  $\sigma^{28}$ -driven CI expression. The Hill coefficient is consistent with the known cooperativity of CI repression.

Protein degradation of both  $A$  and  $R$  is mediated by a shared ClpXP protease pool and is modeled through a concentration-dependent effective lifetime, which captures competition for finite protease capacity, and is described by the following equation:

$$\tau_{XP}(A, R) = \frac{K_{deg} + A + R}{V_{max}}.$$

Here,  $V_{max}$  is the total zero-order degradation capacity of ClpXP (typically 20-100 nM/min for SsrA-tagged substrates), and  $K_{deg}$  is the Michaelis constant for ClpXP-mediated degradation. This formulation is algebraically equivalent to the competitive Michaelis-Menten degradation of  $A$  and  $R$  by a shared enzyme pool.

**2.4 Flow concentration model:** To relate gene-circuit output to active microtubule transport, we model the mean MT flow speed  $v(t)$  as a deterministic function of the instantaneous concentration of force-generating motors and passive mechanical regulators.

In all circuits, kinesin activity is assumed to generate extensile stresses that drive MT motion. The baseline flow speed is taken to increase monotonically with kinesin concentration and to saturate at high motor density:

$$v_0(K) = v_{max} \cdot \Phi(K). \quad (11)$$

$$\Phi(K) = \left( \frac{K^{n_v}}{K_{\frac{1}{2}}^{n_v} + K^{n_v}} \right)$$

where  $K$  is the concentration of active kinesin motors (nM),  $v_{max}$  is the maximal attainable MT speed,  $K_{1/2}$  is the motor concentration at half-maximal speed, and  $n_v$  controls the sharpness of the transition and is empirically reported. This Hill-type form captures cooperative force generation at low motor density and saturation due to filament crowding, motor interference, or force balance at high density (Extended Data Fig. 2-3).

**2.5 Addition of PRC1 protein:** When passive crosslinkers (PRC1) are present, kinesin-generated stresses are mechanically filtered by crosslink-mediated coupling and friction. The effective flow speed is written as

$$v(K, P) = v_0(K) \cdot S(P) \quad (12)$$

with a PRC1-dependent mechanical multiplier

$$S(P) = \frac{1}{1 + \left(\frac{P}{P_s}\right)^h}. \quad (13)$$

This function satisfies:

- $S(P = 0) = 1$ , indicating that no PRC1 implies no mechanical slowing;
- $0 < S(P) < 1$  for all  $P$ , ensuring that the slowing factor remains bounded;
- $S(P) \rightarrow 0$  as  $P \rightarrow \infty$ , corresponding to load-induced stalling.

The parameter  $P_S$  sets the PRC1 concentration at which flow is reduced by half, and  $h$  controls the sharpness of the transition.

### 3. Tables

| Construct Name                              | Promoter      | Gene / Feature                                                                                                            | Source / Construction Method                                                                                                                                                                                              |
|---------------------------------------------|---------------|---------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| p <sub>T7</sub> -KIF3A                      | T7            | Chimeric kinesin KIF3A expresses mouse kinesin-2 KIF3A head and neck linker, dimerized through kinesin-1 neck-coil to 406 | KIF3A406 was a gift from William Hancock (Addgene plasmid # 129768 ).                                                                                                                                                     |
| p <sub>70</sub> -deGFP                      | $\sigma^{70}$ | deGFP reporter                                                                                                            | 4                                                                                                                                                                                                                         |
| p <sub>28</sub> -CI-SsrA                    | $\sigma^{28}$ | CI repressor of $\sigma^{70}$ promoter                                                                                    | 2                                                                                                                                                                                                                         |
| p <sub>70</sub> - $\sigma^{28}$             | $\sigma^{70}$ | Transcription factor $\sigma^{28}$ of <i>E.coli</i>                                                                       | 2                                                                                                                                                                                                                         |
| p <sub>T7</sub> -deGFP                      | T7            | deGFP reporter                                                                                                            | This study. Cloned p <sub>T7</sub> promoter via QuikChange mutagenesis on #40019.                                                                                                                                         |
| p <sub>T7</sub> -KIF3A-deGFP                | T7            | Kinesin-deGFP fusion                                                                                                      | This study. Gibson assembly of KIF3A from #129768 fused to deGFP from #40019.                                                                                                                                             |
| p <sub>70</sub> -KIF3A- $\sigma^{28}$       | $\sigma^{70}$ | Kinesin- $\sigma^{28}$ transcription factor fusion                                                                        | This study. p <sub>T7</sub> replaced with p <sub>70</sub> promoter via QuikChange mutagenesis on (#129768), followed by Gibson assembly of the $\sigma^{28}$ transcription factor from p <sub>70</sub> - $\sigma^{28}$ 2. |
| p <sub>70</sub> -KIF3A- $\sigma^{28}$ -SsrA | $\sigma^{70}$ | Kinesin- $\sigma^{28}$ fusion tagged with SsrA                                                                            | This study. C-terminal SsrA addition via PCR on p <sub>70</sub> -KIF3A- $\sigma^{28}$ .                                                                                                                                   |
| p <sub>70</sub> -PRC1                       | $\sigma^{70}$ | PRC1                                                                                                                      | Constructed by PCR. PRC1 gene taken from 5.                                                                                                                                                                               |
| p <sub>70</sub> -GFP-SsrA                   | $\sigma^{70}$ | GFP-SsrA                                                                                                                  | 5                                                                                                                                                                                                                         |

**Table S1: Plasmids and DNA constructs used in this study.** List of all plasmids used, including promoter type, encoded gene or functional element, and source or cloning method. Constructs were either obtained from Addgene or generated in this study using QuikChange mutagenesis, Gibson assembly, or PCR-based modifications.

| Parameter     | Unit                                   | Definition                               | Value  | Reference |
|---------------|----------------------------------------|------------------------------------------|--------|-----------|
| $\tau_m$      | <i>min</i>                             | mRNA lifetime                            | 12 min | 6         |
| $k_{TX}^{T7}$ | $\text{min}^{-1} \cdot \text{nM}^{-1}$ | Transcription from T7-dependent promoter | 0.15   | 6,7       |

|                      |                                        |                                                                                                        |                                     |              |
|----------------------|----------------------------------------|--------------------------------------------------------------------------------------------------------|-------------------------------------|--------------|
| $k_{TX}^{28}$        | $\text{min}^{-1} \cdot \text{nM}^{-1}$ | Transcription from $\sigma^{28}$ -dependent promoter                                                   | 0.1                                 | 2            |
| $k_{TX}^{70}$        | $\text{min}^{-1} \cdot \text{nM}^{-1}$ | Transcription from $\sigma^{70}$ -dependent promoter                                                   | 0.15                                | 6            |
| $k_{TL}$             | $\text{min}^{-1}$                      | Protein production from mRNA (all circuits)                                                            | 1                                   | 6            |
| $D_k, D_P, D_A, D_R$ | $\text{nM}$                            | DNA concentrations                                                                                     | 1-changing                          | Current work |
| $\gamma_{K1,2}$      | $\text{min}^{-1}$                      | Linear loss for kinesin protein $K$ (circuits 1-2)                                                     | 0                                   | 6            |
| $\gamma_P$           | $\text{min}^{-1}$                      | Linear loss for PRC1 protein $P$ (circuit 2)                                                           | 0                                   | Current work |
| $K_A$                | $\text{nM}$                            | MM activation for $R$ transcription: $\frac{A}{K_A+A}$ , reduced because of the binding to the kinesin | 50                                  | 6,7          |
| $K_I$                | $\text{nM}$                            | Hill repression scale in $\text{Rep}(R)$                                                               | 2                                   | 2,3,6        |
| $n$                  |                                        | Cooperativity of repression of the CI protein                                                          | 4                                   | 2,6          |
| $V_{max}$            | $\frac{\text{nM}}{\text{min}}$         | Range for shared degradation pool construction                                                         | 10-60 set at 20 to get oscillations | 7            |
| $K_{deg}$            | $\text{nM}$                            | Shared-substrate competition scale in $\tau_{XP}$                                                      | 200                                 | 7            |

**Table S2 : Model parameters used in the gene circuit simulations.** List of parameter definitions, values, and literature references used in the protein-level gene circuit model. Parameter names correspond to those implemented in the simulation code.

| parameter | Value | Units           |                                        |
|-----------|-------|-----------------|----------------------------------------|
| $v_{max}$ | 0.5   | $\mu\text{m/s}$ | Maximum MT flow speed                  |
| $K_{1/2}$ | 300   | $\text{nM}$     | Kinesin level for half-maximal flow    |
| $n_v$     | 2     | —               | Cooperativity of kinesin-driven flow   |
| $P_f$     | 600   | $\text{nM}$     | PRC1 level giving half-stall $S = 1/2$ |
| $h$       | 3     | —               | Sharpness of stall transition          |

**Table S3: Parameter values used for numerical simulations in figures.** List of parameter values used to generate the simulation results shown in Figs. S13-S16. Parameter names correspond to those defined in Table S2.

## 4. References

1. Tayar, A. M., Lemma, L. M. & Dogic, Z. Assembling Microtubule-Based Active Matter. *Methods in Molecular Biology* **2430**, 151–183 (2022).
2. Tayar, A. M., Karzbrun, E., Noireaux, V. & Bar-Ziv, R. H. Synchrony and pattern formation of coupled genetic oscillators on a chip of artificial cells. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 11609–11614 (2017).
3. Karzbrun, E., Tayar, A. M., Noireaux, V. & Bar-Ziv, R. H. Programmable on-chip DNA compartments as artificial cells. *Science (1979)*. **345**, 829–832 (2014).
4. Shin, J. & Noireaux, V. Efficient cell-free expression with the endogenous E. Coli RNA polymerase and sigma factor 70. *Journal of Biological Engineering* **2010 4:1** **4**, 8- (2010).
5. Subramanian, R. *et al.* Insights into anti-parallel microtubule crosslinking by PRC1, a conserved non-motor microtubule binding protein. *Cell* **142**, 433 (2010).
6. Karzbrun, E., Shin, J., Bar-Ziv, R. H. & Noireaux, V. Coarse-Grained Dynamics of Protein Synthesis in a Cell-Free System. *Phys. Rev. Lett.* **106**, 048104 (2011).
7. Garamella, J., Marshall, R., Rustad, M. & Noireaux, V. The All E. coli TX-TL Toolbox 2.0: A Platform for Cell-Free Synthetic Biology. *ACS Synth. Biol.* **5**, 344–355 (2016).

## 5. Supplementary Figures

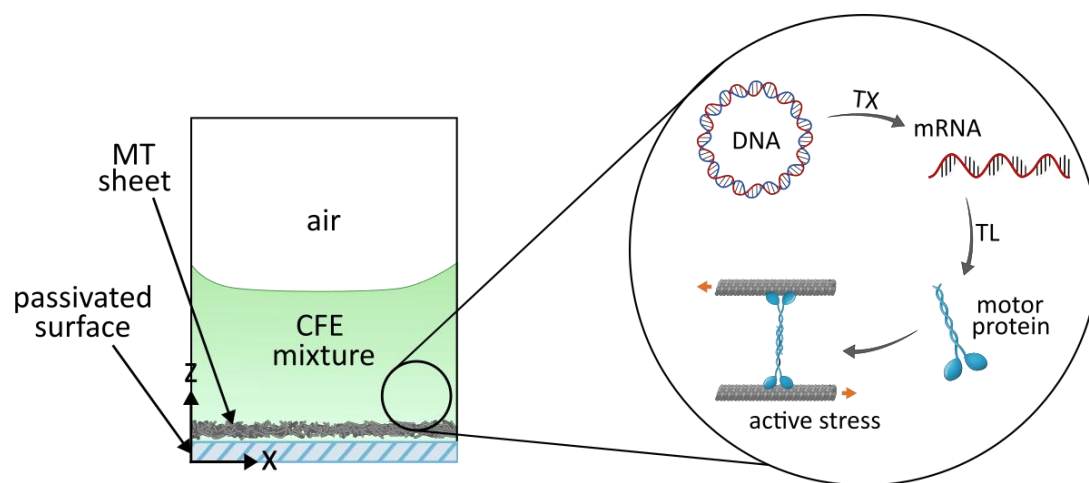


**Figure S1: Experimental platform for cell-free microtubule-associated protein synthesis.** Schematic of the CFE-MT system geometry. The CFE mixture contains DNA templates encoding MT-associated proteins, including motor proteins. The bottom surface of the well is passivated to reduce nonspecific protein adsorption. A dense, quasi-two-dimensional MT layer forms at the base of the well, establishing an active interface where newly synthesized proteins can directly interact with the MT network.

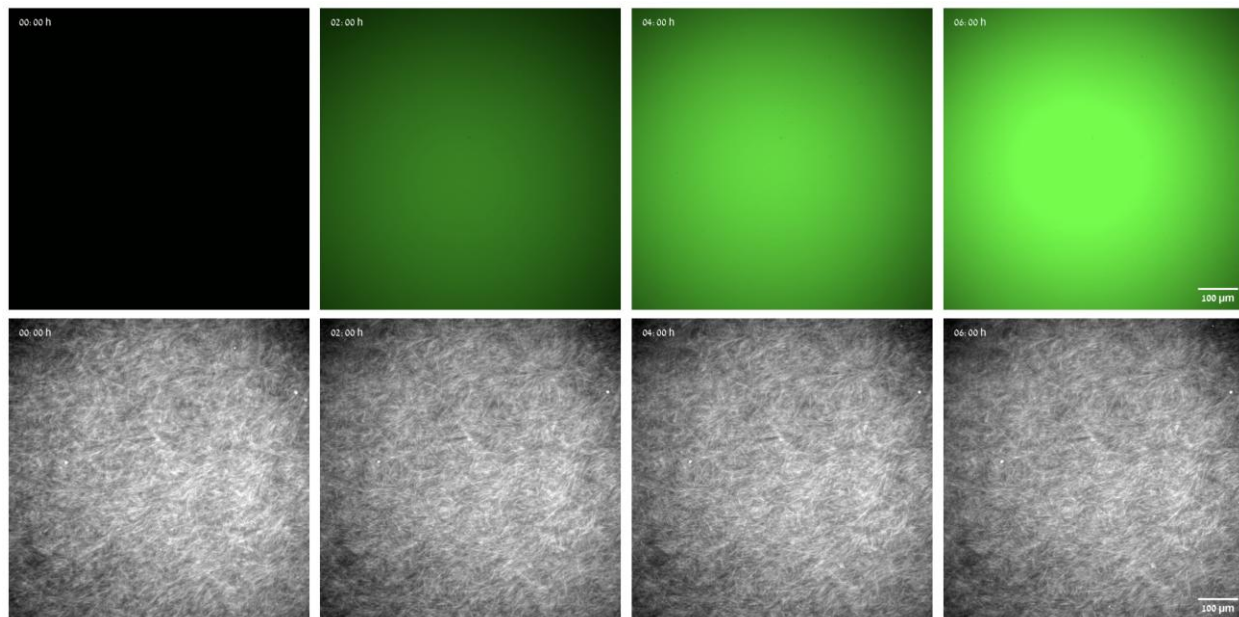


**Figure S2: GFP protein expression in the presence of a microtubule sheet does not generate flow.** Time-lapse images of GFP expression (green) driven by the T7 promoter in the presence of a microtubule sheet (grayscale) sedimented at the bottom of the well. No kinesin was expressed in this experiment. Scale bar: 100  $\mu\text{m}$ .

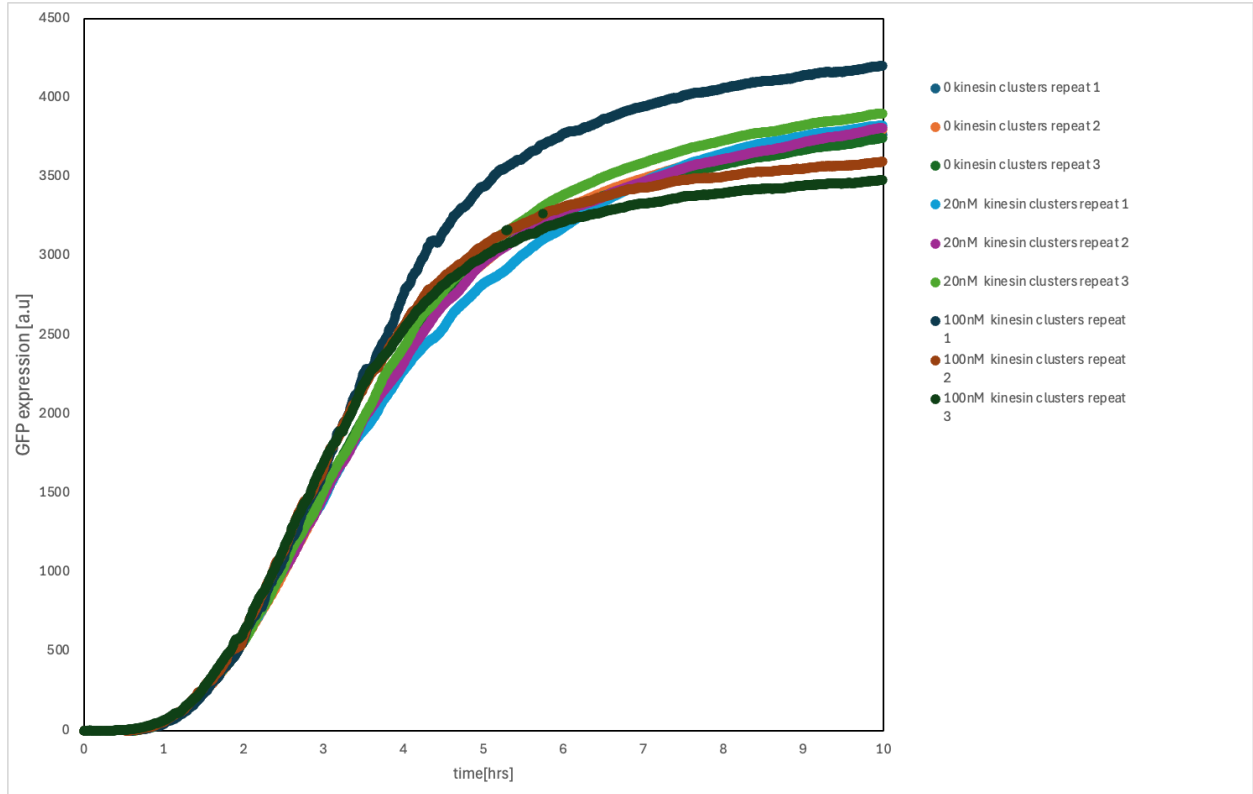


**Figure S3: Active microtubule mixing does not alter cell-free gene expression.** Purified streptavidin-clustered kinesin motors were added to the TXTL reaction at final concentrations of 0, 20, or 100 *nM* together with a 5 *nM* T7-driven GFP reporter plasmid. GFP fluorescence was measured over 10 *h* under each condition. Data represent three independent experiments. Active microtubule flow was observed throughout the experiment in the reactions containing kinesin motor clusters.

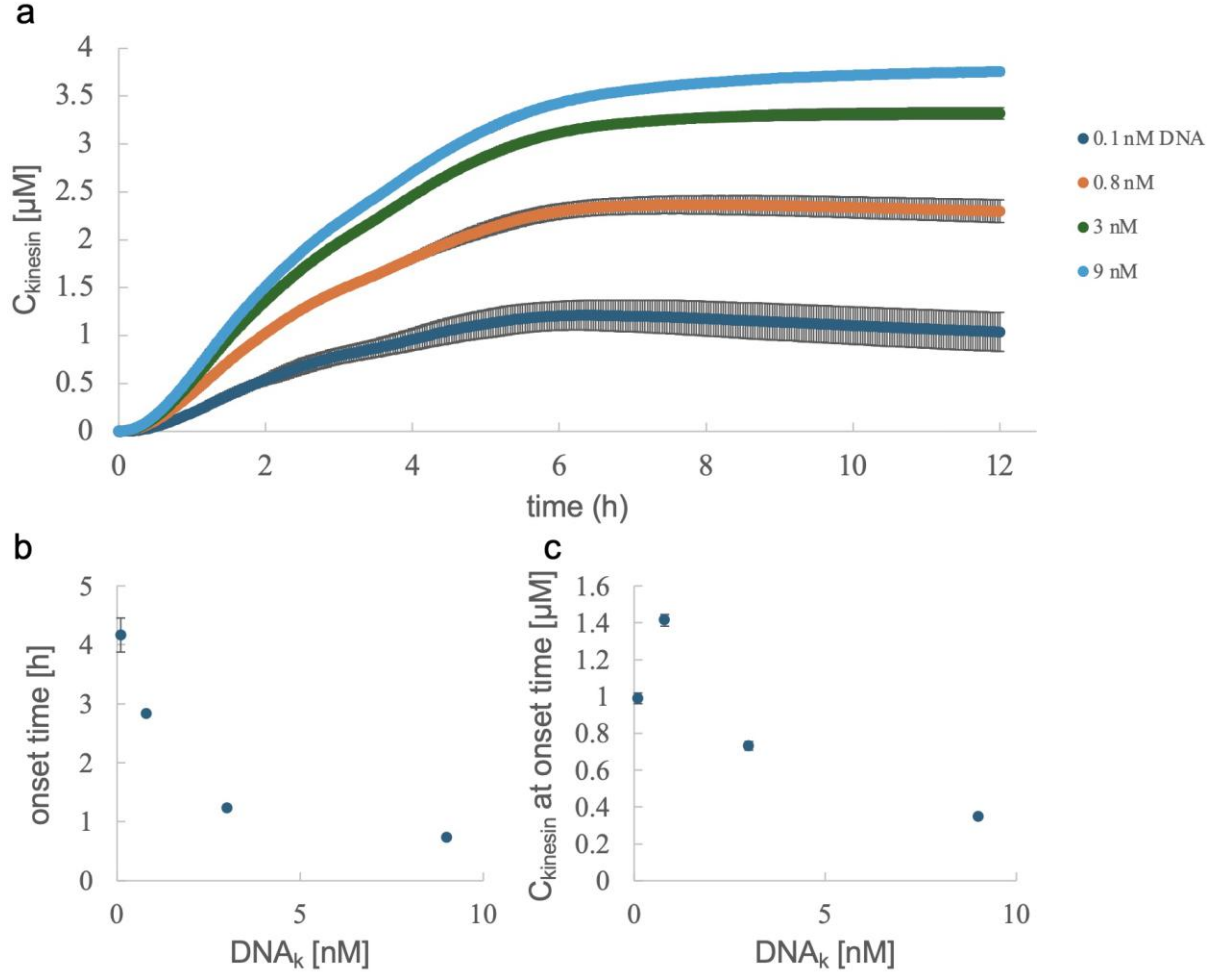


**Figure S4: Kinetics of kinesin production as a function of DNA concentration in a cell-free system.** (a) Kinesin monomer concentration estimated from the reduction in GFP expression in the presence versus absence of kinesin co-expression, with both constructs driven by a T7 promoter. Measurements were performed at four kinesin DNA concentrations (0.1 – 9 nM) with a constant GFP plasmid concentration of 0.4 nM. Fluorescence was converted to absolute kinesin concentration using a purified GFP calibration curve and corrected for differences in molecular weight between GFP and kinesin (SI). (b) Onset time of active flow in the microtubule sheet as a function of kinesin DNA concentration. (c) Kinesin concentration at the onset of flow, estimated from (a), plotted as a function of kinesin DNA concentration. Error bars represent the standard deviation from three independent repeats.

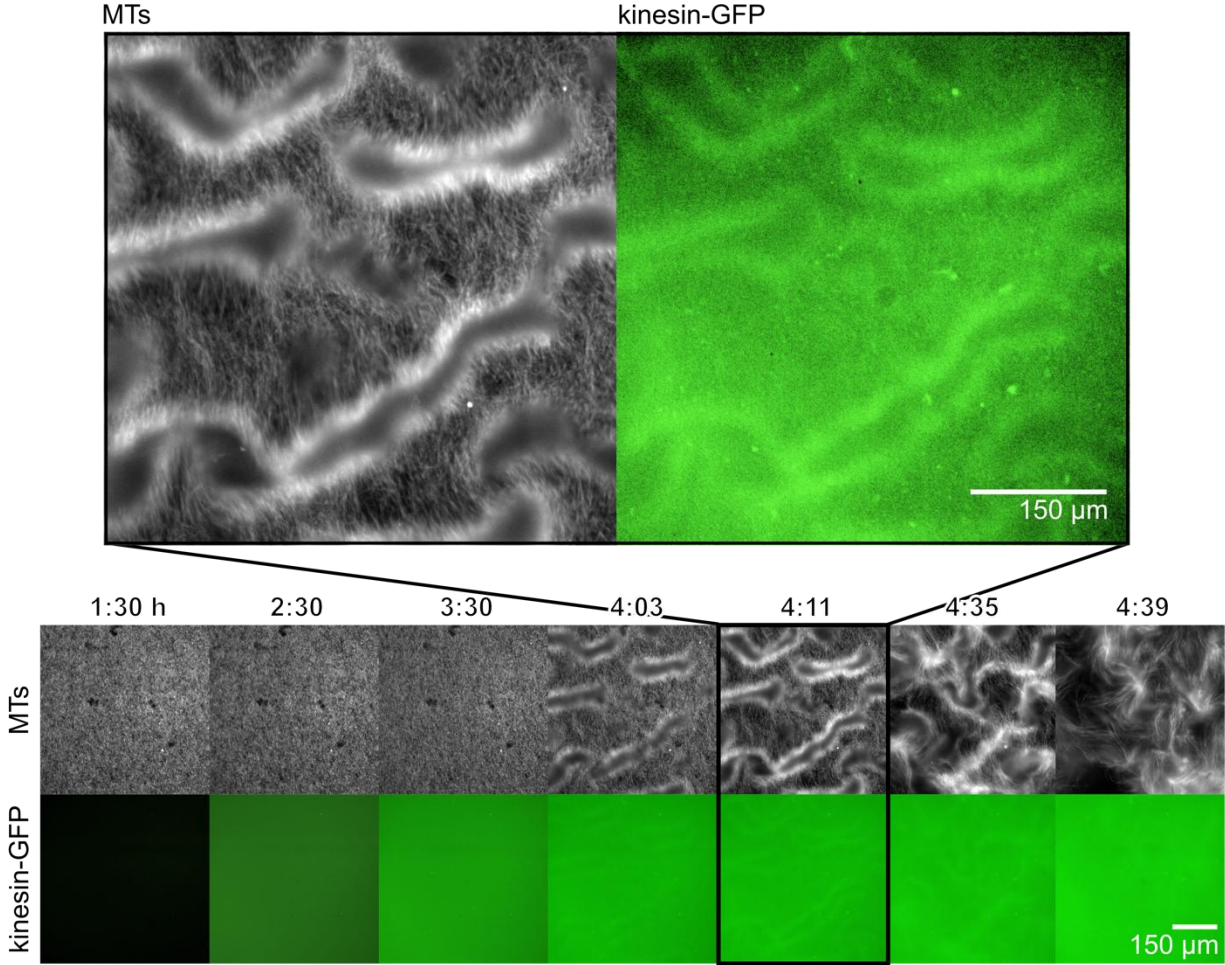


**Figure S5: Time-lapse imaging of kinesin-driven active microtubule (MT) flows.** Expression of a kinesin-GFP fusion (green) is shown alongside corresponding MT sheet images (grayscale), illustrating the emergence and evolution of active flows over time. The kinesin-GFP fusion was expressed under a T7 promoter, constituted ~13% of the total kinesin population, and was co-expressed with unlabeled kinesin to promote tetramer formation and motor activity. The experiment was performed at 0.7 *nM* kinesin DNA and 0.1 *nM* kinesin-GFP DNA. The initially homogeneous MT network evolved into aligned bundles and subsequently developed dynamic, chaotic flow patterns characteristic of MT-based active matter. Scale bar: 150  $\mu\text{m}$ .

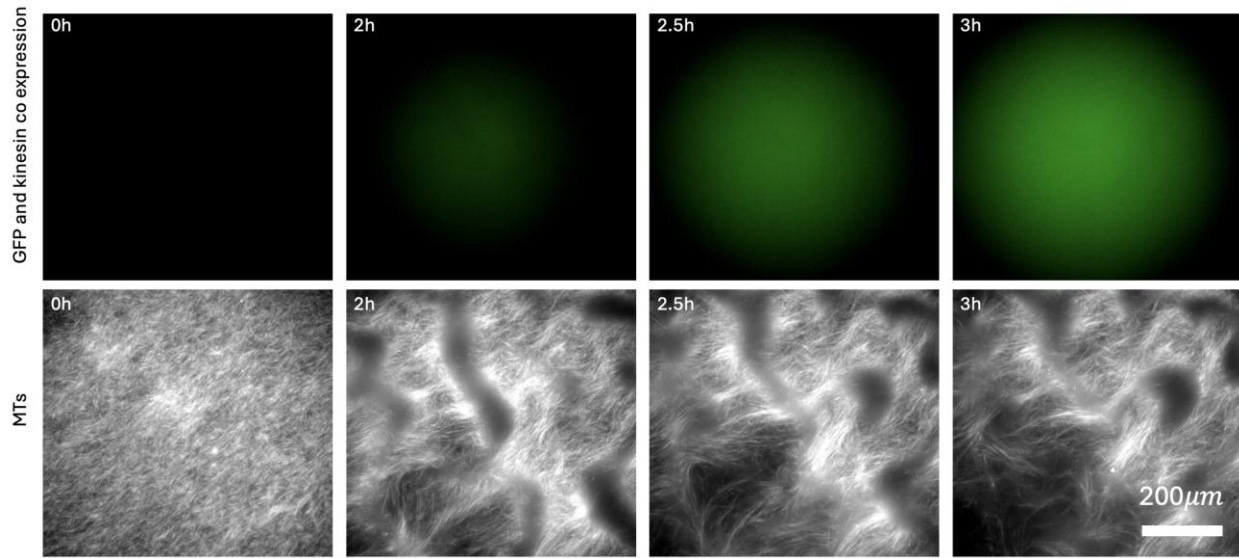


**Figure S6: Time-lapse imaging of GFP co-expression with kinesin (no fusion).** Expression of GFP (green) is shown alongside corresponding MT sheet images (grayscale), illustrating the emergence and evolution of active flows over time without detectable localization of GFP to the MT sheet. Both kinesin and GFP were expressed under a T7 promoter. The experiment was performed at 0.5 *nM* GFP DNA and 2.1 *nM* kinesin DNA. Scale bar: 200  $\mu\text{m}$ .

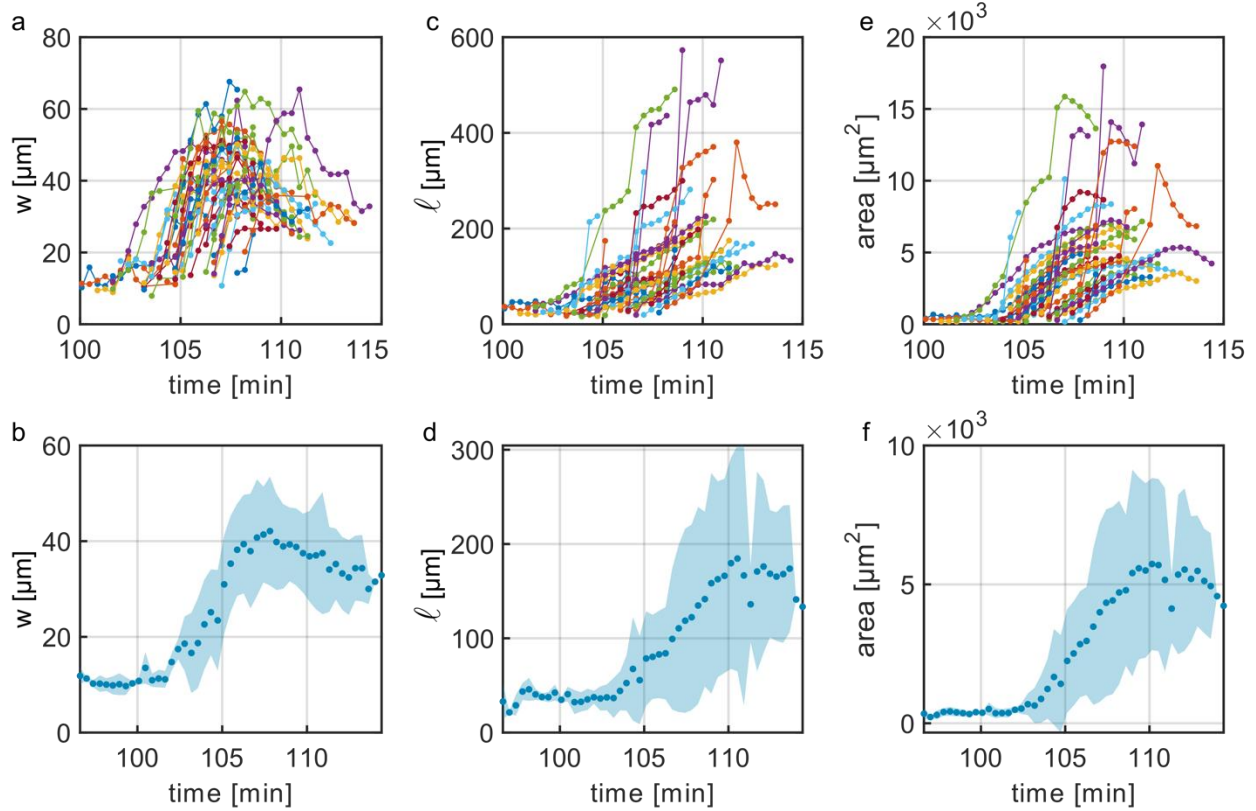


**Figure S7: Dynamics of bending instability growth.** All panels show measurements from the same dataset acquired for  $DNA_k = 3 \text{ nM}$  expression in a large well (no geometric confinement). (a) Individual blister widths as a function of time, with each color representing a single blister event. (b) Mean blister width calculated from the data in (a), showing a well-defined peak followed by relaxation; the shaded region indicates the standard deviation. (c) Individual blister lengths as a function of time. (d) Mean blister length as a function of time, with standard deviation (shaded region), showing sustained growth until blisters close. Data are averaged over all traces in (c). (e) Individual blister areas as a function of time. (f) Mean blister area calculated from the data in (e), with the shaded region indicating the standard deviation, showing an initial increase followed by saturation before blister closure.

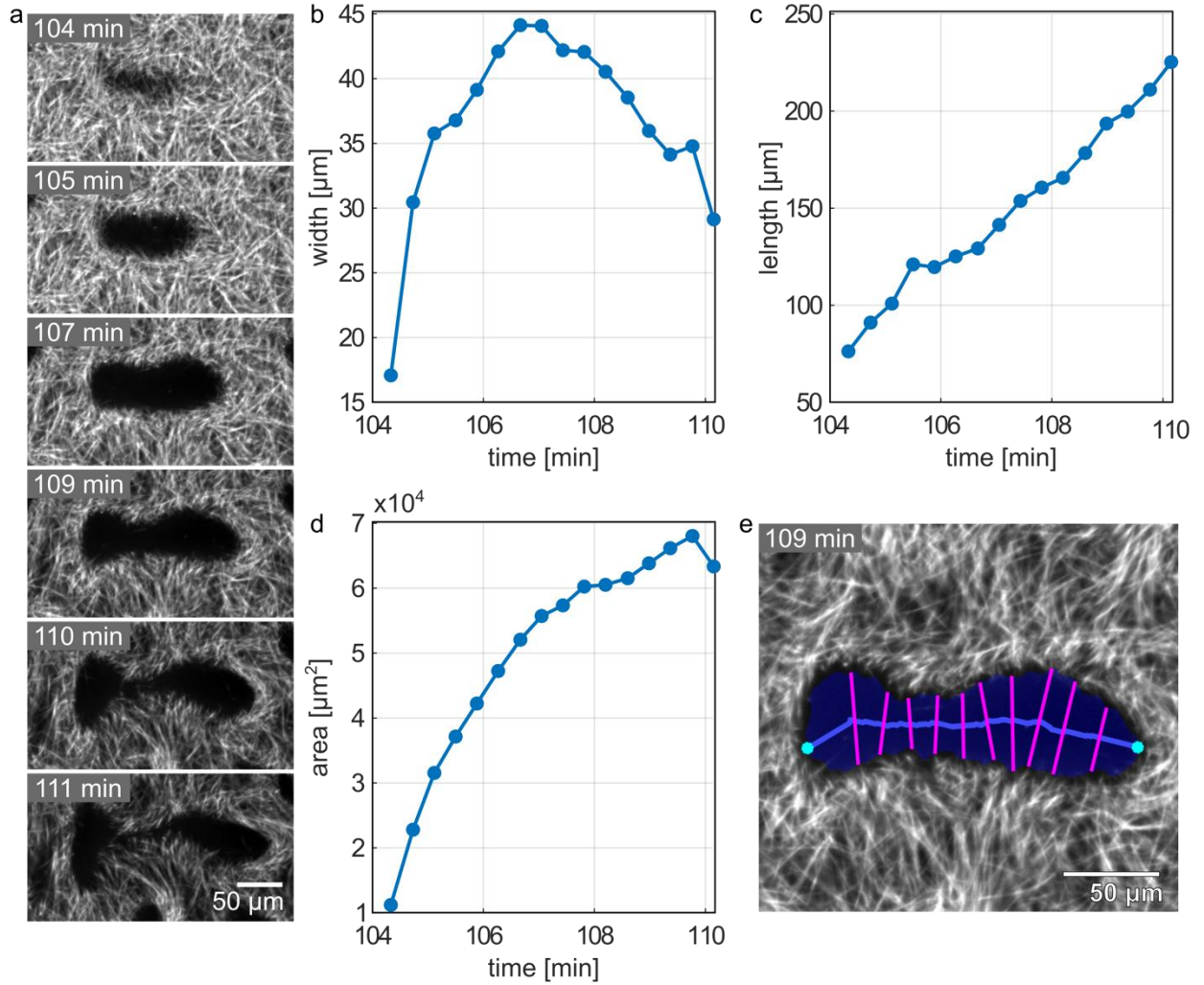


**Figure S8: Planar dynamics of a single blister.** All panels show measurements from the same blister acquired for  $DNA_k = 3 \text{ nM}$  expression in a large well (no geometric confinement). (a) Time-lapse imaging showing the growth of a localized buckling instability in a microtubule network, evolving from an initially small deformation into an elongated blister-like structure, driven by constitutive expression of kinesin under a T7 promoter, and culminating in blister closure. Quantitative analysis tracks the temporal evolution of the blister (b) width, (c) length, and (d) area. (e) The segmented contour of the blister interface geometry at 109 min, with local normals (magenta) used to characterize the spatial structure of the deformation. Scale bar: 50  $\mu\text{m}$ .

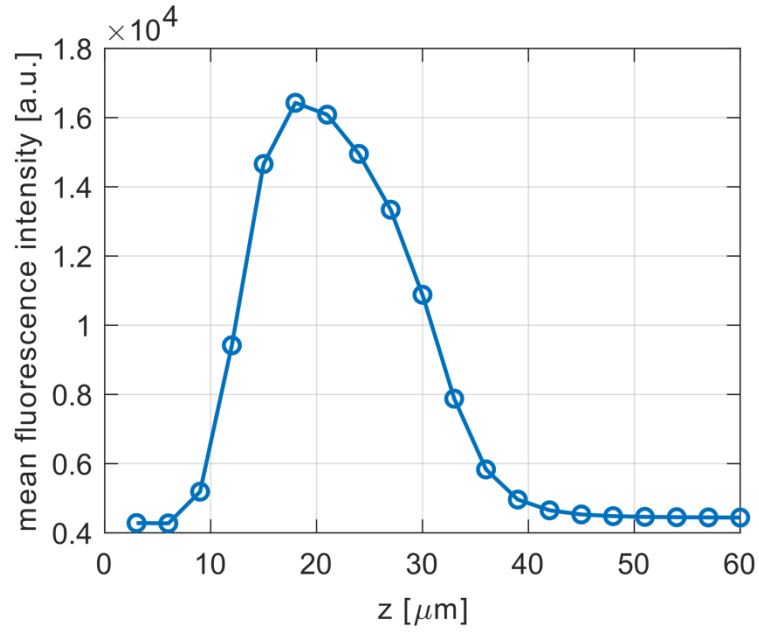


**Figure S9: Estimation of MT sheet thickness.** Confocal side-view fluorescence intensity profile of a microtubule sheet, used to estimate a sheet thickness of  $h \sim 10 \mu\text{m}$ .

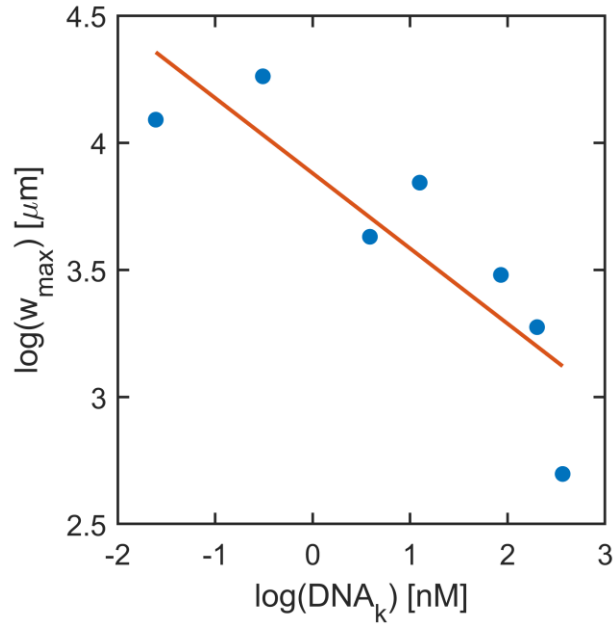


**Figure S10: Scaling of bending instability width with DNA concentration.** Log-log plot of the characteristic blister width,  $w$ , as a function of DNA concentration,  $c_{DNA_k}$ . Data points represent the mean of 20 blister widths from a single sample at each condition. The solid line shows a linear fit in log-log space, corresponding to a scaling  $w \propto c_{DNA_k}^{-0.29}$ . This weak dependence indicates a sublinear relationship between kinesin DNA concentration and the selected instability length scale.

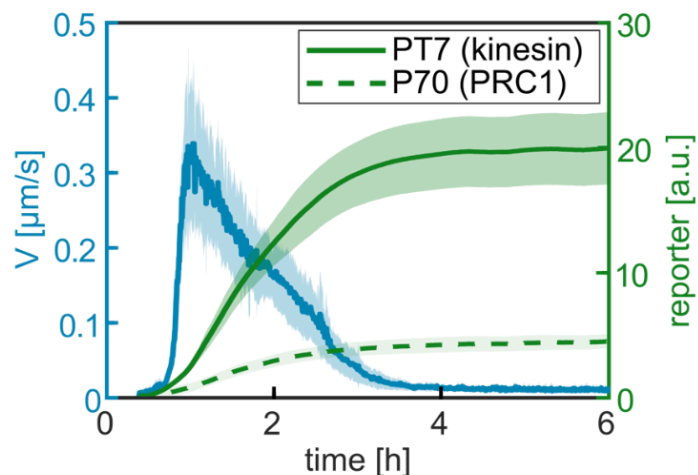


**Figure S11: Mean velocity as a function of time during co-expression of kinesin and PRC1, complementary to the data shown in Fig. 3c.** The blue curve represents the mean velocity magnitude profile. Green curves show protein expression levels measured via GFP reporters: the solid line corresponds to GFP under the T7 promoter (reporting kinesin production), and the dashed line corresponds to GFP under the p<sub>70</sub> promoter (reporting PRC1 expression). Experiments were performed separately for each construct with three replicates each. Shaded regions indicate standard error across replicates.

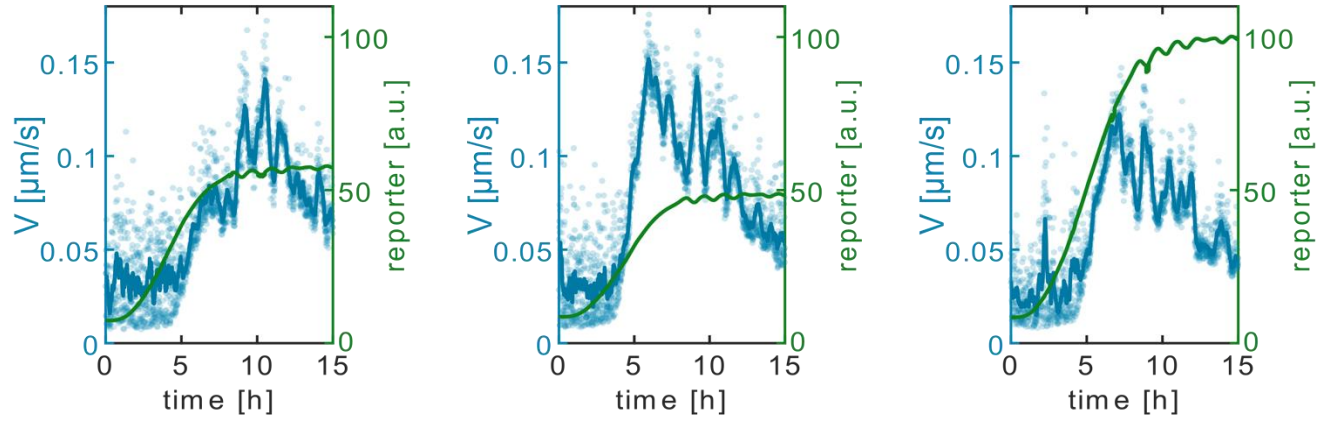


**Figure S12: Oscillations in kinesin gene circuit activity with reporter expression.** Three independent repeats showing oscillatory dynamics of the kinesin gene circuit, plotted as flow velocity (blue) and reporter expression (green) over time. Each panel corresponds to a single experiment. The circuit consists of kinesin fused to the  $\sigma^{28}$  activator, which drives expression of its own repressor, CI, forming a negative feedback loop. Both regulatory proteins carry SsrA degradation tags and are degraded by the endogenous ClpXP protease present at basal levels in the lysate. A co-expressed p<sub>70</sub>-GFP-SsrA reporter is used to track circuit activity. Oscillations in flow velocity correlate with reporter expression, although the presence of the reporter impacts circuit dynamics due to competition for the shared ClpXP degradation machinery. Consequently, full phase-space mapping was performed in the absence of the reporter.

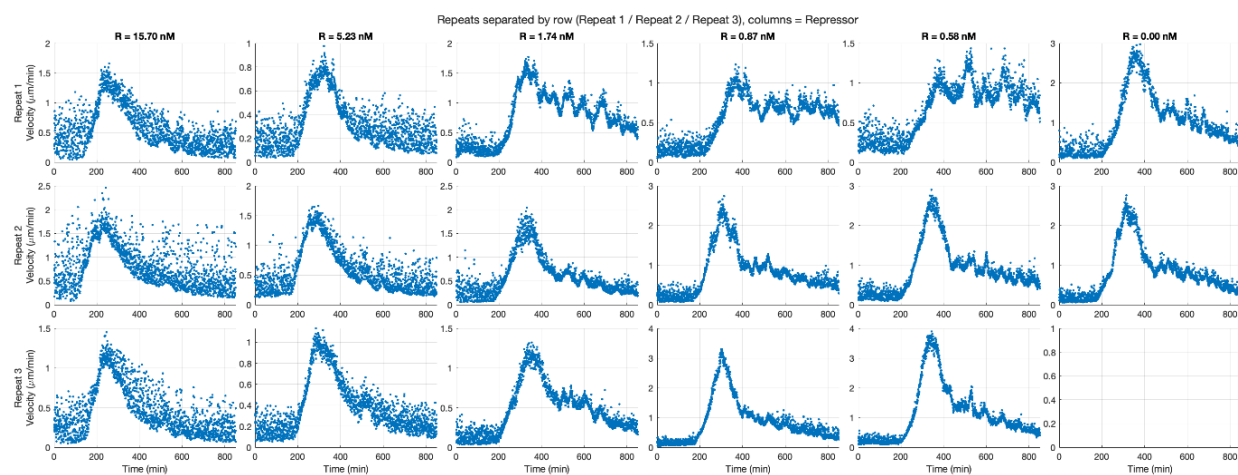


**Figure S13: Velocity magnitude as a function of time for the activator-repressor gene circuit (data shown in Fig. 3e).** Rows correspond to increasing concentrations of repressor DNA ( $R$ ), while columns represent independent experimental repeats (Repeats 1-3). The activator-kinesin fusion gene was kept constant at a concentration of  $20\text{ nM}$ . Data is shown for individual experiments without averaging to preserve the temporal dynamics. Variability between repeats arises from fluctuations in the ClpXP degradation machinery within the lysate.

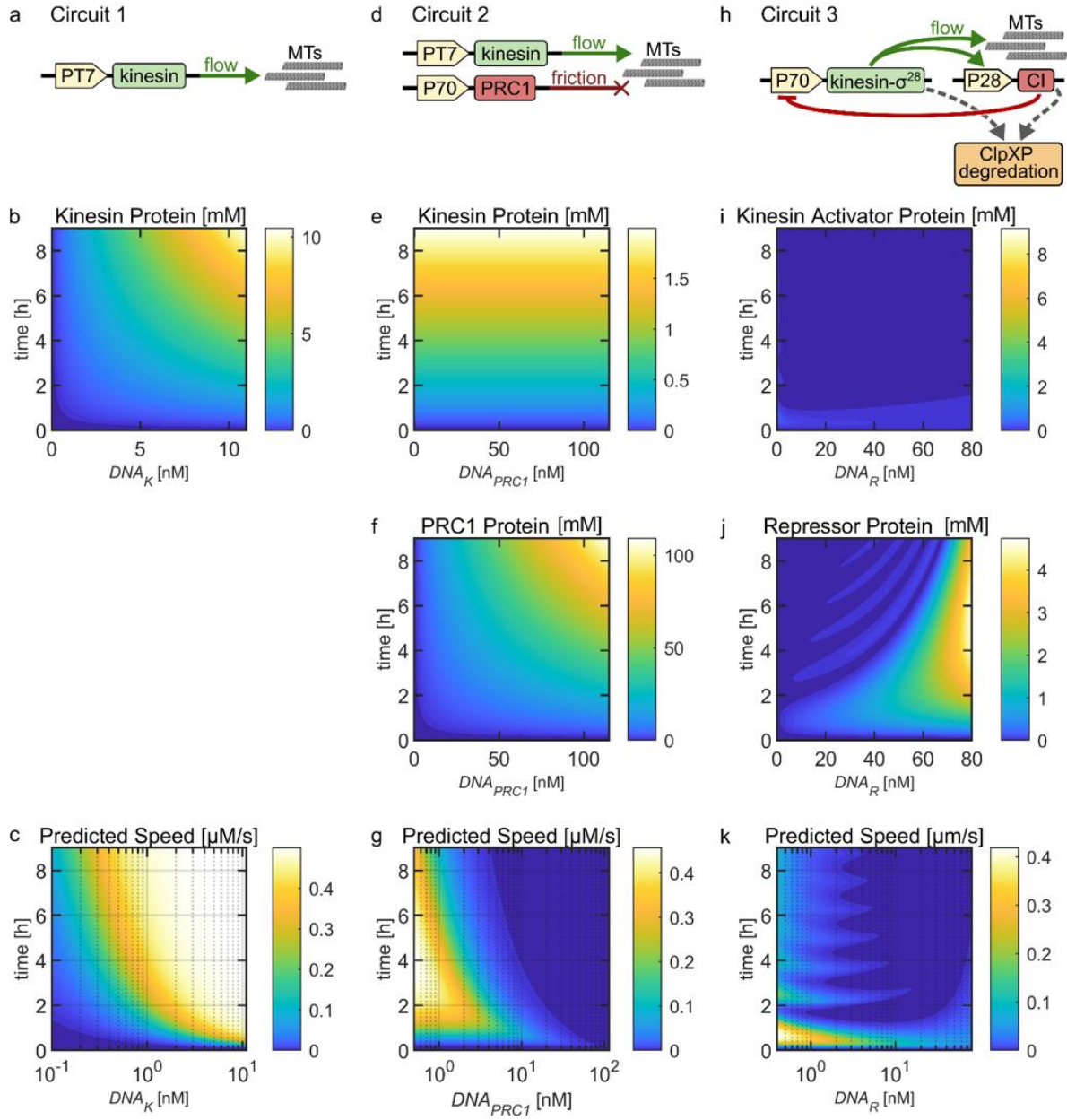


**Figure S14. Complementary version of Extended Data Fig. 3 showing the full dynamic range of the predicted gene expression levels.**

## 6. Video Captions

**Video 1:** 3D reconstruction of confocal time-lapse data showing constitutive kinesin expression from a T7 promoter. DNA concentration: 0.6 *nM*.

**Video 2:** Time-lapse of kinesin expressed from a T7 promoter in a 3D-printed well patterned with 10  $\mu\text{m}$  surface protrusions. DNA concentration: 3 *nM*.