High-density single-molecule analysis of cell surface dynamics
in *C. elegans* embryos

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**Supplementary Figure 1.** Near-homogeneity of the illumination field.

(a) Plot of the average intrinsic intensity vs axial position, measured across the field of illumination in a GFP::Utrophin embryo during maintenance phase. We imaged the embryo for 2000 frames, rotated it by 180 degrees and then imaged for another 2000 frames. Graph shows the first 2000 frames in the initial orientation, second 2000 frames rotated by 180° and all 4000 frames averaged. Error bars indicate the standard error of the mean (n > 20 000 detections for each bin). Image acquistion was performed using 100% laser intensity, 30 ms exposure time and the same laser angle as in all other experiments.

(b) Single frames displaying the intensity of speckles in both orientation.
**Supplementary Figure 2.** Rapid decay to quasi-steady state in GFP::Actin embryos.

Biphasic response for GFP::Actin under illumination conditions that allow accurate single molecule detection and tracking. Graph shows the actin::GFP count and the corresponding fit to the model. Data is the same as in **Fig. 2c**, with uninterrupted time.
Supplementary Figure 3. Tunable and high-density sampling of single molecule behaviors in PAR-6::GFP.

(a) (Top) Biphasic response for PAR-6::GFP under illumination conditions that allow accurate single molecule detection and tracking. (Bottom) Fraction of monomer as a function of time estimated over 100 sequential frames, determined as shown in Fig. 1f. Note that an accurate single molecule count for t < 10 s is impossible because only ~50% of the detection events represent single molecules. (b) Top: Surface density vs time at various laser exposures, shown as a fraction of the initial unobserved density. Error bars indicate standard error of the mean, (n=10,12,13,11,12, low to high laser). Bottom: Corresponding estimates of per molecule photobleaching (k_{ph}) rate as a function of laser.
exposure. Error bars indicate standard deviation. Solid line shows a linear regression against the data. $k_{ph}$ increases linearly with laser exposure. (c) Top: Anterior vs posterior densities during an initial weak exposure to laser (left, 0.75%), followed by a 4-fold stronger exposure to laser (right, 3%), showing an initial “unobserved” quasi-steady state, and the equilibration to a new quasi-steady state under “high” laser exposure. Bottom: laser exposure graph. Note that both the anterior and the posterior cortex equilibrate around the same ratio. 100% laser power $\sim 1.6 \, \mu W.\mu m^{-2}$. 

Supplementary Figure 4. Analysis of PAR-6::GFP mobility during maintenance phase.

(a) Scatter plot of diffusion coefficient $D$ versus exponent $\alpha$, measured by fitting $MSD = 4Dt^\alpha$ for the first 10 lag times for real data, or simulations of brownian diffusion for $D = 0.1$–$0.6 \, \mu m^2.s^{-1}$. Note that the best match between simulated and real data is $D = 0.15$, but the cloud of real data points is still wider than simple diffusion with a single diffusion coefficient (see Supplementary Note 7 for details). (b) Increasing the diffusion coefficient only stretches the vertical axis, but for no diffusion coefficient can simulations of brownian diffusion capture the portion of the real data where $D < 0.7 \, \mu m^2.s^{-1}$. 
**Supplementary Figure 5.** Dependence of estimated turnover values on frame averaging and allowable gap size.

(a) Estimates of assembly and disassembly rates vs axial position during cleavage in a Myosin-depleted embryos for a range of tracking conditions. (b) Same data as above; specific comparison of the 1s average tracked at 1s intervals for gap size = 1–3. The fractional change in estimates of assembly and disassembly rates for different gap sizes is relatively small and is nearly the same for all axial positions, indicating that measurements of relative values should be very insensitive to the small errors caused by disallowing gap closing.
**Supplementary Figure 6.** Measurement of an upper bound on axial localization precision.

Histograms of positional fluctuations of GFP::Utrophin in embryos arrested during meiosis. Imaging conditions were 100% laser power, 30 ms exposure times as for tracking of PAR-6::GFP. Histograms (blue bars) represent (a) $x$ and (b) $y$ particle positions for 4784 single molecule trajectories, aligned to the center-of-mass of each trajectory. Gaussian fits to these distributions yield standard deviations of $24 \pm 1$ nm in $x$ and $y$. Note that no stage de-drifting was performed on the data.
Table 1. Parameters and statistics for single particle tracking in GFP::Actin and PAR-6::GFP.

| Strain       | Exposure time (ms) | Laser power (%) | SNR average | SNR 5th percentile | Nearest neighbor average (pixels) | Nearest neighbor 5th percentile (pixels) | Step size average (pixels) | Step size 95th percentile (pixels) | Tracklength average (frames) | Tracklength > 80 frames (%) |
|--------------|--------------------|-----------------|-------------|--------------------|-----------------------------------|------------------------------------------|-------------------------------|-----------------------------------|--------------------------------|-----------------------------|
| PAR-6::GFP   | 30                 | 100             | 5.4         | 3.2                | 40                                | 8.7                                      | 0.92                         | 2.3                               | 15                             | 3.6                         |
| GFP::Actin   | 100                | 30              | 5.2         | 2.5                | 14                                | 5.4                                      | 0.49                         | 1.1                               | 37                             | 14.8                        |

Imaging parameters and tracking statistics for accurate single molecule particle detection and tracking in PAR-6::GFP and GFP::Actin strains. Column (1) shows the exposure time, (2) laser power, (3) and (4) average and 5th-percentile of signal-to-noise ratio (SNR), (5) and (6) the average and 5th-percentile of the distance to the nearest particle (in pixels), (7) and (8) the average and 95th-percentile of the frame-to-frame step size (in pixels), (9) the average track length (in frames) and (10) fraction of all tracks longer than 80 frames (in percents). Note that in both conditions, the frame-to-frame step size is much smaller than the distance to the closest particle.
Note 1. smPReSS equations: Measuring turn-over from single molecule loss during photobleaching.

Here we present the kinetic model and fitting procedures that underlie our measurements of photobleaching and turnover rates for GFP-tagged proteins at the cell surface in early C. elegans embryos. We consider a population of GFP-tagged proteins that undergo dynamic exchange between a bulk cytoplasmic pool and a patch of the cell surface that is exposed to oblique (near-TIRF) laser illumination. We make the following basic assumptions:

1. The cytoplasmic pool is well mixed
2. The underlying population is kinetically homogeneous; that is the rate constants for local exchange and photobleaching are identical for all molecules
3. The GFP-tagged proteins are representative of the endogenous population; that is they have identical exchange kinetics
4. Photobleaching is restricted to the surface layer, i.e. the photobleaching of cytoplasmic proteins is negligible
5. The diffusive exchange of bleached and unbleached molecules at the edge of the illuminated patch can be neglected.

With these assumptions, we can write a pair of ordinary differential equations that describe the time-evolution of the number of GFP-tagged molecules in the surface patch \( R(t) \) and in the bulk cytoplasm \( Y(t) \):
\[
\frac{dR}{dt} = k_{on} \cdot Y - (k_{off} + k_{ph}) \cdot R
\]
\[
\frac{dY}{dt} = -k_{on} \cdot Y + k_{off} \cdot R
\]

where \(k_{on}Y\) is the observable rate (written as \(k_{\text{app}}\) in the main text) at which molecules appear within the surface patch, \(k_{off}\) is the rate constant for dissociation from the cell surface and \(k_{ph}\) is the rate constant for irreversible photobleaching. We use numbers instead of surface density and cytoplasmic concentration to emphasize conservation of mass as molecules flux between pools. In consequence, the parameter \(k_{on}\) used here differs from the corresponding \(k_{on}\) used in the main text and in Fig. 2a by a scale factor equal to the cytoplasmic volume. Note that the values of \(k_{off}\) and \(k_{ph}\), the targets of our measurements, do not depend on our choice of units or the size of the GFP tagged pool.

The general solution to equations (1) can be written in the form:

\[
R(t) = A_r \cdot e^{r_1 t} + B_r \cdot e^{r_2 t}
\]
\[
Y(t) = A_y \cdot e^{r_1 t} + B_y \cdot e^{r_2 t}
\]

where

\[
r_1 = \frac{-\sigma - \sqrt{\sigma^2 - 4\kappa}}{2}
\]
\[
r_2 = \frac{-\sigma + \sqrt{\sigma^2 - 4\kappa}}{2}
\]
\[
\sigma = k_{on} + k_{off} + k_{ph}
\]
\[
\kappa = k_{on} \cdot k_{ph}
\]
Our primary goal was to extract estimates of $k_{\text{off}}$ and $k_{\text{ph}}$ by fitting the solution for $R(t)$ to experimental measurements in an initially unobserved cell following a step change in laser illumination. In the solution for $R(t)$, there are 4 parameters to fit, namely $r_2$, $r_1$, $A_r$, $B_r$. We reduce this to 3 by writing $A_r$ and $B_r$ in terms of $k_{\text{ph}}$ and the initial value $R(0)$ as follows:

For $t < 0$ the laser is off; thus $k_{\text{ph}} = 0$ and the steady state solution to (1) yields a relationship between initial values $R(0)$ and $Y(0)$:

\begin{equation}
Y(0) = \frac{k_{\text{off}}}{k_{\text{on}}} \cdot R(0)
\end{equation}

For $t > 0$, the laser is on and now we have:

\begin{equation}
\left( \frac{dR}{dt} \right)_{t=0} = k_{\text{on}} \cdot Y(0) - (k_{\text{off}} + k_{\text{ph}}) \cdot R(0)
\end{equation}

Combining (4) and (5) gives:

\begin{equation}
\left( \frac{dR}{dt} \right)_{t=0} = -k_{\text{ph}} \cdot R(0)
\end{equation}

From (2) and (6), we then have:

\begin{equation}
A_r + B_r = R(0)
\end{equation}
Solving for $A_r$ and $B_r$ in terms of $r_1$, $r_2$ and $R(0)$ and substituting into (2) gives:

$$R(t) = R(0) \cdot \left( \frac{k_{ph} + r_2}{r_2 - r_1} \cdot e^{r_1 t} + \frac{k_{ph} + r_1}{r_1 - r_2} \cdot e^{r_2 t} \right)$$

Fitting (8) to the measured response $R(t)$ yields robust estimates for $r_1$, $r_2$ and $k_{ph}$.

Finally, we can rearrange (3) to obtain:

$$k_{on} = \frac{r_1 \cdot r_2}{k_{ph}}$$

$$k_{off} = -(r_1 + r_2) - \left( k_{on} + k_{ph} \right)$$
Note 2: smPReSS experimental procedures

We used a standard MATLAB implementation of non-linear least square curve fitting (lsqcurvefit) to fit equations (8) to measured values for $R(t)$ in defined regions of the embryo surface following a step change in illumination. We extracted standard errors for the fitted values from the fit covariance matrix to estimate the quality of the parameter estimation. In general, these errors (which reflect measurement noise) were negligible when compared to inter-experiment variability. We therefore fit multiple experiments independently and then used the compiled parameter estimates across experiments to estimate parameter means and standard deviations.

A key requirement for obtaining accurate estimates of $k_{ph}$ and $k_{off}$ is that the system must be at its unobserved steady state prior to the onset of illumination. In practice, the illumination required to find and position specimens and fine-tune GFP levels unbalances this steady state and thus once these operations have been achieved it is essential to allow embryos to recover unobserved for a period of time that is significantly greater than $k_{off}$. For GFP::Actin, which has an estimated lifetime of ~12 s we used a recovery interval of $> 30$ s. For Par-6, with an estimated lifetime of ~100 s, we allowed at least 180s. In both cases, we obtained similar estimates using longer recovery times.
In addition, it was important that the quasi-steady state value for $R_{qs} \approx \frac{k_{off}}{k_{ph} + k_{off}} \cdot R(0)$ is sufficiently large to avoid counting errors. Thus in practice, we chose imaging conditions such that $k_{ph}$ was comparable in magnitude of $k_{off}$. For GFP::Actin, this could be achieved under continuous imaging with laser power 30% and 100ms exposure time (Fig. 4), or with time-lapse imaging at laser power 100%, 100ms exposure time, for exposures ranging from 12.5% (100 ms exposure every 800 ms) to 100% (continuous exposure) (Fig. 2). For PAR-6::GFP, we used an illumination duty ratio of ~3% (1 frame every second, at 100% laser power, 30ms exposure time) to obtain the results presented in Supplementary Fig. 2c.

For comparisons of actin turnover, we measured $R(t)$ in rectangular regions at the equator or paired rectangular regions at the poles in the same embryos. For anterior vs posterior comparisons of Par-6 turnover in single embryos, we measured $R(t)$ in the anterior (resp. posterior) cortex by manually delimiting the regions corresponding to high (resp. low) density in the same embryo. We first fit equation (8) to anterior $R(t)$ to estimate $k_{ph}$ and anterior $k_{off}$. The corresponding measurement for posterior Par-6 was considerably noisier because the posterior density of Par-6 is nearly 10-fold lower than in the anterior, leading to considerably more error in the estimation of $k_{off}$ by curve-fitting. To obtain a more robust comparison, we exploited the fact that with an illumination duty ratio of ~3%, $k_{ph} \approx 0.01 \text{ s}^{-1}$ and cytoplasmic depletion was negligible; thus $R(t)$ approaches a stable value after ~ 250 seconds of illumination and can be averaged to obtain good estimates of the
quasi-steady state values $R_{qs}^{\text{ant}}$ and $R_{qs}^{\text{post}}$. Since $R_{qs} \sim \frac{k_{\text{on}}}{k_{\text{ph}} + k_{\text{off}}}$ and $\frac{R_{qs}}{R(0)} \sim \frac{k_{\text{off}}}{k_{\text{ph}} + k_{\text{off}}}$

when the depletion rate is very small, we could use the measured value of $k_{\text{ph}}$ to get good estimates of $k_{\text{off}}^{\text{ant}}$ and $k_{\text{off}}^{\text{post}}$, and thence $k_{\text{on}}^{\text{ant}}$ and $k_{\text{on}}^{\text{post}}$. The values reported in the text were obtained by estimating on and off rates for individual embryos and then averaging the anterior::posterior ratios of these values over 10 embryos.

A key assumption is that the diffusive flux of unbleached GFP from un-illuminated to illuminated regions of the cell surface could affect our measurements. In the case of GFP::actin, the mobility is so low that that this is not an issue. For Par-6, given our estimates of diffusivity $D \sim 0.15 \, \mu m^2.s^{-1}$ and $k_{\text{off}} \sim 0.01 \, s^{-1}$, we estimate a diffusive length scale for Par-6 of $L = \sqrt{\frac{2D}{k_{\text{off}}}} \approx 5 \, \mu m$. Thus local flux near the boundary could influence our measurements. To test this, compared estimates of $k_{\text{off}}$ for the entire anterior region and a smaller subregion obtained by subtracting an outer 3 $\mu m$ shell of the original, and measured a small difference of less than 10%. These errors are systematic and could be corrected, but we chose not to do so here because doing so would not significantly change the main conclusion of our analysis, namely that anterior vs posterior differences in $k_{\text{off}}$ are small and thus the large (10x) differences in anterior vs posterior density must be mainly due to differences in recruitment.
**Supplementary Video 1. Single molecule movie of PAR-6::GFP.** A one cell embryo during maintenance phase expressing PAR-6::GFP at single molecule levels and imaged for 20 s at 30 frames per second, at 100% laser power. Replay speed is 1x. Image size = \(331 \times 231\) pixels, pixel size = 107 nm. Anterior is to the left. Note the range of particle behaviors and mobilities are readily apparent (see main text, and **Supplementary Video 3,4**).

**Supplementary Video 2. Single molecule movie of GFP::Actin.** A one-cell embryo expressing GFP::Actin at single molecule levels and depleted of NMY-2 by RNAi. The embryo was imaged for 500s at 10 frames per second, at 30% laser power, beginning in maintenance phase and proceeding through anaphase and into telophase. Replay speed is 12x. Image size = \(424 \times 267\) pixels, pixel size = 107nm.

**Supplementary Video 3. Single molecule movie of GFP::Actin.** Same embryo as in Video 2, showing the entire acquisition sequence. Replay speed is 48x. Image size = \(424 \times 267\) pixels, pixel size = 107 nm. Note that actin is initially evenly distributed along the anterior posterior axis, but during anaphase and telophase, it accumulates at the equator and is depleted from the poles.

**Supplementary Video 4. A single PAR-6::GFP molecule displaying simple diffusive behavior.** Original image size: \(45 \times 45\) pixels, pixel size: 107nm. Imaging conditions as in **Supplementary Video 1.** Transient failures to detect the particle in a given frame due to motion blur are indicated by the absence of a red circle.
Supplementary Video 5. A single PAR-6::GFP molecule displaying sub-diffusive behavior. Original image size: 45 × 45 pixels, pixel size: 107nm. Imaging conditions as in Supplementary Video 1. For this slower moving particle, there are no detection failures.