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Supporting Material

Rapid Actin-Dependent Viral Motility in Live Cells

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SUPPLEMENTAL MATERIALS

Viruses, Reagents and Plasmids

PV serotype 1, Mahoney strain, was prepared as previously described (1). Cy5 labeling of PV was performed by incubation with amine-reactive dye in a carbonate buffer (pH 9.3) at room temperature and nutated for 1 h. Unbound Cy5 was removed via buffer exchange into 50 mM Hepes buffer (pH 7.4, 145 mM NaCl) using gel filtration columns (Nap5; GE Healthcare). Cy5 labeled virus was titered by plaque assay (1) on adherent HeLa S3 cells and compared to unlabeled virus to confirm that Cy5 labeling did not reduce infectivity. Seneca Valley Virus strain SVV-001, generously provided by Neotropix Corporation, was labeled, purified, and titered using the identical protocol. R18 (octadecyl rhodamine B chloride), DiI, Alexa 647 labeled transferrin, and Alexa 647 labeled phalloidin were obtained from Invitrogen. Head-labeled NBDPS was obtained from Avanti Polar Lipids. A plasmid encoding GFP tagged myosin IIc was obtained from Robert Adelstein (Addgene plasmid 11347); myosins Va-GFP and VI-GFP were generously provided by James Spudich (Stanford University); myosin Vb-GFP was provided by James Goldenring (Vanderbilt University); and myoxin X was provided by Richard Cheney (UNC Medical School).

Cell Culture

The HeLa S3 (human cervical cancer), H446 (human lung carcinoma), and Hep 3B (human hepatocellular carcinoma) cell lines were obtained from American Type Culture Collection. The stable GFP-tubulin HeLa cell line and YFP-actin HeLa cell line were generously provided by Michael Way (Cancer Research UK) (2, 3). All cell types were maintained in a 5% CO_2 environment at 37°C in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 1 mM nonessential amino acids, and antibiotics (25 U/mL penicillin and 25 μg/mL streptomycin). For myosin colocalization experiments, HeLa S3 cells were transfected with GFP-tagged myosins using Fugene HD (Roche) according to the manufacturer's protocol.

SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. Colocalization of PV particles with R18-labeled vesicles. HeLa S3 cells were incubated with 60 μM nocodazole for 50 min followed by PV incubation for 10 min. Cellular membranes and vesicles were nonspecifically labeled with R18 (left panel, green in the right panel). PV fluorescence is displayed in the middle panel and in magenta in the right panel. Red arrow heads indicate colocalization between PV and R18 labeled vesicles. Images were taken 30 min past infection (see also Movie S2). In this experiment, the cell membrane was labeled with R18 for 20 min prior to imaging to allow substantial internationalization of the R18-labeled membrane before imaging. Therefore many intracellular vesicles are also marked with R18.

Fig. S2. Three-dimensional confocal images of cells infected with PV. (*A*) HeLa S3 cells were incubated with virus for 10 min, washed, and stained with R18 immediately prior to imaging. The short incubation time with R18 prior to imaging does not allow substantial internalization of R18-labeled membrane. To construct the 3D image, successive z-stacks spaced by 200 nm were

recorded. The imaging plane used for recording movies of PV motility in all experiments is approximately 900 nm thick as indicated by the dashed lines in the XZ and YZ cross-section. This image plane is located approximately 3 μm above the bottom of the cell in a plane which optically cuts through the nucleus. (*B*) Example XY cross-section of the cell shown in (*A*) at various z positions clearly indicating the bottom, the middle and the top of the cell. (*C*) Cells incubated with 60 μM nocodazole 10 min past PV infection. (*D*) Cells incubated with 60 μM nocodazole and 20 μM CCD 10 min past PV infection.

Fig. S3. Depolymerization of microtubules and actin filaments by treatment with nocodazole and cytochalasin D, respectively. (*A*) Untreated HeLa cells stained for beta-tubulin displayed a dense network of microtubules. (*B*) No microtubule filaments were observed in nocodazole treated cells (60 μM nocodazole, 45 min incubation), indicating the full depolymerization of microtubules under these conditions. (*C*) Untreated HeLa cells stably expressing actin-GFP showed actin filaments and stress fibers. (*D*) No actin filaments or stress fibers were observed in cytochalasin D treated cells (20 μM cytochalasin D, 45 min). Scale bar: 10 μm.

Fig. S4. High frame rate measurement of PV motility. (*A*) Overlay of PV trajectories (gray and red) identified in a representative movie recorded at 20 Hz of nocodazole treated cells (60 μM for 45 min). The white cell boundary map indicates the interior of the cell determined from confocal images of the plasma membrane marker R18, and the dashed line represents the outline of the nucleus. Trajectories not contained entirely within the white cell map are excluded from the plot. Four sample trajectories are highlighted in red and magnified in (*B*). (*C*) Sample trajectories after the application of a four-point vectorial moving average filter. (*D*) Speed plots calculated from low-pass-filtered trajectories shown in *C*.

Fig. S5. Motility of PV in cells depleted of ATP after viral entry. (*A*) PV motility in cells incubated with virus for 10 min followed by 50 min incubation with 60 μM nocodazole (Noco) or 20 μM sodium azide (NaAz). The percent of virus particles with peak speed greater than 0.5 μm/s is plotted. (*B*) Confocal image of PV (magenta) in live cells labeled with R18 (green; see also Movie S5). (*C*) All trajectories identified during a representative 500-frame movie taken at 50 min past NaAz incubation were displayed. The colored trajectories are superimposed on white cell maps indicating the interior of the cells and the dashed lines represent the outlines of the nuclei. Trajectories not contained entirely within the white cell maps are rendered in gray and were excluded from further analysis. PV was able to enter cells before the ATP level dropped significantly. Under these conditions, only static or slow moving particles were observed inside the cells. Error bars represent standard error of the mean.

Fig. S6. PV motility time course in nocodazole treated HeLa S3 cells. In these experiments, cells were pretreated with 60 μM nocodazole for 50 min before incubation with PV for 10 min. Movies were then recorded at various times past infection in the presence of nocodazole. Plotted here is the percent of virus particles with peak speed greater than 0.5 μm/s, indicating that the rapid motility is persistent and occurs from 15 min past infection (mpi) to at least 90 mpi. Error bars represent standard error of the mean.

Fig. S7. Trajectory sorting results for 5 different sets of parameters used in the sorting criteria. A trajectory is considered "directed" if there are at least *c* consecutive steps with an angle of less than *a* degrees between steps. Shown in the plot is the percentage of fast trajectories which are directed in untreated (green) and nocodazole treated cells (blue) for different values of *a* and *c*. Also shown is an estimate of the false positive rate (gray) of falsely identifying an undirected trajectory as being directed. The false positive rates were calculated with two approaches, which give identical results. In the first approach, we simulated a pool of 1000 random-walk trajectories (50 steps each) and determined the percentage of these trajectories which fulfill the directedness criteria as stated above. In the second approach, we derived an analytical solution of the false positive rate $P(l,a,c)\approx 1-exp[-l(1-a/180)(a/180)^c]$ where *a* and *c* are as defined above and *l* is the total number of steps per trajectory.

Figure S8. Ensemble mean square displacement (MSD) analysis. Plotted here are the average MSDs of all PV trajectories in untreated (green), nocodazole (blue), and NaAz (red) treated cells.

Fig. S9. Survival function plots of the speed distributions corresponding to Fig. 3. Survival function plots of the speed distributions for different cargos in non-treated or nocodazole (60 μM for 45 min) treated cells. (*A*) PV infected HeLa cells, (*B*) HeLa cells incubated with transferrin (TFN), (*C*) PV infected H446 cells, (*D*) Seneca valley virus (SVV) infected H446 cells, and (*E*) PV infected Hep3B cells.

Fig. S10. Time course of transferrin motility in nocodazole treated HeLa S3 cells. HeLa S3 cells were incubated with fluorescently labeled transferrin followed by incubation with 60 μM nocodazole. (*A*) The percent of transferrin containing vesicles with peak speed greater than 0.5 μm/s is plotted at various time points after nocodazole treatment. The motility of transferrin was determined for time windows between 0 and 60 min past nocodazole incubation, showing that the progressive depolymerization of microtubules strongly inhibits transferrin motility. (*B* and *C*) All trajectories (displayed in color) identified during the 500-frame movies in untreated (*B*) and 60 min nocodazole treated (*C*) cells. Trajectories not contained entirely within the white cell maps were not rendered here. Error bars represent standard error of the mean.

Fig. S11. Survival function plots of the speed distributions corresponding to Fig. 4A. The curves correspond to PV infected HeLa cells treated 10 min post infection with 60 μM nocodazole (green), 200 μM blebbistatin (blue), 1 μM jasplakinolide (black), or 20 μM cytochalasin D (red). The light-color shades indicate the standard error of the mean.

Fig. S12. PV motility at various concentration of CCD. HeLa S3 cells were infected with PV for 10 min and then incubated with 60 μM nocodazole and the specified concentrations of CCD for 50 min prior to observation. Shown here is the fraction of particles with peak speed above 0.5 μm/s.

Fig. S13. Positive control experiments showing the efficacy of blebbistatin treatment. Representative spinning disk confocal fluorescence images of HeLa S3 cells which have been fixed in 3% paraformaledhyde and stained with Alexa 647 labeled phalloidin. (*A*) Untreated cells show many stress fibers. (*B*) Cells incubated with 200 μM blebbistatin for 45 min in the dark show no actin stress fibers (4). Cells incubated with 200 μM blebbistatin for 45 min and low power 514 nm illumination (1.6×10^{-4} W/cm^2) also lack actin stress fibers. (*D*) Cells incubated

with 200 μM blebbistatin for 45 min and higher power 488 nm laser light (4.3 x 10^{-3} W/cm^2) show a partial recovery of actin stress fibers due to photoinactivation of blebbistatin at this illumination wavelength and power. For each condition, the number of imaged cells was > 20. Note that the total radiation dose per unit area of 514 nm light in (*C*) was identical to that used in our motility assay.

Fig. S14. PV colocalization with myosins. HeLa S3 cells were transfected with GFP tagged myosins (green), infected with PV (magenta) and imaged 20-30 min past infection. (*A*) myosin Vb. (*B*) myosin VI (See also Movie S8). (*C*) myosin Va (See also Movie S9). (*D*) myosin X. Red arrowheads indicate examples where mostly slow or static PV colocalizes with myosin structures. Yellow arrowheads indicate examples where PV travels along actomyosin rich filopodia outlined by GFP-labeled myosin Va or X.

SUPPLEMENTAL MOVIE LEGENDS

Movie S1. Poliovirus (PV, magenta) motility in HeLa S3 cells without drug treatment (movie corresponds to Figs. 1 *A* and 2 *A*). Cellular membrane and vesicles were nonspecifically labeled with octadecyl rhodamine B (R18, green). The movie was recorded at 4 Hz for PV and at 0.4 Hz for R18, and played back at 20 frames per second (123 s, actual duration).

Movie S2. PV (magenta) motility in HeLa S3 cells that have been pretreated with 60 μM nocodazole for 50 min in order to depolymerize microtubules (movie corresponds to Fig. S1). Cellular membranes and vesicles were nonspecifically labeled with R18 (green).The movie was recorded 30 min past infection at 4 Hz for both PV and R18 in order to allow a clear view of the colocazlization between PV and vesicles. The movie is played back at 20 frames per second (123 s, actual duration).

Movie S3. PV (magenta) motility in HeLa S3 cells that have been pretreated with 60 μM nocodazole for 50 min in order to depolymerize microtubules (movie corresponds to Figs. 1 *B* and 2 *B*). The movie was recorded at 4 Hz for PV and at 0.4 Hz for R18, and played back at 20 frames per second (123 s, actual duration).

Movie S4. PV (magenta) motility in HeLa S3 cells that have been pretreated with 20 μM sodium azide and 25 μM deoxyglucose for 50 min in order to deplete cells of ATP (movie corresponds to Figs. 1 *C* and 2 *C*). The movie was recorded at 4 Hz for PV and at 0.4 Hz for R18, and played back at 20 frames per second (123 s, actual duration).

Movie S5. PV (magenta) motility in HeLa S3 cells that have been incubated with 20 μM sodium azide and 25 μM deoxyglucose 10 min past incubation with PV, allowing PV to enter before the depletion of ATP (movie corresponds to Figs. S5 *B* and S5 *C*). The movie was recorded at 4 Hz for PV and at 0.4 Hz for R18, and played back at 20 frames per second (123 s, actual duration).

Movie S6. SVV (magenta) motility in H446 cells that have been incubated with 60 μM nocodazole 10 min past virus inoculation allowing SVV to enter the cell prior to drug treatment (movie corresponds to Fig. 3). The movie was recorded 60 min past infection at 4 Hz for SVV and at 0.4 Hz for R18, and played back at 20 frames per second (88 s, actual duration).

Movie S7. PV (magenta) motility in HeLa S3 cells that have been incubated with 20 μM cytochalasin D and 60 μM nocodazole 10 min past inoculation of PV, allowing PV to enter before the inhibitory effect prevents entry. The movie was recorded 60 min past infection at 4 Hz for PV and at 0.4 Hz for R18, and played back at 20 frames per second (123 s, actual duration).

Movie S8. HeLa S3 cells were transfected with GFP tagged myosin VI (green), infected with PV (magenta), and imaged 20-30 min past infection (movie corresponds to Fig. S14 *B*). The movie was recorded at 4 Hz and played back at 25 frames per second (75 s, actual duration).

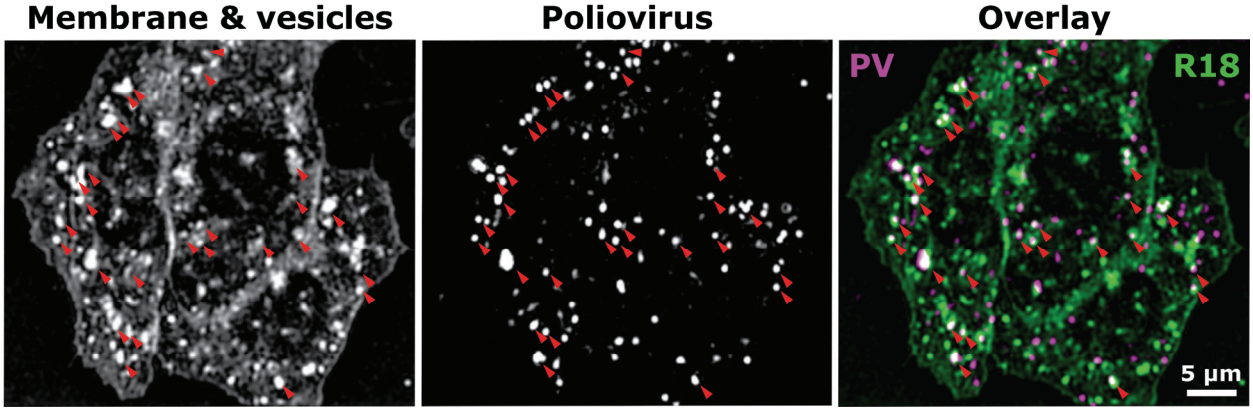
Movie S9. HeLa S3 cells were transfected with GFP tagged myosin Va (green), infected with PV (magenta), and imaged 20-30 min past infection (movie corresponds to Fig. S14 *C*). The movie was recorded at 4 Hz and played back at 25 frames per second (92 s, actual duration).

REFERENCES

1. Rueckert, R. R., and M. A. Pallansch. 1981. Preparation and characterization of encephalomyocarditis (EMC) virus. Methods Enzymol 78:315-325.
2. Arakawa, Y., J. V. Cordeiro, and M. Way. 2007. F11L-mediated inhibition of RhoA-mDia signaling stimulates microtubule dynamics during vaccinia virus infection. Cell Host Microbe 1:213-226.
3. Arakawa, Y., J. V. Cordeiro, S. Schleich, T. P. Newsome, and M. Way. 2007. The release of vaccinia virus from infected cells requires RhoA-mDia modulation of cortical actin. Cell Host Microbe 1:227-240.
4. Brzeska, H., J. Szczepanowska, F. Matsumura, and E. D. Korn. 2004. Rac-induced increase of phosphorylation of myosin regulatory light chain in HeLa cells. Cell Motil Cytoskeleton 58:186-199.

SUPPLEMENTAL FIGURES

Supplemental figure 1



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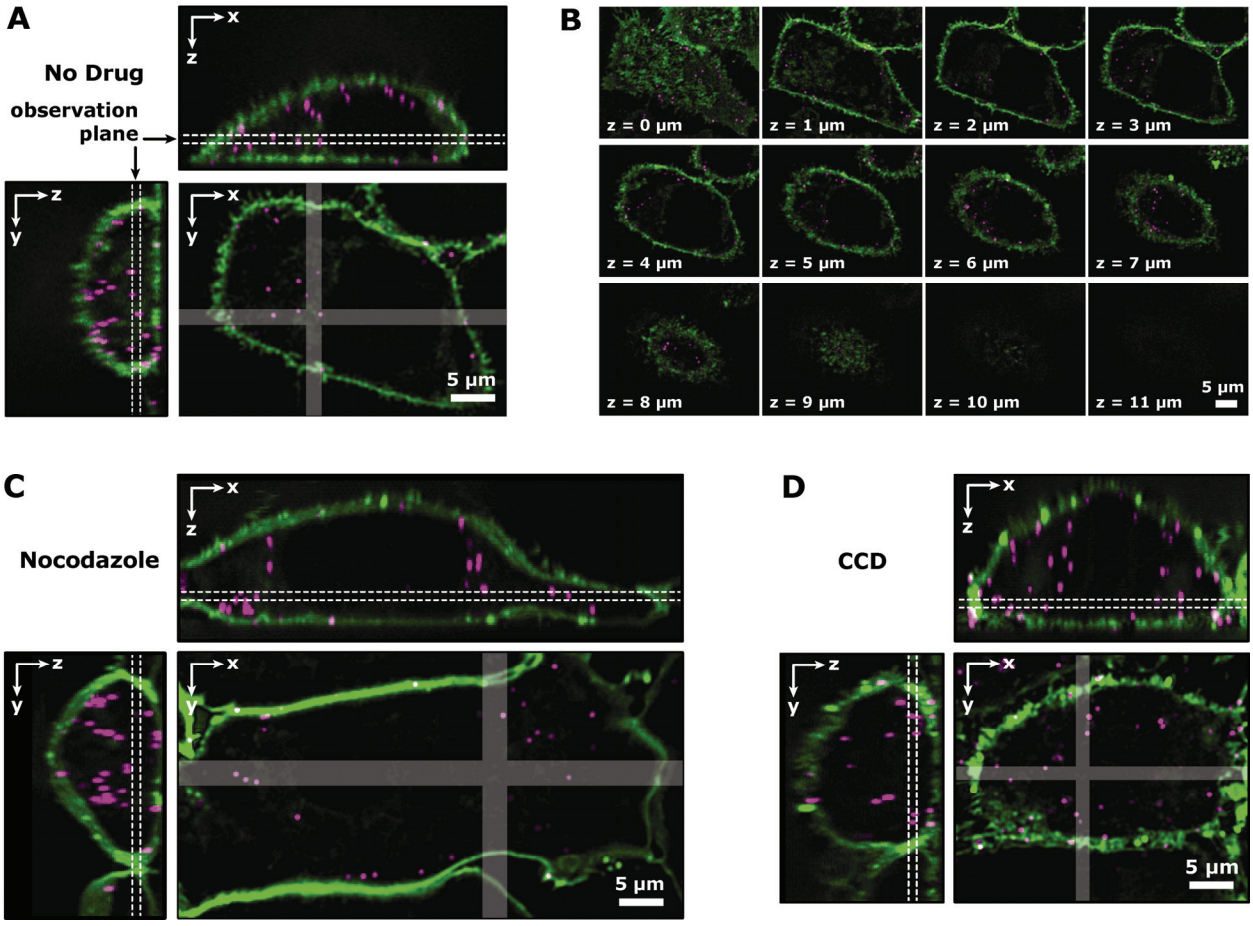

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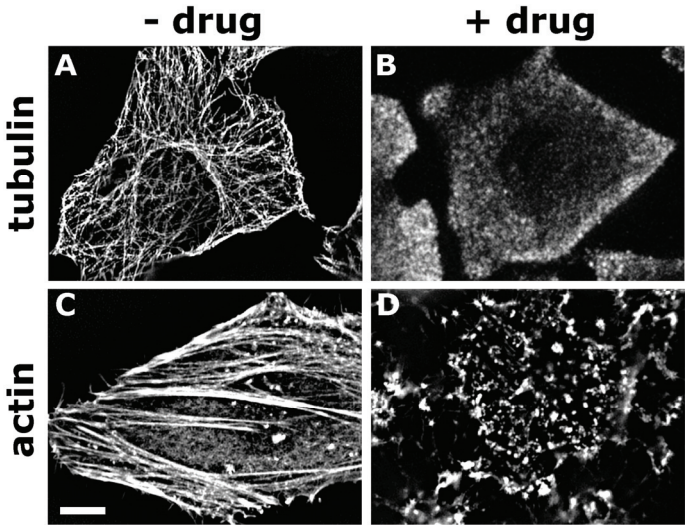

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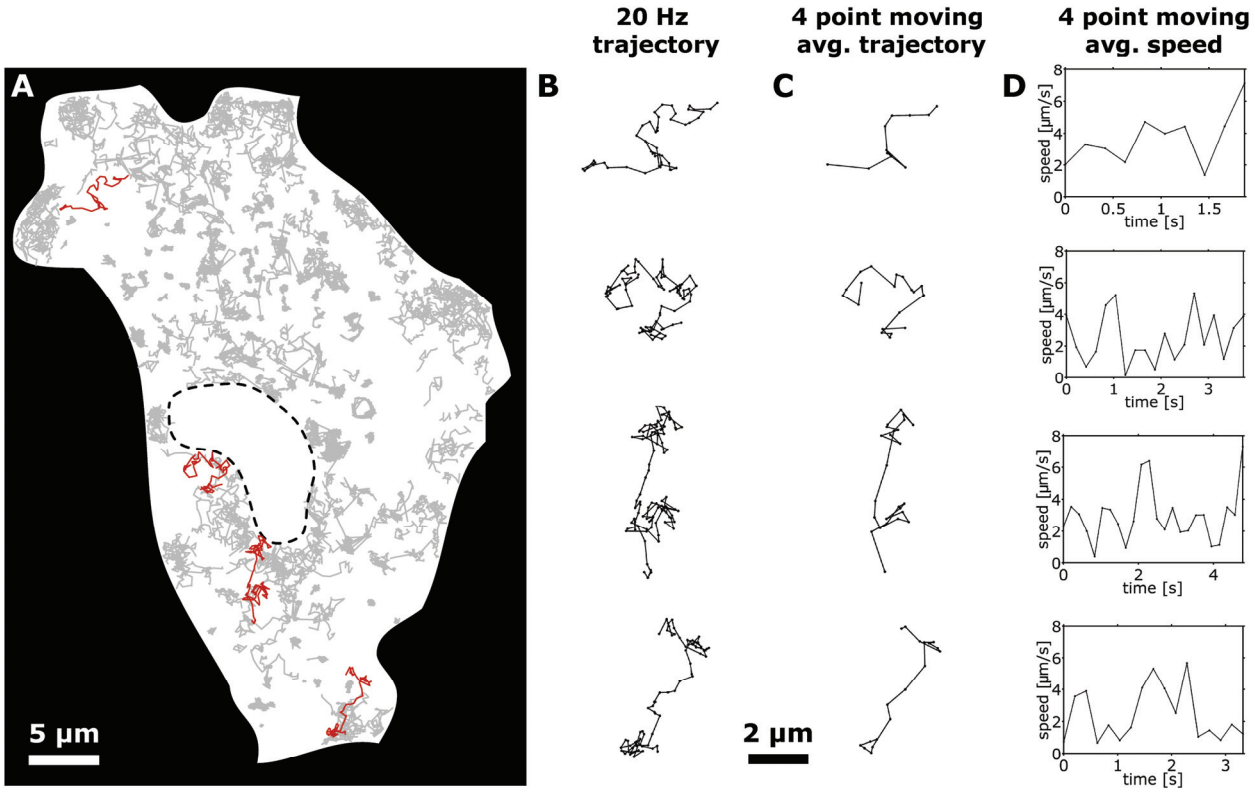

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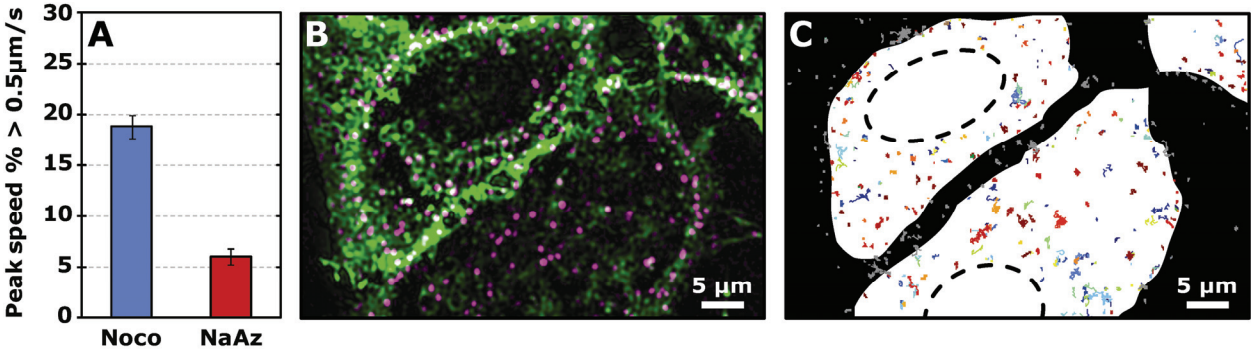

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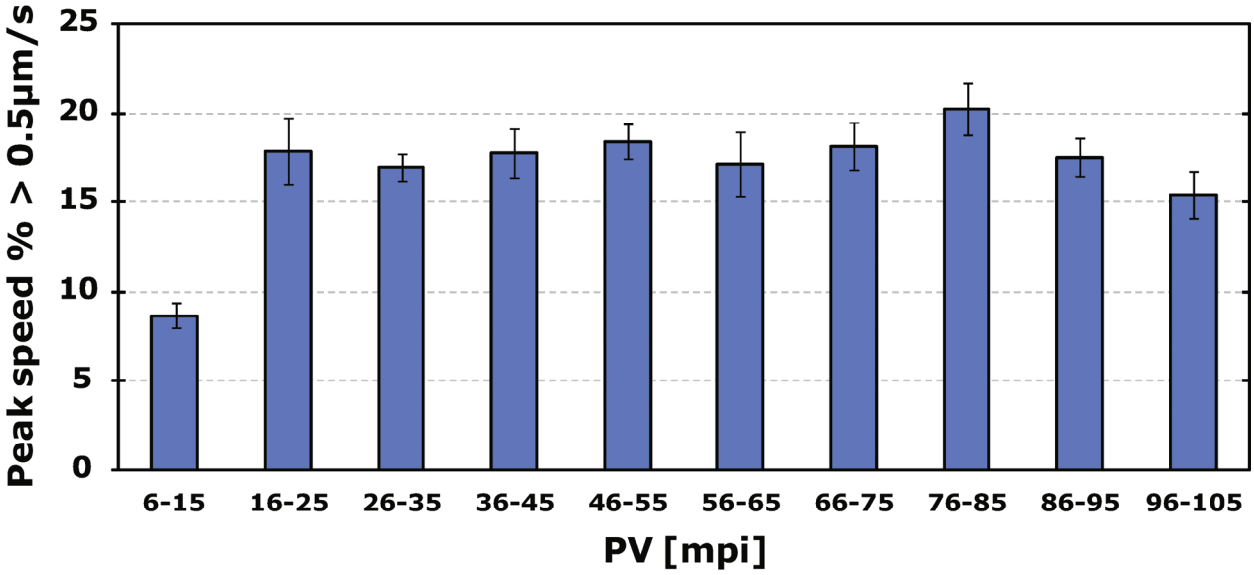

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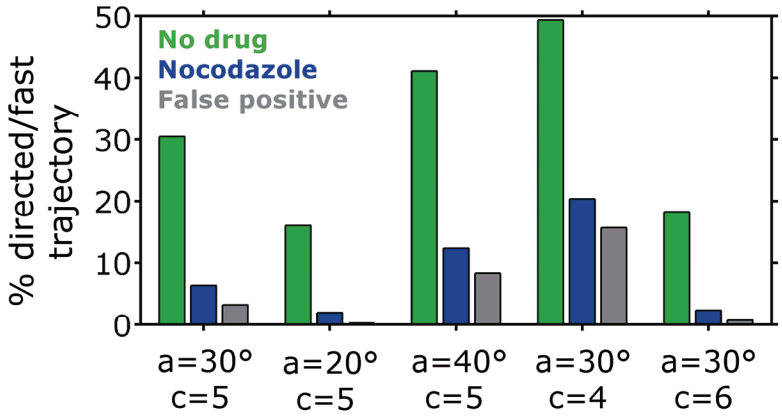

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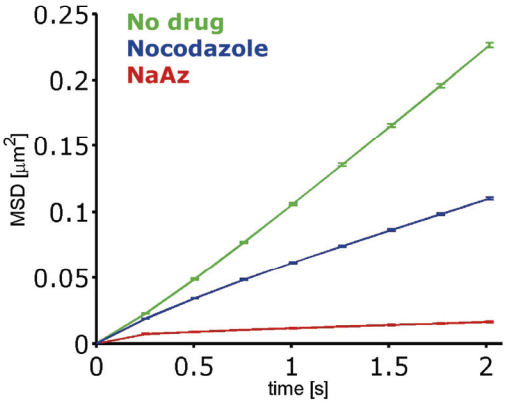

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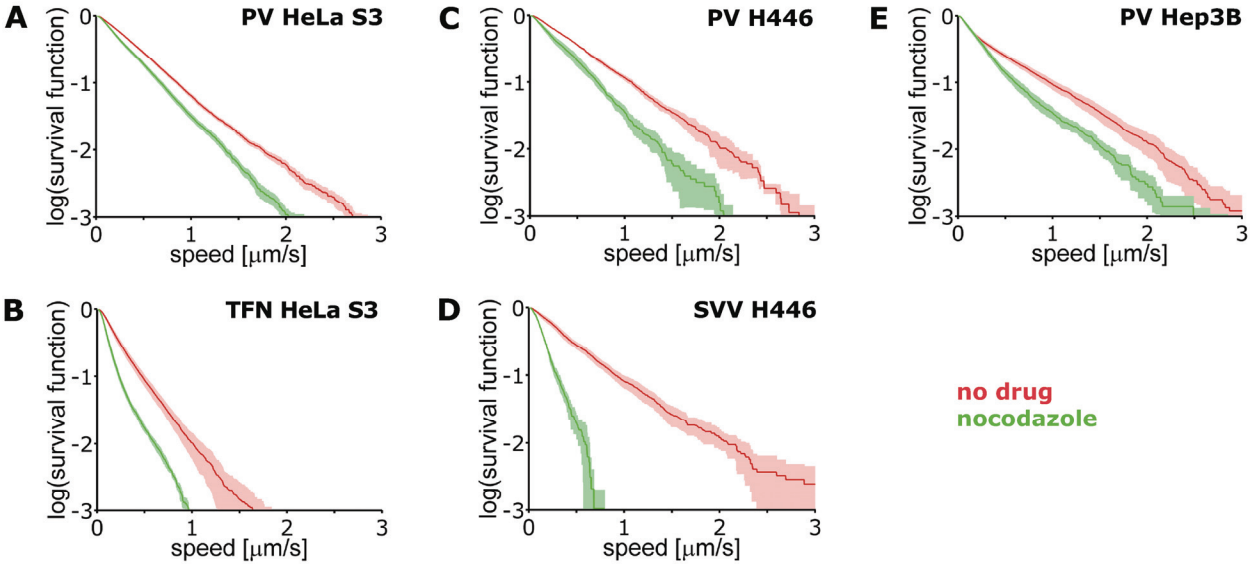

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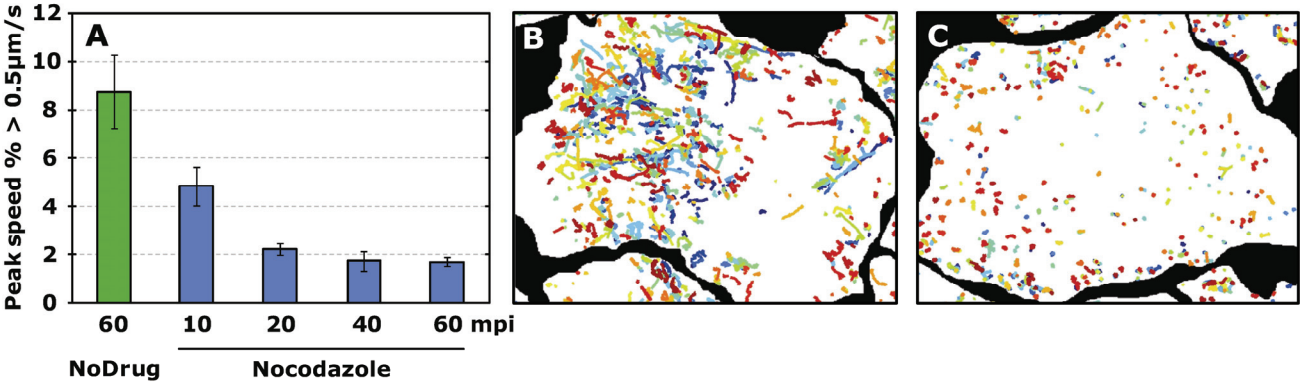

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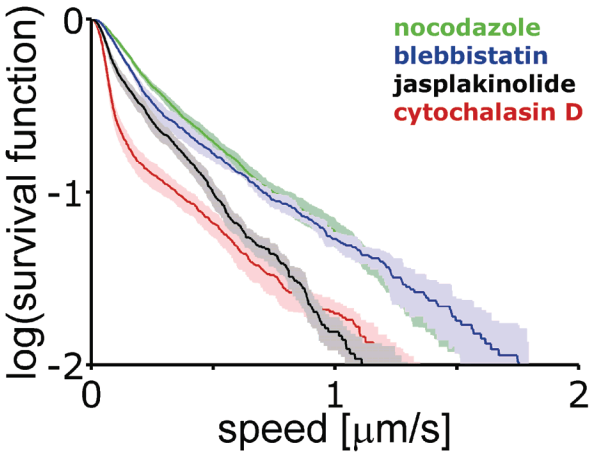

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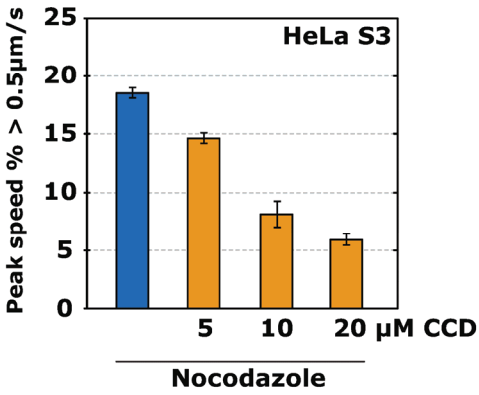

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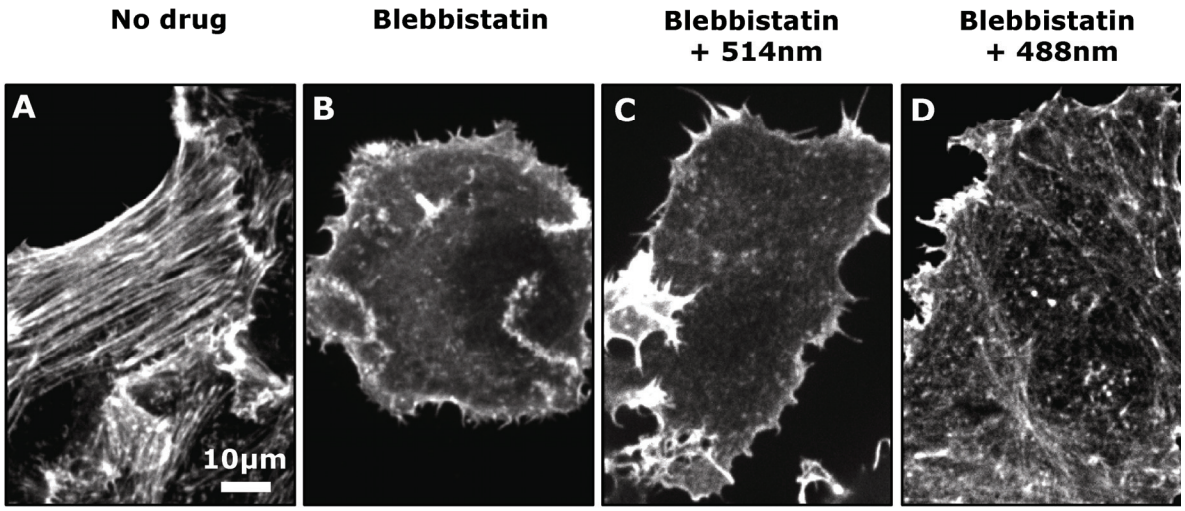

Supplemental figure 14