

Human dynein-dynactin is a fast processive motor in living cells

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Abstract

Minus-end directed transport along microtubules in eukaryotes is primarily mediated by cytoplasmic dynein and its cofactor dynactin. Significant advances have been made in recent years characterizing human dynein-dynactin structure and function using in vitro assays, however, there is limited knowledge about the motile properties and functional organization of dynein-dynactin in living human cells. Total internal reflection fluorescence microscopy (TIRFM) of CRISPR-engineered human cells is employed here to visualize fluorescently tagged dynein heavy chain (DHC) and p50 with high spatio-temporal resolution. We find that p50 and DHC exhibit indistinguishable motility properties in their velocities, run lengths, and run times. The dynein-dynactin complexes are fast ($\sim 1.2 \mu\text{m/s}$) and typically run for several microns ($\sim 2.7 \mu\text{m}$). Quantification of the fluorescence intensities of motile puncta reveals that dynein-dynactin runs are mediated by at least one DHC dimer while the velocity is consistent with that measured for double dynein (two DHC dimers) complexes in vitro.

eLife assessment

In their short technical report, Verma et al. describe how endogenously-tagged dynein and dynactin molecules localize to growing microtubule plus-ends and move processively along microtubules in cells. The authors present **solid** evidence that cytoplasmic dynein is a processive motor that takes long excursions prior to dissociating from microtubules. However, there are concerns about the robustness of the imaging and analysis protocols, which should be more clearly defined. This is a **useful** study that will be of interest to cell biologists and biochemists in the motor protein field.

Introduction

The eukaryotic microtubule cytoskeleton plays a critical role in the organization, positioning and motility of organelles, mRNA, and proteins. Intracellular motility is mediated by molecular motor proteins that move cargoes along polarized microtubule tracks (for reviews see (Cason and Holzbaur, 2022 ; Reck-Peterson et al., 2018 ). The kinesin superfamily includes motors that are responsible for plus-end directed transport, and others that contribute to minus-end motility and regulation of microtubule dynamics (Hirokawa et al., 2009 ). In contrast, cytoplasmic dynein 1

motor complexes (hereafter dynein) (Canty et al., 2021 [↗](#); Pfister et al., 2005 [↗](#)) are exclusively minus-end directed. The dynein complex is composed of two catalytic heavy chains, and additional light, light intermediate and intermediate chains (Carter et al., 2016 [↗](#); Cason and Holzbaur, 2022 [↗](#)). The isolated dynein motor complex displays predominantly diffuse motility along microtubules in vitro, an observation that led to the identification of the dynactin complex, which is important for dynein motility (Gill et al., 1991 [↗](#); McKenney et al., 2014 [↗](#); Schlager et al., 2014 [↗](#); Schroer and Sheetz, 1991 [↗](#)). In addition, a number of adaptor complexes that link dynein and dynactin to specific cargoes and activate the motor have been identified and characterized (Canty et al., 2021 [↗](#); Cason and Holzbaur, 2022 [↗](#); Reck-Peterson et al., 2018 [↗](#); Splinter et al., 2012 [↗](#)).

Vesicular transport has been directly visualized by live-cell imaging in diverse cells including highly polarized axons (Canty et al., 2021 [↗](#)). Motility of vesicles is bidirectional, both plus- and minus-end directed motors co-purify with cargoes, and adaptor complexes have been shown to associate with both kinesin and dynein motors (Ali et al., 2023 [↗](#); Hancock, 2014 [↗](#); Hendricks et al., 2010 [↗](#); Maeder et al., 2014 [↗](#); Vale, 1987 [↗](#)). Together these observations indicate that the number and activity of cargo associated motors must be regulated to ensure efficient delivery to appropriate cellular destinations (Cason and Holzbaur, 2022 [↗](#)). In vitro studies in which the number of motors bound to artificial cargoes can be precisely controlled demonstrate that cargoes with both plus and minus motors often stall or pause, or show directed motion that is distinct from either individual motor acting alone; however, once directed motion is initiated, reversals are infrequent (Belyy et al., 2016 [↗](#); Derr et al., 2012 [↗](#)). Cryo-EM of dynein-dynactin complexes revealed that the stoichiometry of dynein relative to dynactin is determined by the activating adaptors that link them. Specifically, the adaptor BICD2 recruited a single dynein to dynactin while BICDR1 and HOOK3 supported assembly of a “double dynein” complex (Chaaban and Carter, 2022 [↗](#); Grotjahn et al., 2018 [↗](#); Urnavicius et al., 2018 [↗](#); Urnavicius et al., 2015 [↗](#)). Interestingly, the processivity and velocity of dynein-dynactin differs depending on the number of dyneins such that the double dynein complexes assembled with BICDR1 and HOOK3 had a higher frequency of processive motility events and moved faster than the single dynein complexes assembled with BICD2 (Urnavicius et al., 2018 [↗](#)).

In contrast to the relative ease of visualizing motor proteins in in vitro motility assays, the crowded three-dimensional intracellular environment poses a challenge for imaging and quantifying motility events in living cells. In human cells constitutively over-expressing a GFP-tagged intermediate chain of dynein both dot-like, bidirectional motility and comet-like motile events, which co-localized with EB1, were observed, confirming a role for dynein in both tip-tracking and vesicle motility (Kobayashi and Murayama, 2009 [↗](#)). Quantification of organelle motility in the highly polarized filamentous fungus *Ustilago maydis* expressing endogenously tagged fluorescent dynein revealed that binding of a single dynein to an anterograde-directed early endosome was sufficient to trigger directional reversal of its transport (Schuster et al., 2011 [↗](#)).

In this study, HeLa cells expressing CRISPR/Cas9 modified dynein heavy chain (DHC) or the p50 subunit of dynactin were visualized using high-resolution fluorescence microscopy approaches to gain insights into the behaviors of dynein and dynactin in vivo. As a result, we were able to directly observe and quantify core motility parameters of dynein-dynactin while analyses of their fluorescence intensities relative to a known standard was informative of the stoichiometry of dynein in motile dynein-dynactin complexes in proliferating human cells.

Results and discussion

Human HeLa cells were engineered using CRISPR/Cas9 to insert a cassette encoding FKBP and EGFP tags in frame at the 3' end of the dynein heavy chain (DYNC1H1) gene (SF1). A clonal DHC-EGFP-expressing HeLa cell line was generated and subjected to live-cell fluorescence imaging to directly visualize DHC activities in interphase cells. The most striking dynein behavior was tip-tracking on polymerizing MTs as an abundance of DHC comets traveling at the speed of MT polymerization (Cassimeris et al., 1988 [\[1\]](#); Rusan et al., 2001 [\[2\]](#); Walker et al., 1988 [\[3\]](#)) were readily observed by both spinning disc confocal microscopy and total internal reflection fluorescence microscopy (TIRFM) (**Fig. 1 A, B** [\[4\]](#), **Videos 1 and 2**). SiR-Tubulin was next introduced to visualize DHC localization and behaviors relative to MTs (**Fig. 1 C** [\[5\]](#)). Interestingly, because SiR-Tubulin is a docetaxol derivative, its addition suppressed plus-end MT polymerization resulting in a significant reduction in the DHC tip-tracking population and a much clearer view of a different population of MT-associated DHC puncta (**Fig. 1 C** [\[5\]](#)). The SiR-Tubulin-treated cells were subjected to two-color TIRFM to determine if the DHC puncta exhibited motility and; indeed, puncta were observed streaming along MTs, which was especially evident via TIRFM on MTs that were pinned between the nucleus and the plasma membrane (**Video 3**). DHC puncta were next visualized with higher temporal resolution by acquiring single color TIRFM time-lapses of the EGFP channel at a rate of 5 frames per second (fps) (**Fig. 1 D** [\[6\]](#), **Video 4**). The TIRFM time-lapses were assessed by eye and several parameters of the motile puncta were measured using kymographs (**Fig. 1 E** [\[7\]](#)). The average DHC puncta moved at 1.2 ± 0.05 (mean $\pm$ SEM) $\mu\text{m}/\text{sec}$ over a distance of $2.8 \pm 0.2 \mu\text{m}$ for 2.6 ± 0.2 seconds (**Fig. 1 F-H** [\[8\]](#)). While some motile puncta appeared to switch “tracks” at MT intersections, which was inferred from observations of sudden high angle turns, directional switches on the same MT were infrequent ($\sim 5\text{-}10\%$ of runs). It should also be noted that the run lengths and run times for DHC are likely underestimates as the puncta could only be tracked while they remained in the TIRFM evanescent field and prior to their photobleaching.

Activation of dynein motility requires its interaction with the dynactin complex (Chowdhury et al., 2015 [\[9\]](#); Grotjahn et al., 2018 [\[10\]](#); Urnavicius et al., 2018 [\[11\]](#); Zhang et al., 2017 [\[12\]](#)). CRISPR/Cas9-based genomic engineering was used to insert the cassette encoding FKBP and EGFP tags at the 3' end of the p50/dynamitin gene and a clonal p50-EGFP-expressing HeLa cell line was subjected to live-cell fluorescence imaging to visualize dynactin in interphase cells. Consistent with prior observations of dynactin localization (Vaughan et al., 1999 [\[13\]](#); Vaughan et al., 2002 [\[14\]](#)) and like dynein, dynactin exhibited robust tip-tracking activity on growing MT plus-ends that was clearly visualized via both spinning disc confocal imaging and TIRFM (**Fig. 2 A, B** [\[15\]](#), **Videos 5 and 6**). Suppression of plus-end polymerization dynamics upon introduction of SiR-Tubulin caused a loss of the tip-tracking pool of dynactin, which made a second pool of motile, MT-associated puncta of p50 more pronounced especially when visualized with TIRFM (**Fig. 2 C** [\[16\]](#), **Video 7**). The p50-EGFP-expressing cells were then subjected to single color TIRFM at 5 fps and kymograph analysis to measure the motility parameters of the dynactin complex (**Fig. 2 D** [\[17\]](#)). The mean velocity of the p50 puncta was $1.2 \pm 0.07 \mu\text{m}/\text{sec}$ (**Fig. 2 E** [\[18\]](#)) while the mean run length was $2.6 \pm 0.2 \mu\text{m}$ (**Fig. 2 F** [\[19\]](#)) and the mean run time was 2.2 ± 0.2 seconds (**Fig. 2 G** [\[20\]](#)). Similar to dynein, dynactin was observed to switch MT “tracks” but infrequently switched direction.

In comparing the motility parameters of the motile dynein and dynactin puncta, their velocities (**Fig. 3 A** [\[21\]](#)), run lengths (**Fig. 3 B** [\[22\]](#)), and run times (**Fig. 3 C** [\[23\]](#)) were statistically indistinguishable. Thus, we inferred that the motile population of dynein was associated with the dynactin complex. The fluorescence intensities of motile DHC and p50 puncta were next compared to EGFP-tagged kinesin-1 dimer as a standard to assess the stoichiometries of the motile dynein and dynactin complexes (**Fig. 3 D-F** [\[24\]](#)). Interestingly, the intensity of motile DHC-EGFP puncta was not statistically significantly different from the intensity of motile kinesin-1-EGFP dimers; however, the intensity of motile p50 puncta had a mean fluorescence intensity that was about half that of the motile kinesin-1-EGFP puncta (**Fig. 3 G** [\[25\]](#)). Thus, the motile dynein and dynactin complexes visualized here via high-speed TIRFM were comprised, on average, of two EGFP-tagged

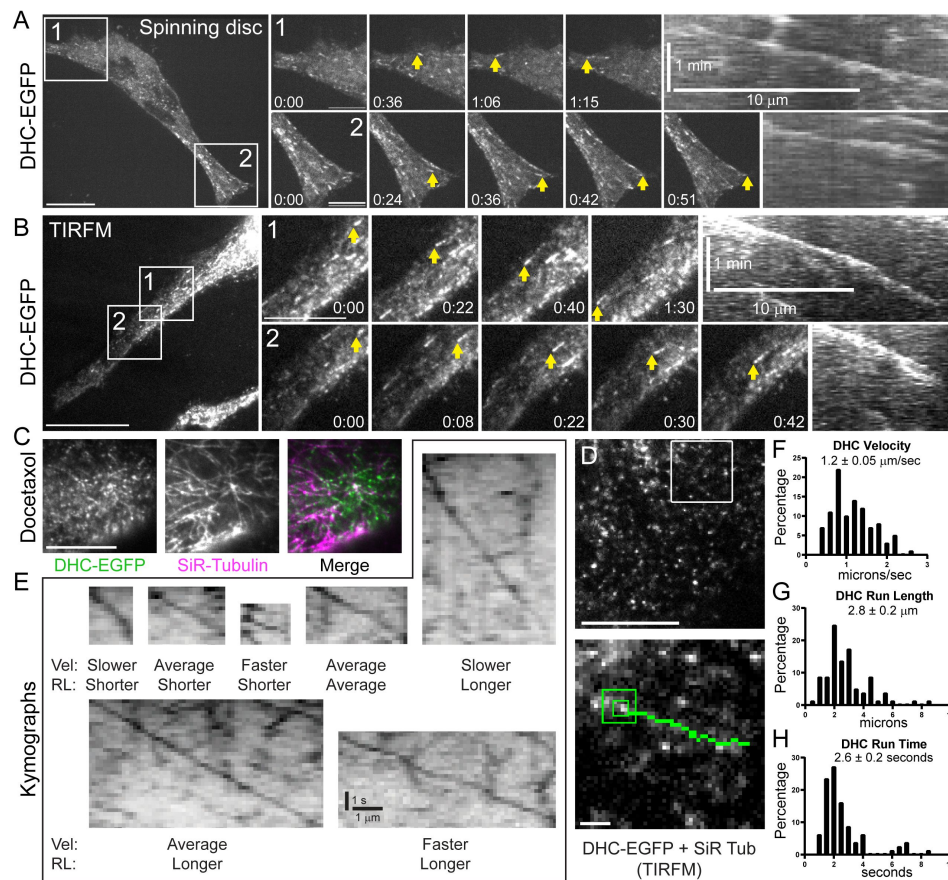


Figure 1.

Dynein tip-tracks on polymerizing MTs and walks processively at high velocities in interphase HeLa cells. (A)

Representative spinning disc confocal time-lapse of a DHC-EGFP-expressing HeLa cell showing robust tip-tracking. Zoomed views of the numbered boxed regions are shown to the right with kymographs of the tip-tracking events highlighted with yellow arrows in the zoomed panels. **(B)** Representative TIRFM time-lapse of a DHC-EGFP-expressing HeLa cell showing the tip-tracking population. Zoomed views of the numbered boxed regions are shown with kymographs of the tip-tracking events highlighted with yellow arrows. **(C)** Still frame from a representative TIRFM time-lapse of a DHC-EGFP-expressing HeLa cell treated with SiR-Tubulin. In the merge image DHC is green and SiR-Tubulin labeled MTs are magenta. **(D)** Still frame from a representative high temporal resolution (5 fps) TIRFM time-lapse of a DHC-EGFP-expressing HeLa cell treated with SiR-Tubulin. Boxed region is shown as a zoomed inset in the lower panel with the track of a motile DHC puncta highlighted in green. **(E)** Representative kymographs of motile puncta spanning the range of measured velocities (Vel) and run lengths (RL). **(F)** Distribution of DHC velocities ($n = 100$ puncta). **(G)** Distribution of DHC run lengths ($n = 81$ puncta). **(H)** Distribution of DHC run times ($n = 81$ puncta). Scale bars, $10\ \mu\text{m}$ (A-D); $1\ \mu\text{m}$ (all insets), $10\ \mu\text{m}$ (horizontal); $1\ \text{min}$ (vertical) in the kymographs in A and B; and $1\ \mu\text{m}$ (horizontal); $1\ \text{second}$ (vertical) in E. Displayed times are min:sec.

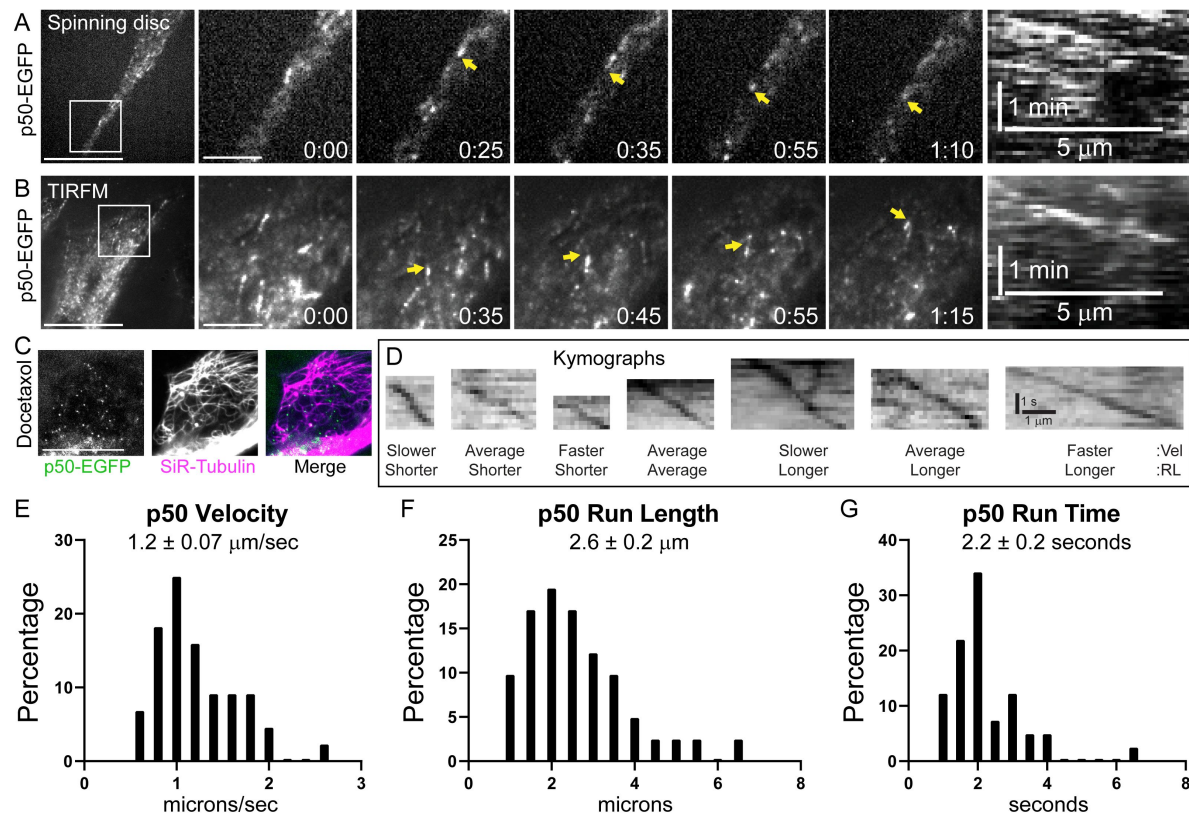


Figure 2.

The dynactin complex component p50 tip-tracks on polymerizing MTs and walks processively at high velocities in interphase HeLa cells. (A)

Still frames from a representative spinning disc confocal time-lapse of a p50-EGFP-expressing HeLa cell. Zoomed view of the boxed region is shown with a kymograph of the tip-tracking event highlighted with the yellow arrow. **(B)** Still frames from a representative TIRFM time-lapse of a p50-EGFP-expressing HeLa cell showing the tip-tracking population. Zoomed view of the boxed region is shown with a kymograph of the tip-tracking event highlighted by the yellow arrow. **(C)** Still frame from a representative TIRFM time-lapse of a p50-EGFP-expressing HeLa cell treated with SiR-Tubulin. In the merge image p50 is green and SiR-Tubulin labeled MTs are magenta. **(D)** Representative kymographs of motile p50 puncta spanning the range of measured velocities (Vel) and run lengths (RL). **(E)** Distribution of p50 velocities ($n = 44$ puncta). **(F)** Distribution of p50 run lengths ($n = 41$ puncta). **(G)** Distribution of p50 run times ($n = 41$ puncta). Scale bars, 10 μ m (A-C); 1 μ m (all insets), 5 μ m (horizontal); 1 min (vertical) in the kymographs in A and B; and 1 μ m (horizontal); 1 second (vertical) in D. Displayed times are min:sec.

DHC molecules and a single p50-EGFP molecule. When considering how these values relate to physiological stoichiometries of the motile dynein-dynactin complexes, it is important to note that both the DHC-EGFP and p50-EGFP HeLa cell line clones are heterozygotes (**Fig. 3 H, I**). Dynein motility requires DHC dimerization and if there is an equal likelihood of EGFP-tagged and untagged DHC incorporation into a functional dimer then the motile dynein puncta we visualized would actually contain two DHC dimers. While we were unable to assess the relative expression levels of tagged versus untagged DHC for technical reasons, we can confidently conclude from our data that there is at least one DHC dimer in the motile puncta. Interestingly, western blotting of cell extract prepared from the p50-EGFP clone revealed that the cells expressed a ~5-6-fold molar excess of the untagged p50 compared to p50-EGFP (**Fig. 3 I**). Differential allele regulation has been observed for endogenously tagged proteins (Mann and Wadsworth, 2018; Roberts et al., 2017) suggesting that regulation of gene expression may help avoid deleterious effects when cells can tolerate a fraction of the total protein pool being modified. The relative levels of EGFP-tagged versus untagged p50 most likely explains why motile p50 puncta only had a single EGFP molecule despite the fact that the dynactin complex is known to have four copies of p50 (Eckley et al., 1999; Urnavicius et al., 2015). Thus, we conclude that the motile dynactin complexes visualized here are typically comprised of one p50-EGFP molecule and 3 untagged copies of p50.

While several groups have recently imaged endogenously tagged dynein in mitotic HeLa cells and iNeurons (Fellows et al., 2023; Ide et al., 2023), to our knowledge - this is the first direct visualization of motile endogenously-tagged human dynein-dynactin complexes in proliferating, non-neuronal cells. Two distinct motile populations of dynein-dynactin exist in human interphase cells: one pool that tip-tracks on polymerizing MT plus-ends and a second pool that moves processively at high velocities (~7X faster than the tip-tracking population) along MTs. We propose that the ~3 μ m run lengths measured here are an underestimate of what the dynein-dynactin complex is capable of due to the relatively short and dynamic MT tracks available to it in interphase HeLa cells and because motile puncta could only be visualized while they were in the TIRF field and prior to their photobleaching. Indeed, dynein was recently shown to be incredibly processive in iNeurons where its mean run length was ~35 μ m and runs >100 μ m were observed (Fellows et al., 2023). It is possible that the tip-tracking pool of dynein-dynactin is regulated in a cell-cycle dependent manner because dynein-dynactin tracking events were less robust in mitotic cells than in interphase cells. The dynein-dynactin pool that exhibited directional motility on MTs was capable of switching MT tracks but rarely switched directions suggesting that there is not a constant tug-of-war between dynein-dynactin and kinesins in proliferating cells as compared to observations of bidirectional vesicular transport in neurons (Hancock, 2014; Hendricks et al., 2010; Maeder et al., 2014). The low directional switching frequency observed here is consistent with in vitro studies reconstituting the tug-of-war phenomenon showing that one type of motor dominates once directional movement begins and that reversal events are rare (Ali et al., 2023; Belyy et al., 2016; Derr et al., 2012). The velocities measured here in HeLa cells were comparable to those measured for unopposed dynein and; therefore, did not suggest that there was significant resistive drag from associated kinesin motors as has been observed in vitro. This suggests that the cargos, which we presume motile dynein-dynactin puncta are bound to, any kinesins associated with the cargos are inactive and/or cannot effectively bind the MT lattice during dynein-dynactin-mediated transport in interphase HeLa cells. Interestingly, directional switching was also uncommon during retrograde dynein-dynactin movements in iNeuron axons (Fellows et al., 2023) and in retrograde trafficking of early endosomes in *Ustilago maydis* mediated by dynein-dynactin containing the HOOK3 adaptor (Bielska et al., 2014; Schuster et al., 2011).

Dynein-dynactin motility is also affected by tubulin modifications (Barisic and Maiato, 2016; Roll-Mecak, 2020; Sirajuddin et al., 2014) most notably by the tyrosination state of α -tubulin such that dynein-dynactin preferentially associates with tyrosinated MTs to promote the initiation of motility (McKenney et al., 2016; Nirschl et al., 2016; Peris et al., 2006). Since all of the motility parameters reported here were measured for dynein-dynactin walking on docetaxol-

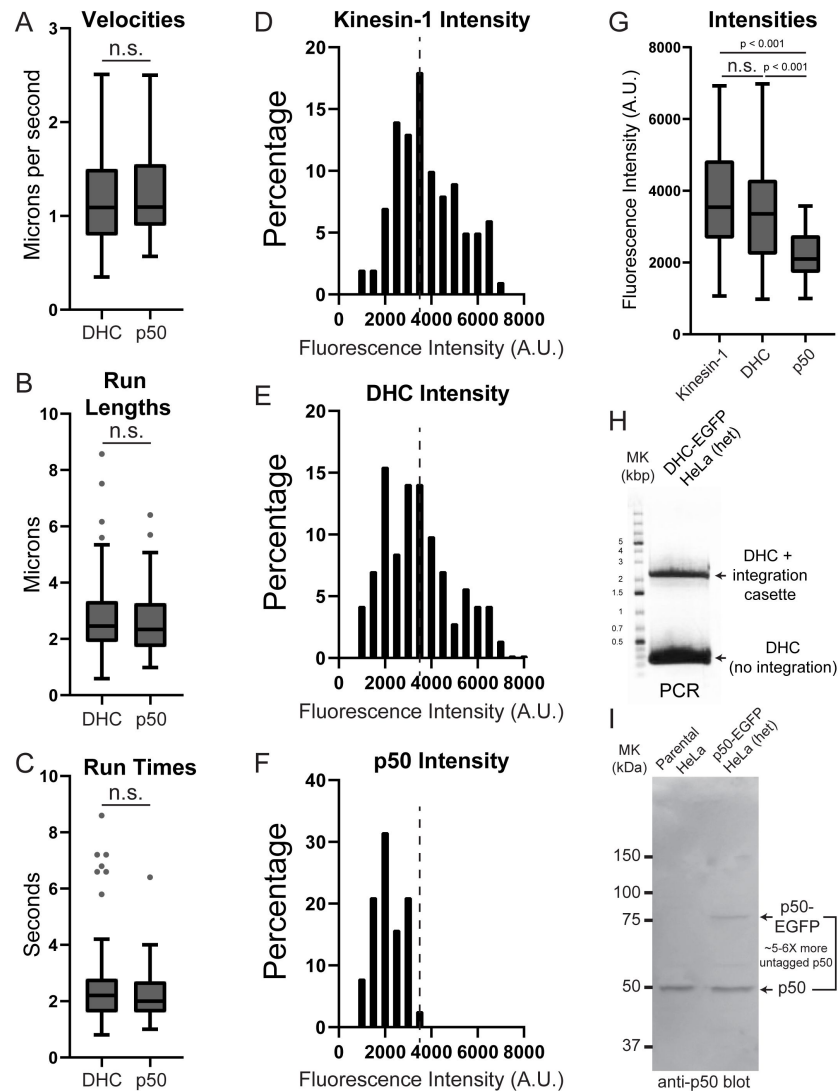


Figure 3.

Motile DHC and p50 puncta exhibit identical motility parameters but different fluorescent intensities. (A-C)

Box and whisker plots of DHC and p50 velocities (A), run lengths (B), and run times (C). (D-F) Distributions of kinesin-1-EGFP (D), DHC-EGFP (E), and p50-EGFP background corrected fluorescence intensities. The dashed line in each histogram denotes the mean value of the kinesin-1-EGFP data set. (G) Box and whisker plots of the kinesin-1, DHC, and p50 fluorescence intensities. (H) PCR of genomic DNA from the DHC-EGFP clone used in this study using PCR primers flanking the integration site of the repair cassette. The upper band was extracted and subjected to sequencing the results of which are shown in **Figure 1 – supplemental figure 1**. (I) Western blot for p50 of cell lysates from the parental HeLa cell line and the p50-EGFP clone used in this study. The tagged p50 runs ~30 kDa larger than the untagged p50 and is expressed at ~5-6-fold lower levels than the endogenous p50. In the box and whisker plots, whiskers indicate min-max, box is 25th-75th percentile, and bar is median. The reported p-values were determined by a randomization method: n.s. is not significant ($p > 0.05$).

stabilized MTs it is likely that the MT lattice was more detyrosinated than normal in our experimental conditions (Gundersen et al., 1987 [↗](#); Webster et al., 1990 [↗](#)). Thus, it would be informative to apply our high resolution TIRFM imaging of dynein-dynactin in cells where tubulin modifications – starting with the tyrosination state – can be experimentally manipulated (Barisic et al., 2015 [↗](#); Ferreira et al., 2020 [↗](#)).

Finally, our cell-based data are consistent with recent in vitro characterizations of dynein-dynactin structure, function, and regulation (Belyy et al., 2016 [↗](#); Chaaban and Carter, 2022 [↗](#); Urnavicius et al., 2018 [↗](#)). Based on our fluorescence intensity measurements, we favor the interpretation that the motile dynein-dynactin complexes visualized here are typically comprised of a single dynactin complex bound to a tandem array of two dyneins. Our measured dynein-dynactin velocity of 1.2 $\mu\text{m}/\text{sec}$ further supports this conclusion since this speed is consistent with in vitro velocities of dynein-dynactin complexes containing activating adaptors that recruit two dyneins although we do not exclude that the range of DHC velocities and intensities measured here may include sub-populations of dynein-dynactin containing a single dynein dimer. It would be valuable to test whether dynein-dynactin regulation mechanisms that have been characterized in vitro also apply to the physiological regulation of dynein-dynactin activity in living cells by measuring DHC motility parameters and intensities in cells depleted of different cargo adaptors. Ultimately, we hope that the direct visualization of human dynein-dynactin and quantification of its motility parameters and stoichiometries in living cells will be an important physiological complement to in vitro assays, which altogether will better inform mechanistic models of the many cellular processes that rely on dynein-dynactin.

Materials and methods

Cell culture

Parental HeLa and CRISPR-edited HeLa clones expressing DHC-EGFP or p50-EGFP were grown in standard DMEM medium (Gibco, USA) supplemented with 10% non-heat-inactivated FBS (Gibco, USA) and 0.5 $\times$ antibiotic/antimycotic cocktail (Sigma-Aldrich) and maintained at 37°C with 5% CO₂. Human-Kinesin-1-EGFP-expressing *Drosophila* S2 cells were grown in Schneider's medium (Life Technologies) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 0.5 $\times$ antibiotic/antimycotic cocktail (Sigma), and maintained at 25°C. Kinesin-1-EGFP expression was induced with 500 μM CuSO₄ for 16-18 hours.

CRISPR-engineered cell line production

CRISPR gene editing

FKBP-EGFP tags were added to the C-terminus of human dynein heavy chain (DHC) and p50 using methods described previously (Sheridan and Bentley, 2016 [↗](#); Stewart-Ornstein and Lahav, 2016 [↗](#)). In brief, repair cassettes comprised of FKBP-EGFP linked to a cleavable peptide (T2A) followed by a selectable marker (Neomycin) were cloned into pCMV and used for PCR reactions. Glycine-Alanine linkers were included between proteins in the construct. Guide sequences were selected using the CRISPR design tool (<http://crispr.mit.edu/> [↗](#)) from the Zhang lab at MIT (Ran et al., 2013 [↗](#)) using “other regions” and the human target genome (hg19). The search tool was used to select guides close to the C-terminus of the protein of interest (~100nt surrounding and including the stop codon). Top and bottom oligos were obtained for each guide with the bases 5'-CACC -3' added to the top oligo and 5'-AAAC-3' added to the complement of the bottom oligo.

Guides were cloned into a Cas9 containing plasmid (PX459) Addgene #62988, (Cambridge, MA) following methods previously outlined (Moyer and Holland, 2015 [↗](#)). Top and bottom oligos were annealed and then phosphorylated by T4 PNK (NEB, Ipswich, MA). Guides were then cloned into

PX459 that was cut using BbsI (NEB, Ipswich, MA) and ligated using T4 ligase (NEB Ipswich, MA). Guide-Cas9 containing plasmids were then sequenced using the U6 promotor primer (Ran et al., 2013) and purified using either endotoxin free mini-preps or midi-preps according to manufacturer protocol (Promega, Madison, WI). Repair cassettes were amplified using primers designed to be homologous to the C-terminal genomic DNA surrounding the STOP codon. In all cases, the guide target sequence was mutated to prevent Cas9 from recognizing the repair cassette.

Cells were grown in DMEM medium (ThermoFisher Scientific) with 10% Fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA) and 0.5X antibiotic/antimycotic solution (final concentrations 50 U/mL penicillin, 0.05 mg/mL streptomycin, 0.125 ug/mL amphotericin B; Sigma-Aldrich, St. Louis, MO) at 37 °C and 5% carbon dioxide (CO₂). For long term storage cells were frozen in DMEM medium with 5% FBS and 0.5X antibiotic/antimycotic solution and 15% DMSO and held at -80°C for 1-2 days before moving to liquid nitrogen.

To generate CRISPR modified cell lines, parental cells were nucleofected using an Amaxa Nucleofector (Lonza, Portsmouth, NH) program I-013 and Mirus nucleofection reagent (Mirus Bio LLC, Madison, WI) according to the manufacturer's recommendations. Plasmids and Repair cassettes were used at ratio of 1:1 at a concentration of 1 µg DNA each. Following nucleofection, cells were grown in regular growth media in 100mm dishes for 48-72 Hrs. and then 0.2g/L Neomycin/G418 (InvivoGen, San Diego, CA) selection media was added. Media was then changed daily for 10-14 days and colonies of green cells were picked using cloning rings and returned to regular media for further screening and experiments.

Genotyping

Genomic DNA was isolated from clonal CRISPR tagged cells using Genomic DNA Mini Kit (Invitrogen) according to manufacturer's recommendations. DNA was then amplified by PCR using genomic primers targeting the Neomycin cassette- and downstream of the Stop codon. Phusion polymerase (NEB) was used to amplify 1 µL of isolated genomic DNA in a 50 µL reaction for 30 cycles. The resulting PCR products were analyzed using 0.8% agarose gel electrophoresis. The expected band was excised, and gel extracted using Qiagen gel extraction kit and sequenced to verify proper integration of the tag.

Live-cell TIRF microscopy (TIRFM)

Cells were seeded onto 35 mm glass bottom petri dishes (Cellvis) 2-3 days prior to imaging. With the exception of the visualization of tip-tracking DHC-EGFP, cells were incubated in MEDIA supplemented with 1 µM SiR-Tubulin (Cytoskeleton, Inc) for 30-60 minutes prior to washing out the SiR-Tubulin with fresh DMEM before imaging. The cells were visualized on a Nikon Ti-E microscope equipped with a TIRF illuminator, a 100X 1.49-NA differential interference contrast TIRF Apochromat oil-immersion objective, Nikon perfect focus system (PFS), a Hamamatsu ORCA-Flash 4.0 LT digital complementary metal-oxide semiconductor camera (C11440), four laser lines (447, 488, 561, and 641 nm), and MetaMorph software (Molecular Devices). Flat interphase cells were identified and the initial focal plane was set by focusing on the far-red SiR tubulin signal with a particular emphasis on finding cells in which individual microtubules could be seen between the nucleus and the glass-adhered plasma membrane. Individual snap shots were then taken on the EGFP channel to refine the focal plane on the DHC or p50 puncta. Once individual puncta were well-resolved the PFS was engaged and the cell was subjected to streaming TIRFM for 12 seconds at an acquisition rate of 5 frames per second (200 ms exposure) and 2×2 camera binning.

Quantifications of motility parameters and fluorescence intensities

High temporal (5 fps) TIRFM time-lapses were examined by eye to identify evident motile puncta, the paths of which were traced manually in MetaMorph using the Multi-Line drawing function. A kymograph of the line segment with a 5-pixel line width was then generated and the velocity was determined by measuring the slope of the motility event on the kymograph from start to end using the Single Line drawing function. The run time was measured by defining the start and end frame of evident movement in the TIRFM time-lapse and the run length was then calculated by multiplying the run time by the velocity measured in the kymograph of the motility event. The run times and run lengths are likely underestimates of what dynein-dynactin can achieve due to the fact that the puncta sometimes bleached or exited the TIRFM evanescent field.

The number of EGFP molecules per motile puncta of DHC and p50 was determined by comparing their background-corrected fluorescence intensities to that of a known dimer standard: human kinesin-1 tagged with EGFP. As previously described (Ye et al., 2018 [DOI](#)) *Drosophila* S2 cells expressing inducible human Kinesin-1 (in this case tagged with EGFP) were induced with 500 μ M CuSO₄ for 16 hours to induce expression. The next day, the induced S2 cells were seeded onto a concanavalin A coated glass bottom petri dish and allowed to adhere for ~1 hour prior to visualization by TIRFM. The DHC-EGFP and p50-EGFP HeLa clones and the human Kinesin-1-EGFP-expressing S2 cells were each visualized sequentially via live-cell TIRF microscopy using identical imaging parameters (5 fps acquisition rate, 200 ms exposure, 2X2 binning) within the same region of the camera chip. The region-in-region method (Ye and Maresca, 2018 [DOI](#)) was then used to background correct and quantify the integrated fluorescence intensities of motile puncta of DHC-, p50-, and kinesin-1-EGFP in the same TIRFM time-lapses that were used to measure the motility parameters. The intensity of each motile puncta was measured for a single time-point from each run in which the spot was most clearly resolved and did not have significant local background signal from nearby EGFP puncta.

Western blotting

Twenty μ g of total protein from cell lysates prepared from parental HeLa cells or the p50-EGFP clone were loaded onto a 10% SDS-PAGE gel, run out, and transferred to a nitrocellulose membrane on the Trans-Blot® Turbo™ transfer system (Bio-Rad Laboratories) using the preprogrammed “HIGH MW” 10-minute protocol. After blocking for 1 hour in TBS with 0.1% Tween and 5% milk, the membrane was incubated overnight at 4°C with mouse anti-dynactin p50 (BD Transduction Laboratories, Cat. # 611002) diluted at 1:1000 in the block. The membrane was washed 3X 5 minutes in TBS + 0.1% Tween and then incubated for 1 hour in milk containing donkey-anti-mouse IgG secondary antibodies conjugated with HRP (Jackson ImmunoResearch Laboratories, Inc.) diluted at 1:5000 in block. Following 3X 5 minutes washes in TBS + 0.1% Tween the blot was incubated with Immobilon Western Chemiluminescent HRP Substrate (Millipore) according to the manufacturer’s recommendations. Imaging of the membrane was done on a G:Box system controlled by GeneSnap software (Syngene).

Statistical analyses

Statistical analyses reporting p-values from two-tailed Student’s t-tests were done in excel while p-values generated with the randomization method were done using the PlotsofDifferences web tool at: <https://huygens.science.uva.nl/PlotsOfDifferences> [DOI](#) (Goedhart, 2019 [DOI](#)). PlotsofDifferences does not rely on assumptions about the distribution of the data (normal versus non-normal) to calculate p-values.

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Conflict of interest

The authors declare no competing financial interests.

Supplemental materials

Supplemental Figures

Supplemental Videos

Video 1. Spinning disk confocal time-lapse of a DHC-EGFP CRISPR-engineered interphase HeLa cell. Zoomed views of the boxed regions in the upper left panel are shown in the right panels. Displayed times are min:sec. Scale bars, 10 μm (upper left panel) and 1 μm (right panels). Relates to **Fig. 1A** [↗](#).

Video 2. TIRFM time-lapse of a DHC-EGFP CRISPR-engineered interphase HeLa cell. Displayed times are min:sec. Scale bar, 10 μm . Relates to **Fig. 1B** [↗](#).

Video 3. TIRFM time-lapse of a DHC-EGFP (green) CRISPR-engineered interphase HeLa cell treated with SiR-Tubulin (magenta). Displayed times are min:sec. Scale bar, 10 μm . Relates to **Fig. 1C** [↗](#).

Video 4. High temporal-resolution (5 frames per second) TIRFM time-lapse of a DHC-EGFP CRISPR-engineered interphase HeLa cell treated with SiR-Tubulin. The boxed regions highlight motile DHC puncta. Displayed times are sec:millisec. Scale bar, 1 μm . Relates to **Fig. 1C** [↗](#).

Video 5. Spinning disk confocal time-lapse of a p50-EGFP CRISPR-engineered interphase HeLa cell. Displayed times are min:sec. Scale bar, 10 μm . Relates to **Fig. 2A** [↗](#).

Video 6. TIRFM time-lapse of a p50-EGFP CRISPR-engineered interphase HeLa cell. Displayed times are min:sec. Scale bar, 10 μm . Relates to **Fig. 2B** [↗](#).

Video 7. TIRFM time-lapse of a p50-EGFP (green) CRISPR-engineered interphase HeLa cell treated with SiR-Tubulin (magenta). Displayed times are min:sec. Scale bar, 10 μm . Relates to **Fig. 2C** [↗](#).

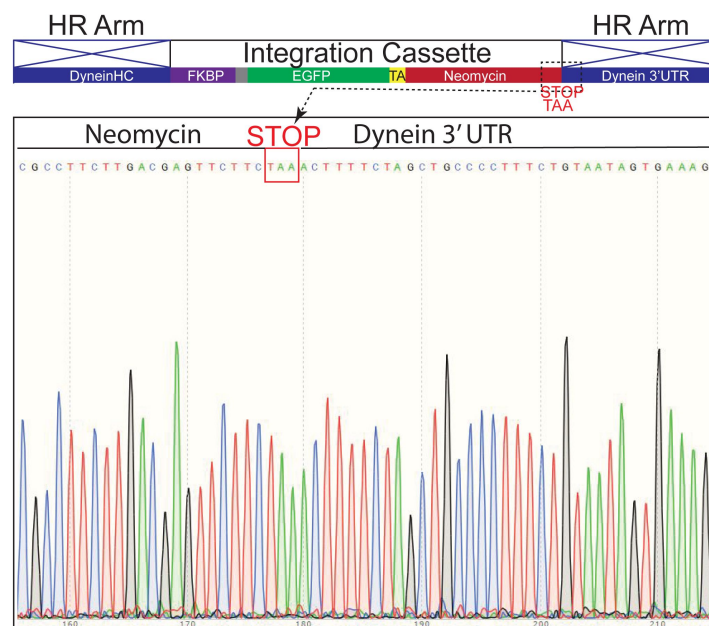


Figure 1 – figure supplement 1.

Repair cassette for generating the DHC-EGFP CRISPR-engineered HeLa cell line with sequencing trace showing its integration at the dynein heavy chain (DYNCH1) gene locus.

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Editors

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Reviewer #1 (Public Review):

Summary:

The manuscript by Verma et al. is a simple and concise assessment of the in-cell motility parameters of cytoplasmic dynein. Although numerous studies have focused on understanding the mechanism by which dynein is activated using a complement of in vitro methodologies, an assessment of dynein motility in cells has been lacking. It has been unclear whether dynein exhibits high processivity within the crowded and complicated environment of the cell. For example, does cargo-bound dynein exhibit short, non-processive motility (as has been recently suggested; Tirumala et al., 2022 bioRxiv)? Does cargo-bound dynein move against opposing forces generated by cargo-bound kinesins? Do cargoes exhibit bidirectional switching due to stochastic activation of kinesins and dyneins? The current work addresses these questions quite simply by observing and quantitating the motility of natively tagged dynein in HeLa cells.

Strengths:

The work uses a simple and straightforward approach to address the question at hand: is dynein a processive motor in cells? Using a combination of TIRF and spinning disc confocal microscopy, the authors provide a clear and unambiguous answer to this question.

Weaknesses:

My only significant concern (which is quite minor) is that the authors focus their analysis on dynein movement in cells treated with docetaxol, which could potentially affect the observed behavior. However, this is likely necessary, as without it, motility would not have been observed due to the 'messiness' of dynein localization in a typical cell (e.g., plus end-tracking in addition to cargo transport).

<https://doi.org/10.7554/eLife.94963.1.sa2>

Reviewer #2 (Public Review):

Summary:

Verma et al. provide a short technical report showing that endogenously tagged dynein and dynactin molecules localize to growing microtubule plus-ends and also move processively along microtubules in cells. The data are convincing, and the imaging and movies very nicely demonstrate their claims. I don't have any large technical concerns about the work. It is perhaps not surprising that dynein-dynactin complexes behave this way in cells due to other reports on the topic, but the current data are among some of the nicest direct demonstrations of this phenomenon. It may be somewhat controversial since a separate group has reported that dynein does not move processively in mammalian cells (<https://www.biorxiv.org/content/10.1101/2021.04.05.438428v3>). Because of this, it might be nice for the authors to comment on this discrepancy in the field, although the aforementioned work is still in pre-print form.

Strengths:

Using state-of-the-art methods to endogenously tag dynein/dynactin subunits and performing

live-cell imaging is convincing and useful for the field.

Weaknesses:

The claims are perhaps not surprising or novel given the extensive data already published in the field. However, there aren't many similar studies using endogenously tagged subunits to date.

<https://doi.org/10.7554/eLife.94963.1.sa1>

Reviewer #3 (Public Review):

Summary:

In this manuscript, Verma et al. set out to visualize cytoplasmic dynein in living cells and describe their behaviour. They first generated heterozygous CRISPR-Cas9 knock-ins of DHC1 and p50 subunit of dynactin and used spinning disk confocal microscopy and TIRF microscopy to visualize these EGFP-tagged molecules. They describe robust localization and movement of DHC and p50 at the plus tips of MTs, which was abrogated using SiR tubulin to visualize the pool of DHC and p50 on the MTs. These DHC and p50 punctae on the MTs showed similar, highly processive movement on MTs. Based on comparison to inducible EGFP-tagged kinesin-1 intensity in *Drosophila* S2 cells, the authors concluded that the DHC and p50 punctae visualized represented 1 DHC-EGFP dimer+1 untagged DHC dimer and 1 p50-EGFP+3 untagged p50 molecules.

Strengths:

The idea and motivation behind this work are commendable.

Weaknesses:

There are several major issues with the characterization of the knock-in lines generated, the choice of imaging and analysis methods, and inadequate discussion of prior findings.

The specific points are below:

1. CRISPR-edited HeLa clones:

- (i) The authors indicate that both the DHC-EGFP and p50-EGFP lines are heterozygous and that the level of DHC-EGFP was not measured due to technical difficulties. However, quantification of the relative amounts of untagged and tagged DHC needs to be performed - either using Western blot, immunofluorescence or qPCR comparing the parent cell line and the cell lines used in this work.
- (ii) The localization of DHC predominantly at the plus tips (Fig. 1A) is at odds with other work where endogenous or close-to-endogenous levels of DHC were visualized in HeLa cells and other non-polarized cells like HEK293, A-431 and U-251MG (e.g.: OpenCell (<https://opencell.czbiohub.org/target/CID001880>), Human Protein Atlas (<https://www.proteinatlas.org/ENSG00000197102-DYNC1H1/subcellular#human>), <https://www.biorxiv.org/content/10.1101/2021.04.05.438428v3>). The authors should perform immunofluorescence of DHC in the parental cells and DHC-EGFP cells to confirm there are no expression artifacts in the latter. Additionally, a comparison of the colocalization of DHC with EB1 in the parental and DHC-EGFP and p50-EGFP lines would be good to confirm MT plus-tip localisation of DHC in both lines.
- (iii) It would also be useful to see entire fields of view of cells expressing DHC-EGFP and p50-EGFP (e.g. in Spinning Disk microscopy) to understand if there is heterogeneity in expression. Similarly, it would be useful to report the relative levels of expression of EGFP (by measuring the total intensity of EGFP fluorescence per cell) in those cells employed for the analysis in the manuscript.
- (iv) Given that the authors suspect there is differential gene regulation in their CRISPR-edited lines, it cannot be concluded that the DHC-EGFP and p50-EGFP punctae tracked are functional

and not piggybacking on untagged proteins. The authors could use the FKBP part of the FKBP-EGFP tag to perform knock-sideways of the DHC and p50 to the plasma membrane and confirm abrogation of dynein activity by visualizing known dynein targets such as the Golgi (Golgi should disperse following recruitment of EGFP-tagged DHC-EGFP or p50-EGFP to the PM), or EGF (movement towards the cell center should cease).

2. TIRFM and analysis:

- (i) What was the rationale for using TIRFM given its limitation of visualization at/near the plasma membrane? Are the authors confident they are in TIRF mode and not HILO, which would fit with the representative images shown in the manuscript?
- (ii) At what depth are the authors imaging DHC-EGFP and p50-EGFP?
- (iii) The authors rely on manual inspection of tracks before analyzing them in kymographs - this is not rigorous and is prone to bias. They should instead track the molecules using single particle tracking tools (eg. TrackMate/uTrack), and use these traces to then quantify the displacement, velocity, and run-time.
- (iv) It is unclear how the tracks that were eventually used in the quantification were chosen. Are they representative of the kind of movements seen? Kymographs of dynein movement along an entire MT/cell needs to be shown and all punctae that appear on MTs need to be tracked, and their movement quantified.
- (v) What is the directionality of the moving punctae?
- (vi) Since all the quantification was performed on SiR tubulin-treated cells, it is unclear if the behavior of dynein observed here reflects the behavior of dynein in untreated cells. Analysis of untreated cells is required.

3. Estimation of stoichiometry of DHC and p50

Given that the punctae of DHC-EGFP and p50 seemingly bleach on MT before the end of the movie, the authors should use photobleaching to estimate the number of molecules in their punctae, either by simple counting the number of bleaching steps or by measuring single-step sizes and estimating the number of molecules from the intensity of punctae in the first frame.

4. Discussion of prior literature

Recent work visualizing the behavior of dyneins in HeLa cells (DOI: 10.1101/2021.04.05.438428), which shows results that do not align with observations in this manuscript, has not been discussed. These contradictory findings need to be discussed, and a more objective assessment of the literature in general needs to be undertaken.

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