

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

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In this **valuable** technical report, Verma et al. provide **convincing** evidence that endogenously tagged dynein and dynactin form processive motor complexes that move along microtubules in living cells. Using quantitative fluorescence microscopy, they directly compare the stoichiometry and motility of these complexes to kinesin-1, revealing distinct transport behaviors and regulatory properties. This study offers key methodological and conceptual advance for understanding the dynamics of native motor proteins within the cellular environment and will be of interest to the cell biology community.

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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 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*.

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 tends to favor single dynein and the BICDR1 and HOOK3 tend to favor two dyneins assembled into 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 vitro, the crowded three-dimensional intracellular environment poses a challenge for imaging and quantifying motility events in living cells. Puncta exhibiting bidirectional movement on MTs and comet-like motile events that co-localized with EB1 were observed in human cells over-expressing a GFP-tagged dynein intermediate chain, supporting a role for dynein in MT plus-end 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 living cells. In doing so, 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 (**Figure 1-figure supplement 1 A**). 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 (**Figure 1-figure supplement 1B**). 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; Rusan et al., 2001; Walker et al., 1988) were readily observed by both spinning disc confocal microscopy and total internal reflection fluorescence microscopy (TIRFM) (**Figure 1A, B**, **Videos 1 and 2**). SiR-Tubulin was next introduced to visualize DHC localization and behaviors relative to MTs (**Figure 1C**). Interestingly, because SiR-Tubulin is a docetaxel 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 (**Figure 1C**). The SiR-Tubulin-treated cells were subjected to two-color TIRFM and DHC-EGFP puncta were clearly observed streaming on SiR-Tubulin labeled MTs, which was especially evident 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) (**Figure 1D**, **Video 4**). The TIRFM time-lapses were assessed by eye and several parameters of the motile puncta were measured using kymographs (**Figure 1E**). 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 (**Figure 1F-H**). 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 ($\sim 3\%$ of runs).

Activation of dynein motility requires its interaction with the dynactin complex (Chowdhury et al., 2015; Grotjahn et al., 2018; Urnavicius et al., 2018; Zhang et al., 2017).

CRISPR/Cas9-based genomic engineering was used to insert the cassette encoding FKBP and EGFP tags at the 3' end of the p50/dynactin gene and a clonal p50-EGFP-expressing HeLa cell line was subjected to live-cell fluorescence imaging to visualize dynactin in interphase cells (**Figure 2 - figure supplement 1 A-C**). Consistent with prior observations of dynactin localization (Vaughan et al., 1999; Vaughan et al., 2002) 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 (**Figure 2A, B**; **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 (**Figure 2C**, **Videos 7 and 8**). 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 (**Figure 2D**). The mean velocity of the p50 puncta was $1.2 \pm 0.07 \mu\text{m}/\text{sec}$ (**Figure 2E**) while the mean run length was $2.6 \pm 0.2 \mu\text{m}$ (**Figure 2F**) and the mean run time was 2.2 ± 0.2 seconds (**Figure 2G**). 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 (**Figure 3A**), run lengths (**Figure 3B**), and run times (**Figure 3C**) 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 motile EGFP-tagged kinesin-1 dimers (**Figure 3 - figure supplement 1 A-C**) as a standard to assess the stoichiometries of the dynein-dynactin complexes. The intensities of motile DHC and p50 puncta were compared to kinesin-1-EGFP expressed in either *Drosophila melanogaster* S2 cells (**Figure 3 -**

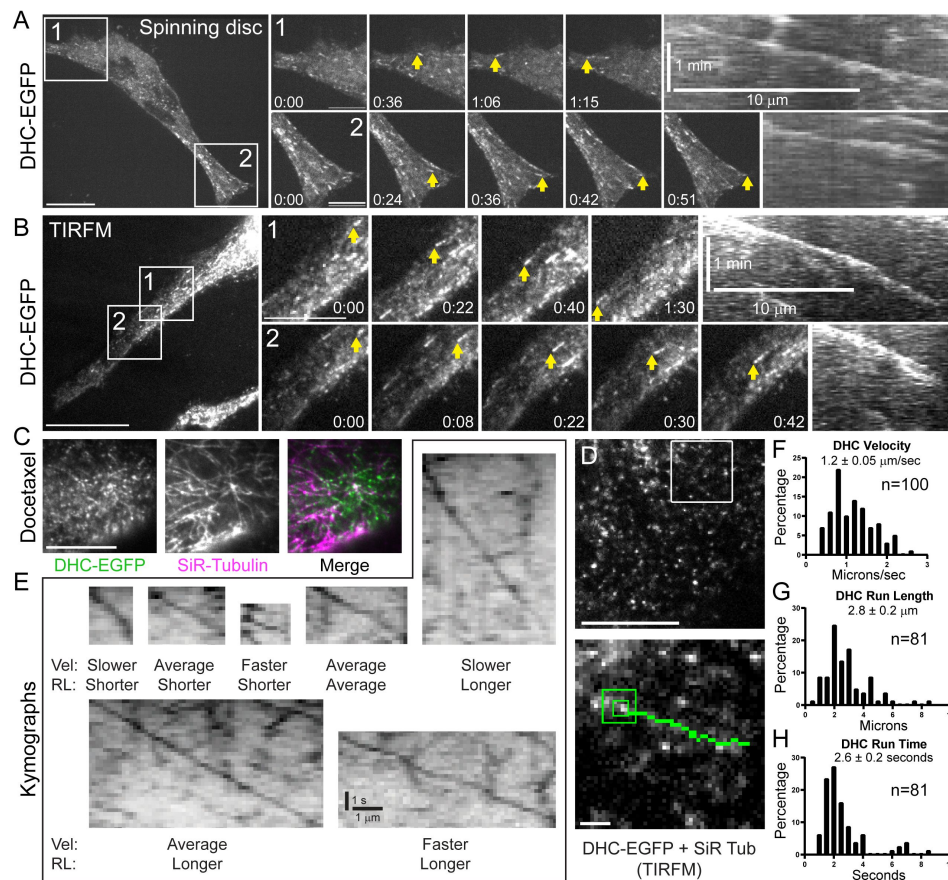


Figure 1.

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 μm (A-D); 1 μm (all insets), 10 μm (horizontal); 1 min (vertical) in the kymographs in A and B; and 1 μm (horizontal); 1 second (vertical) in E. Displayed times are min:sec.

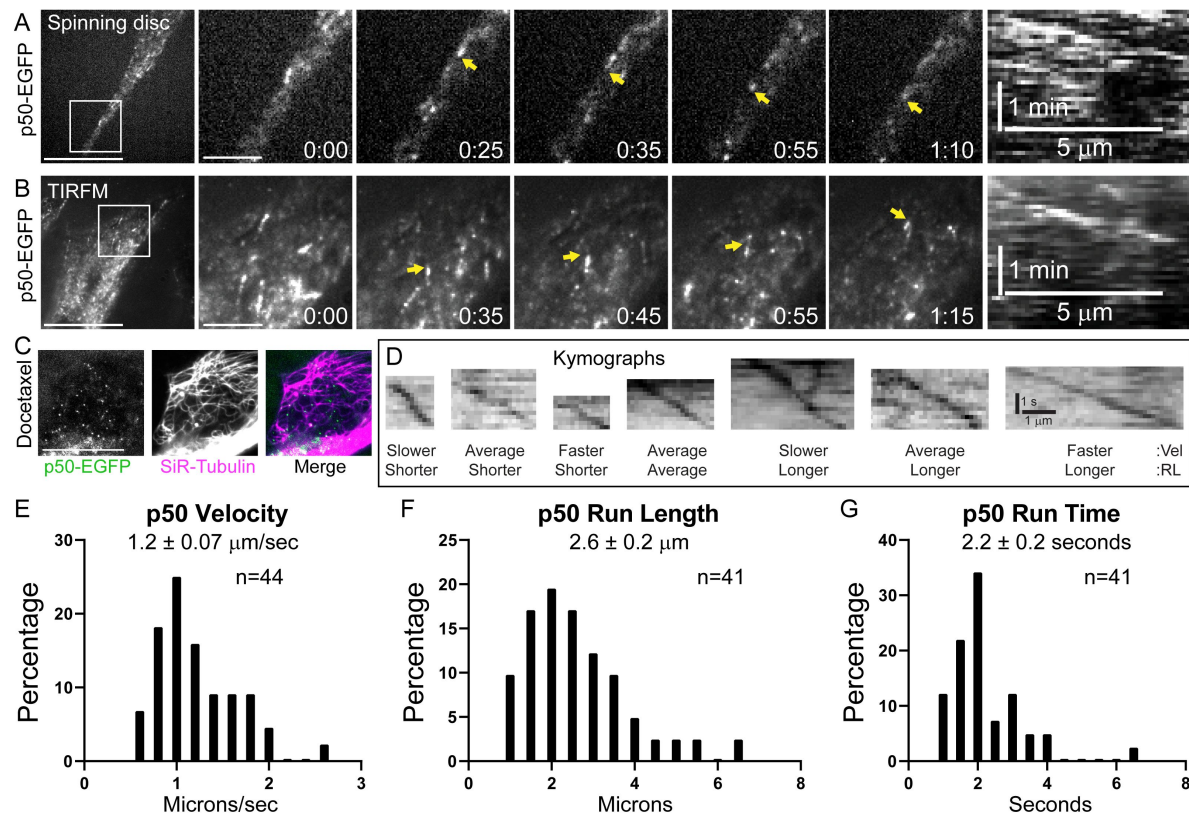


Figure 2.

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 μm (A-C); 1 μm (all insets), 5 μm (horizontal); 1 min (vertical) in the kymographs in A and B; and 1 μm (horizontal); 1 second (vertical) in D. Displayed times are min:sec.

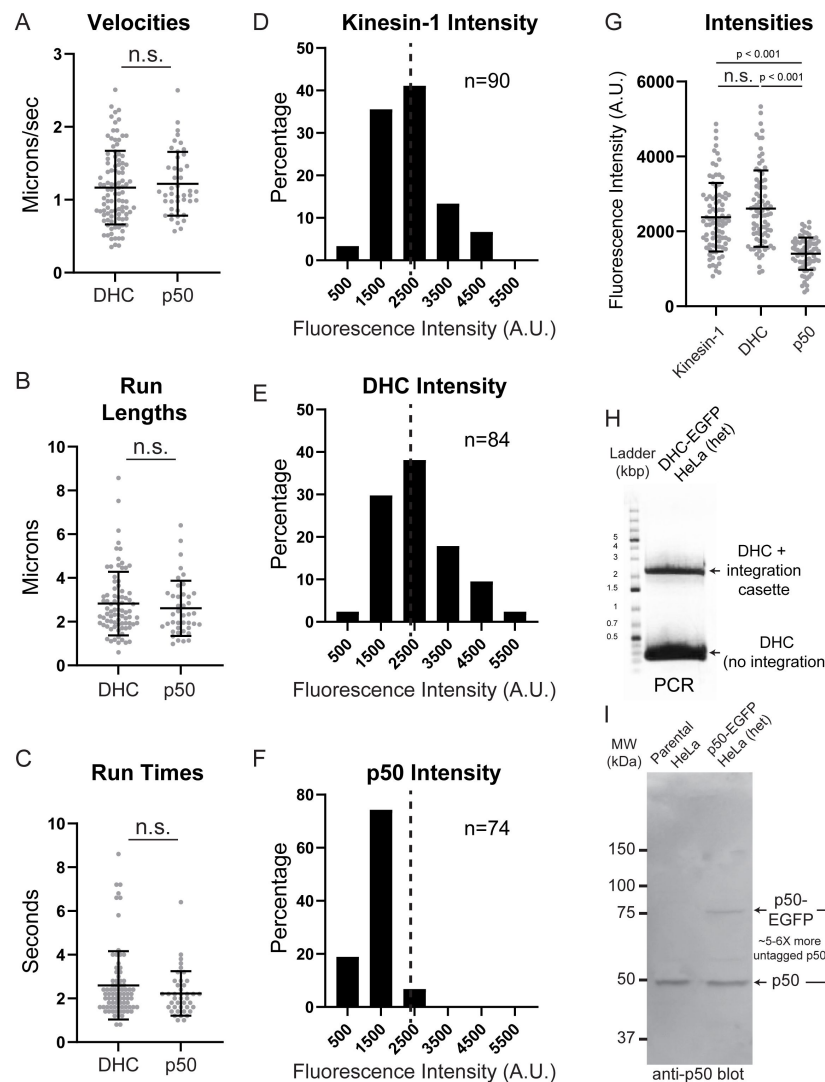


Figure 3.

Motile DHC and p50 puncta exhibit identical motility parameters but different fluorescent intensities.

(A-C) Scatter plots of (A) velocities (DHC, $n = 100$; p50, $n = 44$), (B) run lengths (DHC, $n = 81$; p50, $n = 41$), and (C) run times (DHC, $n = 81$; p50, $n = 41$). (D-F) Distributions of the background corrected fluorescence intensities of motile puncta of (D) kinesin-1-EGFP transiently expressed in HeLa cells, (E) DHC-EGFP, and (F) p50-EGFP. The dashed line in each histogram denotes the mean value of the kinesin-1-EGFP data set. (G) Scatter plots of the kinesin-1, DHC, and p50 fluorescence intensities (kinesin-1, $n = 90$ puncta; DHC, $n = 84$ puncta; p50, $n = 74$ puncta). (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 A. (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. Error bars are mean values $\pm$ standard deviations. The reported p-values were determined by a randomization method: n.s. is not significant ($p > 0.05$).

figure supplement 2 A-C or HeLa cells (**Figure 3 D-F**). 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 (**Figure 3G**, **Figure 3 - figure supplement 2D**). Thus, the motile dynein and dynactin complexes visualized here via high-speed TIRFM were comprised, on average, of two EGFP-tagged 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 (**Figure 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 contain two DHC dimers. We were unable to assess the relative expression levels of tagged versus untagged DHC by western blot due to the very large size of DHC (>500 kDa) relative to the EGFP tag. However, we can 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 (**Figure 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 only 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., 2024; 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. In human interphase cells, two distinct motile populations of dynein-dynactin are observed: one that tip-tracks on polymerizing microtubule (MT) plus-ends, and another that moves processively along MTs at high velocities - approximately seven times faster than the tip-tracking population. The tip-tracking pool may be regulated in a cell cycle-dependent manner, as dynein-dynactin tracking events appear less robust in mitotic cells compared to interphase cells. We propose that the ~3 μm run lengths measured for the motile pool likely underestimate the true processivity of dynein-dynactin. This is due to the relatively short and dynamic MT tracks available in interphase HeLa cells, and the fact that motile puncta could only be visualized while within the TIRF field and prior to photobleaching. Indeed, dynein was recently shown to be highly processive in iNeurons, with a mean run length of ~35 μm and some runs exceeding 100 μm (Fellows et al., 2024). In contrast, a separate study (Tirumala et al., 2024) reported that dynein is not highly processive, typically exhibiting runs of very short duration (~0.6 s) in HeLa cells. A notable technical difference that may account for this discrepancy is that our study visualizes endogenously tagged human DHC, whereas Tirumala et al. characterized over-expressed mouse DHC in HeLa cells. Over-expression of the DHC may result in an imbalance of the subunits that comprise the active motor complex, leading to inactive, or less active complexes. Similarly, mouse DHC may not have the ability to efficiently assemble into active and processive dynein-dynactin-adaptor complexes to the same extent as human DHC. Two-color imaging of SiR-Tubulin-labeled MTs and dynein-dynactin revealed a population of p50-EGFP and DHC-EGFP puncta not evidently associated with MTs, suggesting the presence of a cortical pool of dynein-dynactin. Cortical dynein-dynactin has been clearly visualized in mitotic mammalian cells, where it functions in spindle positioning (Busson et al., 1998; Collins et al., 2012; Faulkner et al., 2000; Kiyomitsu and Cheeseman, 2012; Kobayashi and Murayama, 2009). In interphase mammalian cells, dynein-dynactin also contributes to centrosome positioning (Etienne-Manneville and Hall, 2001; Palazzo et al., 2001), and has been proposed to do so via pulling forces exerted on centrosomal MTs by a cortical pool (Burakov et al., 2003; Dujardin and Vallee, 2002; Gundersen, 2002) that we

likely visualized by TIRFM. While a cortical pool of dynein-dynactin may exhibit lateral diffusion at the plasma membrane, it would, by necessity, be considerably less motile than conventionally tip-tracking or running dynein-dynactin complexes.

The dynein-dynactin pool that exhibited directional motility on MTs was capable of switching MT tracks, which is a known behavior of dynein-dynactin (Ross et al., 2008 [↗](#)), as evidenced by sharp changes in direction sometimes at near perpendicular angles. Dynein-dynactin rarely (~3% of motile puncta) 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, suggest that there was not significant resistive drag from associated kinesin motors as has been observed in vitro. The velocity and low switching frequency of motile puncta suggest that any kinesin motors associated with cargos being transported by the dynein-dynactin visualized here 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., 2024 [↗](#)) 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 [↗](#)).

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 tend to recruit two dyneins. However, we do not exclude that the range of DHC velocities and intensities measured here may also include sub-populations of complexes 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, the direct visualization of human dynein-dynactin and quantification of its motility parameters and stoichiometries in living cells should be an important physiological complement to in vitro assays, which altogether will better inform mechanistic models of the cellular processes that rely on dynein-dynactin function.

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 in S2 cells was induced with 500 μM CuSO₄ for 16-18 hours prior to imaging.

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 [DOI](#); Stewart-Ornstein and Lahav, 2016 [DOI](#)). 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/> [DOI](#)) from the Zhang lab at MIT (Ran et al., 2013 [DOI](#)) 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 [DOI](#)). 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 [DOI](#)) 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 µg/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 larger band was excised, and gel extracted using Qiagen gel extraction kit and sequenced to verify proper integration of the tag.

Live-cell microscopy

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 μ M 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, Borealis (Andor) retrofitted CSU-10 (Yokogawa) spinning disc head, a 100X 1.49-NA differential interference contrast TIRF Apochromat oil-immersion objective, Nikon perfect focus system (PFS), two ORCA-Flash4.0 LT Digital CMOS cameras (Hamamatsu), four laser lines (447, 488, 561, and 641 nm) in a dual-output laser launch system (Andor), and MetaMorph software (Molecular Devices). The imaging mode (spinning disc versus TIRFM) of the system is set by selecting the appropriate fiber optic output and corresponding CMOS camera. For TIRFM imaging, 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 $\times$ 2 camera binning. The population of dynein-dynactin on MTs positioned between the plasma membrane and nucleus would fall within the 100-200 nm excitable range of the evanescent wave produced by TIRF since the plasma membrane is typically $\sim$ 5 nm and a MT is $\sim$ 25 nm. While TIRFM is capable of effectively visualizing dynein-dynactin motility in living cells, in some time-lapses the excitation laser may have been angled to propagate as a highly inclined and laminated optical sheet (HILO) (Tokunaga et al., 2008 [DOI](#)). The experimental design and analyses would be unaffected by whether the motile punta were visualized via TIRFM or HILO microscopy. For consistency, the imaging mode employed in this study is referred to as TIRFM.

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 in both *Drosophila* S2 cells and in HeLa cells. 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 $\sim$ 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, 2 $\times$ 2 binning) within the same region of the camera chip.

To compare DHC-EGFP and p50-EGFP intensities to human kinesin-1-EGFP in HeLa cells, HeLa cells were seeded in a 35 mm dish in 2 mL of complete HeLa medium (DMEM supplemented with 10% FBS and 0.5X antibiotic-antimycotic mix) 18–24 hours prior to transfection at an initial density (0.25×10^6) to achieve ~50% confluency on the day of transfection. On the day of transfection, two sterile 1.5 mL microcentrifuge tubes were prepared and labeled A and B. Each tube received 125 μ L of OPTI-MEM medium. Tube A was supplemented with 7.5 μ L of Lipofectamine 3000 reagent and vortexed briefly. Tube B was supplemented with 250 ng of endotoxin-free plasmid pB80-hsKIF5B(1-560)-L-GFP (human kinesin-1-EGFP) (Addgene plasmid # 193716) and 4 μ L of P3000 reagent, followed by brief vortexing. The contents of tube B were then transferred to tube A, vortexed gently, and incubated at room temperature for 15 minutes to allow complex formation. The resulting DNA-lipid complexes were added dropwise to the HeLa cells, which were then incubated at 37°C in a humidified CO₂ incubator for 24 hours. After incubation, the medium was replaced with 2 mL of fresh HeLa medium, and cells were subjected to imaging over the next 1–2 days (2–3 days post transfection). The kinesin-1-EGFP transfected cells, and DHC-EGFP and p50-EGFP HeLa clones were each visualized sequentially on the same day via live-cell TIRF microscopy using identical imaging parameters within the same region of the camera chip. The region-in-region method (Ye and Maresca, 2018 [\[18\]](#)) was used to background correct and quantify the integrated fluorescence intensities of motile puncta of DHC-, p50-, and kinesin-1-EGFP (for both the S2 cell and HeLa cell comparisons). 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. Cytoplasmic levels of DHC-EGFP and p50-EGFP were quantified from max projections of spinning disk confocal Z-stacks by subtracting the integrated intensity of a 25×25 pixel square ROI in the nucleus from the integrated intensity of a 25×25 pixel ROI placed in a representative region of the cytoplasm.

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

Reported p-values were generated with a randomization method using the PlotsofDifferences web tool at: <https://huygens.science.uva.nl/PlotsOfDifferences> [\[19\]](#) (Goedhart, 2019 [\[19\]](#)). PlotsofDifferences does not rely on assumptions about the distribution of the data (normal versus non-normal) to calculate p-values.

Data availability

Source data used for generating the figures can be provided.

Acknowledgements

We are grateful to Barbara Mann for generating the CRISPR-engineered HeLa cell lines. Thank you to Thomas Laskarzewski for help with R and data visualization. pB80-hsKIF5B(1-560)-L-GFP was a gift from Lukas Kapitein (Addgene plasmid # 193716; <http://n2t.net/addgene:193716>; [RRID:Addgene_193716](https://doi.org/10.12555/RRID:Addgene_193716)). This work was supported by NIH grants (GM107026 and GM156188) to T.J.M. and by an NSF grant (MCB1817926) to P.W.

Additional files

Figure 1 - figure supplement 1. (A) Repair cassette for generating the DHC-EGFP CRISPR-engineered HeLa cell line with sequencing trace showing its integration at the dynein heavy chain (DYNC1H1) gene locus. **(B)** Montage of representative fields of view of the CRISPR-engineered DHC-EGFP cell line used in this study. Expression levels are consistent across the population and do not exhibit significant cell-to-cell variation. Scale bar, 20 μ m. [↗](#)

Figure 2 - figure supplement 1. (A) Montage of representative fields of view of the CRISPR-engineered p50-EGFP clone in comparison to the montage of DHC-EGFP fields from Figure 1 - figure supplement 1B. For the purpose of comparison, the two cell lines were visualized with identical imaging parameters on the same day and displayed identically in the figure. **(B)** Scatter plots of background corrected fluorescence intensities of cytoplasmic pools of DHC-EGFP ($n = 31$ cells) and p50-EGFP ($n = 13$ cells) from (A). **(C)** Montage of fields of view p50-EGFP clonal cells visualized with longer exposure times (500 ms) than in (A). Error bars are mean values $\pm$ standard deviations. Scale bars, 20 μ m. [↗](#)

Figure 3 - figure supplement 1. (A) Representative kymographs of motile kinesin-1-EGFP puncta from transiently transfected HeLa cells. **(B)** Distribution of kinesin-1 velocities (mean $\pm$ SEM; $n = 76$ puncta). **(C)** Distribution of kinesin-1 run lengths (mean $\pm$ SEM; $n = 60$ puncta). Kinesin-1 expressed in HeLa cells exhibited a slower mean velocity and shorter mean run length than the HeLa dynein-dynactin. Scale bars, 1 μ m (horizontal); 1 second (vertical). [↗](#)

Figure 3 - figure supplement 2. Distributions of background corrected fluorescence of (A) kinesin-1-EGFP expressed in *Drosophila melanogaster* S2 cells, (B) DHC-EGFP, and (C) p50-EGFP. The dashed line in each histogram denotes the mean value of the kinesin-1-EGFP data set. **(D)** Box and whisker plots of the kinesin-1, DHC, and p50 fluorescence intensities (kinesin-1, $n = 100$ puncta; DHC, $n = 71$ puncta; p50, $n = 38$ puncta). The reported p -values were determined by a randomization method: n.s. is not significant ($p > 0.05$). [↗](#)

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 μ m (upper left panel) and 1 μ m (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 μ m. 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 μ m. 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 μ m. Relates to Fig. 1D. [↗](#)

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

Video 8. 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 μ m. [↗](#)

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Reviewer #1 (Public Review):

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.

<https://doi.org/10.7554/eLife.94963.2.sa3>

Reviewer #2 (Public Review):

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>).

<https://doi.org/10.7554/eLife.94963.2.sa2>

Reviewer #3 (Public Review):

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.

<https://doi.org/10.7554/eLife.94963.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

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.

Thank you for the recognition of the strength of our work

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).

You are exactly correct that this treatment was required to provided us a clear view of motile dynein and p50 puncta. One concern about the treatment that we had noted in our original submission was that the docetaxel derivative SiR tubulin could increase microtubule detirosination, which has been implicated in affecting the initiation of dynein-dynactin motility but not motility rates (doi: 10.15252/emboj.201593071). In response to a comment from reviewer 2 we investigated whether there was a significant increase in alpha-tubulin detirosination in our treatment conditions and found that there was not. We have removed the discussion of this possibility from the revised version. Please also see response to comments raised by reviewer 2.

Reviewer 1 (Recommendations for the authors):

Major points:

(1) The authors measured kinesin-1-GFP intensities in a different cell line (drosophila S2 cells) than what was used for the DHC and p50 measurements (HeLa cells). It is unclear if this provides a fair comparison given the cells provide different environments for the GFP. Although the differences may in fact be trivial, without somehow showing this is indeed a fair comparison, it should at least be noted as a caveat when interpreting relative intensity differences. Alternatively, the authors could compare DHC and p50 intensities to those measured from HeLa cells treated with taxol.

Thank you for this suggestion. We conducted new rounds of imaging with the DHCEGFP and p50-EGFP clones in conjunction with HeLa cells transiently expressing the human kinesin-1-EGFP and now present the datasets from the new experiments. Importantly, our new data was entirely consistent with the prior analyses as there was not a significant difference between the kinesin-1-EGFP dimer intensities and the DHC-EGFP puncta intensities and there was a statistically significant difference in the intensity of p50 puncta, which were approximately half the intensity of the kinesin-1 and DHC. We have moved the old data comparing the intensities in S2 cells expressing kinesin-1-EGFP to Figure 3 - figure supplement 2 A-D and the new HeLa cell data is now shown in Figure 3 D-G.

(2) Given the low number of observations (41-100 puncta), I think a scatter plot showing all data points would offer readers a more transparent means of viewing the single-molecule data presented in Figures 3A, B, C, and G. I also didn't see 'n' values for plots shown in Figure 3.

The box and whisker plots have now been replaced with scatter plots showing all data points. The accompanying 'n' values have been included in the figure 3 legend as well as the histograms in figures 1 and 2 that are represented in the comparative scatter plots.

(3) Given the authors have produced a body of work that challenges conclusions from another pre-print (Tirumala et al., 2022 bioRxiv) - specifically, that dynein is not processive in cells - I think it would be useful to include a short discussion about how their work challenges theirs. For example, one significant difference between the two experimental systems that may account for the different observations could simply be that the authors of the Tirumala study used a mouse DHC (in HeLa cells), which may not have the ability to assemble into active and processive dynein-dynactin-adaptor complexes.

Thank you for pointing this out! At the time we submitted our manuscript we were conflicted about citing a pre-print that had not been peer reviewed simply to point out the discrepancy. If we had done so at that time we would have proposed the exact potential technical issue that you have proposed here. However, at the time we felt it would be better for these issues to be addressed through the review process. Needless to say, we agree with your interpretation and now that the work is published (Tirumala et al. JCB, 2024) it is entirely appropriate to add a discussion on Tirumala et al. where contradictory observations were reported.

The following statement has been added to the manuscript:

"In contrast, a separate study (Tirumala et al., 2024) reported that dynein is not highly processive, typically exhibiting runs of very short duration (~0.6 s) in HeLa cells. A notable technical difference that may account for this discrepancy is that our study visualizes endogenously tagged human DHC, whereas Tirumala et al. characterized over-expressed mouse DHC in HeLa cells. Over-expression of the DHC may result in an imbalance of the subunits that comprise the active motor complex, leading to inactive, or less active complexes. Similarly, mouse DHC may not have the ability to efficiently assemble into active and processive dynein-dynactin-adaptor complexes to the same extent as human DHC."

Minor points:

(1) "Specifically, the adaptor BICD2 recruited a single dynein to dynactin while BICDR1 and HOOK3 supported assembly of a "double dynein" complex." It would be more accurate to say that dynein-dynactin complexes assembled with Bicd2 "tend to favor single dynein, and the Bicdr1 and Hook3 tend to favor two dyneins" since even Bicd2 can support assembly of 2 dynein-1 dynactin complexes (see Urnavicius et al, Nature 2018).

Thank you, the manuscript has been edited to reflect this point.

(2) "Human HeLa cells were engineered using CRISPR/Cas9 to insert a cassette encoding FKBP and EGFP tags in the frame at the 3' end of the dynein heavy chain (DYNC1H1) gene (SF1)." It is unclear to what "SF1" is referring.

SF1 is supplementary figure 1, which we have now clarified as being Figure 1 – figure supplement 1A.

(3) "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..." This sentence is strangely punctuated (the ";" is likely a typo?).

Thank you for pointing this out, the typo has been corrected and the sentence now reads:

“The SiR-Tubulin-treated cells were subjected to two-color TIRFM and DHC-EGFP puncta were clearly observed streaming on Sir-Tubulin labeled MTs, which was especially evident on MTs that were pinned between the nucleus and the plasma membrane (Video 3)”

(4) I am unfamiliar with the "MK" acronym shown above the molecular weight ladders in Figure 3H and I. Did the authors mean to use "MW" for molecular weight?

We intended this to mean MW and the typo has been corrected.

(5) "This suggests that the cargos, which we presume motile dynein-dynactin puncta are bound to, any kinesins..." This sentence is confusing as written. Did the authors mean "and kinesins"?

Agreed. We have changed this sentence to now read:

“The velocity and low switching frequency of motile puncta suggest that any kinesin motors associated with cargos being transported by the dynein-dynactin visualized here are inactive and/or cannot effectively bind the MT lattice during dynein-dynactin-mediated transport in interphase HeLa cells.”

Reviewer 2 (Recommendations for the authors):

(1) I am confused as to why the authors introduced an FKBP tag to the DHC and no explanation is given. Is it possible this tag induces artificial dimerization of the DHC?

FKBP was tagged to DHC for potential knock sideways experiments. Since the current cell line does not express the FKBP counterpart FRB, having FKBP alone in the cell line would not lead to artificial dimerization of DHC.

(2) The authors use a high concentration of SiR-tubulin (1 μ M) before washing it out. However, they observe strong effects on MT dynamics. The manufacturer states that concentrations below 100nM don't affect MT dynamics, so I am wondering why the authors are using such a high amount that leads to cellular phenotypes.

We would like to note that in our hands even 100 nM SiR-tubulin impacted MT dynamics if it was incubated for enough time to get a bright signal for imaging, which makes sense since drugs like docetaxel and taxol become enriched in cells over time. Thus, it was a trade-off between the extent/brightness of labeling and the effects on MT dynamics. We opted for shorter incubation with a higher concentration of Sir-Tubulin to achieve rapid MT labeling and efficient suppression of plus-end MT polymerization. This approach proved useful for our needs since the loss of the tip-tacking pool of DHC provided a clearer view of the motile population of MT-associated DHC.

(3) The individual channels should be labeled in the supplemental movies.

They have now been labelled.

(4) I would like to see example images and kymographs of the GFP-Kinesin-1 control used for fluorescent intensity analysis. Further, the authors use the mean of the intensity distribution, but I wonder why they don't fit the distribution to a Gaussian instead, as that seems more common in the field to me. Do the data fit well to a Gaussian distribution?

Example images and kymographs of the kinesin-1-EGFP control HeLa cells used for the updated fluorescent intensity analysis have been now added to the manuscript in Figure 3 - figure supplement 1. The kinesin-1-EGFP transiently expressed in HeLa cells exhibited a slower mean velocity and run length than the endogenously tagged HeLa dynein-dynactin. Regarding the distribution, we applied 6 normality tests to the new datasets acquired with DHC and p50 in comparison to human kinesin-EGFP in HeLa cells. While we are confident concluding that the data for p50 was normally distributed ($p > 0.05$ in 6/6), it was more difficult to reach conclusions about the normality of the datasets for kinesin-1 ($p > 0.05$ in 4/6) and DHC ($p > 0.5$ in 1/6). We have decided to report the data as scatter plots (per the suggestion in major point 1 by reviewer 1) in the new Figure 3G since it could be misleading to fit a non-normal distribution with a single Gaussian. We note that the likely non-normal distribution of the DHC data (since it “passed” only 1/6 normality tests) could reflect the presence of other populations (e.g. 1 DHC-EGFP in a motile puncta), but we could also not confidently conclude this since attempting to fit the data with a double Gaussian did not pass statistical muster. Indeed, as stated in the text, on lines 197-198 we do not exclude that the range of DHC intensities measured here may include sub-populations of complexes containing a single dynein dimer with one DHC-EGFP molecule.

Ultimately, we feel the safest conclusion is that there was not a statically significant difference between the DHC and kinesin-1 dimers ($p = 0.32$) but there was a statistically significant difference between both the DHC and kinesin-1 dimers compared to the p50 (p values < 0.001), which was ~50% the intensity of DHC and kinesin-1. Altogether this leads us to the fairly conservative conclusion that DHC puncta contain at least one dimer while the p50 puncta likely contain a single p50-EGFP molecule.

(5) The authors suggest the microtubules in the cells treated with SiR-tubulin may be more deetyrosinated due to the treatment. Why don't they measure this using well-characterized antibodies that distinguish tyrosinated/deetyrosinated microtubules in cells treated or not with SiR-tubulin?

At your suggestion, we carried out the experiment and found that under our labeling conditions there was not a notable difference in microtubule deetyrosination between DMSO- and SiR-Tubulin-treated cells. Thus, we have removed this caveat from the revised manuscript.

(6) "While we were unable to assess the relative expression levels of tagged versus untagged DHC for technical reasons." Please describe the technical reasons for the inability to measure DHC expression levels for the reader.

We made several attempts to quantify the relative amounts of untagged and tagged protein by Western blotting. The high molecular weight of DHC (~500kDa) makes it difficult to resolve it on a conventional mini gel. We attempted running a gradient mini gel (4%-15%), and doing a western blot; however, we were still unable to detect DHC. To troubleshoot, the experiments were repeated with different dilutions of a commercially available antibody and varying concentrations of cell lysate; however, we were unable to obtain a satisfactory result.

We hold the view that even if it had it worked it would have been difficult to detect a relatively small difference between the untagged (MW = 500kDa) and tagged DHC (MW = 527kDa) by western blot. We have added language to this effect in the revised manuscript.

Reviewer #3 (Public Review):

(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.

See response to reviewer 2 above.

(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.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.

The microtubule (MT) plus-tip localization of DHC was already observed in the 1990s, as evidenced by publications such as (PMID:10212138) and (PMID:12119357), which were further confirmed by Kobayashi and Murayama in 2009 (PMID:19915671). We hold the view that further investigation into this localization is not worthwhile since the tip-tracking behavior of DHC-dynactin has been long-established in the field.

(iii) It would also be useful to see entire fields of view of cells expressing DHC-EGFP and p50EGFP (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.

Representative images of fields have been added as Figure 1 - figure supplement 1B and Figure 2 – figure supplement 1 in the revised manuscript. We did not see drastic cell-to-cell variation of expression within the clonal cell lines.

(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).

Despite trying different concentrations and extensive troubleshooting, we were not able to replicate the reported observations of Ciliobrevin D or Dynarrestin during mitosis. We would like to emphasize that the velocity (1.2 $\mu\text{m/s}$) of dynein-dynactin complexes that we measured in HeLa cells was comparable to those measured in iNeurons by Fellows et al. (PMID: 38407313) and for unopposed dynein under in vitro conditions.

(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?

To avoid overcrowding, it was important to image the MT tracks that were pinned between the nucleus and the plasma membrane. It is unclear to us why the reviewer feels that true TIRFM could not be used to visualize the movement of dynein-dynactin on this population of MTs since the plasma membrane is ~ 3-5 nm and a MT is ~25-27 nm all of which would fall well within the 100-200 nm excitable range of the evanescent wave produced by TIRF. While we feel TIRF can effectively visualize dynein-dynactin motility in cells, we have mentioned the possibility that some imaging may be HILO microscopy in the materials and methods.

(ii) At what depth are the authors imaging DHC-EGFP and p50-EGFP?

The imaging depth of traditional TIRFM is limited to around 100-200 nm. In adherent interphase HeLa cells the nucleus is in very close proximity (nanometer not micron scale) to the plasma membrane with some cytoskeletal filaments (actin) and microtubules positioned between the plasma membrane and the nuclear membrane. The fact that we were often visualizing MTs positioned between the nucleus and the membrane makes us confident that we were imaging at a depth (100 - 200nm) consistent with TIRFM.

(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.

Although automated single particle tracking tools offer several benefits, including reduced human effort, and scalability for large datasets, they often rely on specialized training datasets and do not generalize well to every dataset. The authors contend that under complex cellular environments human intervention is often necessary to achieve a reliable dataset. Considering the nature of our data we felt it was necessary to manually process the time-lapses.

(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.

Considering the densely populated environment of a cell, it will be nearly impossible to quantify all the datasets. We selected tracks for quantification, focusing on areas where MTs were pinned between the nucleus and plasma membrane where we could track the movement of a single dynein molecule and where the surroundings were relatively less crowded.

(v) What is the directionality of the moving punctae?

In our experience, cells rarely organized their MTs in the textbook radial MT array meaning that one could not confidently conclude that “inward” movements were minus-end directed. Microtubule polarity was also not able to be determined for the MTs positioned between the plasma membrane and the nucleus on which many of the puncta we quantified were moving. It was clear that motile puncta moving on the same MT moved in the same direction with the exception of rare and brief directional switching events. What was more common than directional switching on the same MT were motile puncta exhibiting changes in direction at sharp (sometimes perpendicular) angles indicative of MT track switching, which is a well-characterized behavior of dynein-dynactin (See DOI: 10.1529/biophysj.107.120014).

(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.*

It was important to quantify SiR tubulin-treated cells because SiR-Tubulin is a docetaxel derivative, and its addition suppressed plus-end MT polymerization resulting in a significant reduction in the DHC tip-tracking population and a clearer view of the motile population of MT-associated DHC puncta. Otherwise, it was challenging to reliably identify motile puncta given the abundance of DHC tip-tracking populations in untreated cells.

(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.

Comparing the fluorescence intensity of a known molecule (in our case a kinesin-1EGFP dimer) to calculate the numbers of an unknown protein molecule (in our case Dynein or p50) is a widely accepted technique in the field. For example, refer to PMID: 29899040. To accurately estimate the stoichiometry of DHC and p50 and address the concerns raised by other reviewers, we expressed the human kinesin-EGFP in HeLa cells and analyzed the datasets from new experiments. We did not observe any significant differences between our old and new datasets.

(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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