Quantitative optical trapping on single organelles in cell extract

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We have developed an optical trapping method to precisely measure the force generated by motor proteins on single organelles of unknown size in cell extract. This approach, termed VMatch, permits the functional interrogation of native motor complexes. We apply VMatch to measure the force, number and activity of kinesin-1 on motile lipid droplets isolated from the liver of normally fed and food-deprived rats.

Cytoskeletal motors transport organelles as cargo along actin and microtubule filaments. A direct measure of motor activity is the force generated to transport cargo, which can be measured by *in vitro* optical trapping of plastic beads coated with purified motors. However, such simplified assays do not reproduce the *in vivo* configuration of motors on the artificial surface of a bead. Although *in vivo* optical trapping has been reported, there are disagreements among the forces measured between different *in vivo* reports as well as between *in vivo* and *in vitro* reports. Issues related to optical-trap centering and the variable sizes of organelles may complicate the interpretation of *in vivo* trapping.

We have chosen to pursue a 'middle path' in which we probe force generation on single organelles in cell extract. Such assays are attractive because they probe motors in a native-like state under conditions in which regulatory factors are unavailable in the extract. Nevertheless, quantitative optical trapping in cell extract could enhance our understanding of how motors work in cells.

The restoring force \( F_{\text{TRAP}} \) on a cargo pulled out to distance \( X \) from the center of a trap functioning as a linear spring of stiffness \( K_{\text{TRAP}} \) is

\[
F_{\text{TRAP}} = K_{\text{TRAP}} \times X
\]

The maximum force exerted by motors \( F_{\text{MOTORs}} \) is

\[
F_{\text{MOTORs}} = K_{\text{TRAP}} \times X_{\text{STALL}}
\]

Here \( X = X_{\text{STALL}} \) when motors 'stall' against the trap. \( K_{\text{TRAP}} \) is determined from thermal fluctuations in the position of a trapped object using the power spectrum or variance methods.

These fluctuations must be sampled at high bandwidth using a quadrant photodiode detector (QPD; *Supplementary Fig. 1*). Both methods require (directly or indirectly) the size of the trapped cargo to be known precisely. However, the size of an organelle in cell extract is variable. \( K_{\text{TRAP}} \) depends on this size, varying approximately sixfold between organelles with diameters between 0.5 \( \mu \)m and 3 \( \mu \)m, which is the size range for many cellular organelles. Hence, the estimation of \( K_{\text{TRAP}} \) for organelles is prone to error.

\[
K_{\text{TRAP}} = \frac{k_B T}{<X^2>}
\]

\( k_B \) is the Boltzmann constant, and \( T \) is temperature in Kelvin. The position fluctuations \( <X> \) must be known in real distance units (nanometers). A calibration factor \( S_{\text{QPD}} \) that converts the QPD voltage fluctuations \( (V_{\text{QPD}}) \) to distance fluctuations \( (X_{\text{QPD}}) \) is therefore required.

\[
<X^2> = <X_{\text{QPD}}^2> = <(V_{\text{QPD}} \times S_{\text{QPD}})^2>
\]

\( S_{\text{QPD}} \) must be determined precisely because the squared term in equation (4) amplifies small errors. A strong dependence of \( S_{\text{QPD}} \) on the size of the trapped object \( (\text{Fig. 1b}) \) precludes the use of the variance method on organelles of unknown size.

Here we describe a simple method \( (\text{Fig. 1a}) \) to determine \( S_{\text{QPD}} \) precisely for individual organelles of unknown size in cell extract. Motor-driven stalls of an organelle are recorded simultaneously with a QPD (as a voltage signal) and a video charge-coupled device (CCD) camera (as a movie). We then trap the organelle away from the microtubule to measure its thermal fluctuations \( (V_{\text{QPD}}) \) in the QPD. The movie of the stalls is then video tracked to obtain the displacement \( (X_{\text{VIDEO}}) \) of the organelle in real distance units. The stall recorded earlier in the QPD is multiplied by that factor, which minimizes the r.m.s. difference between the QPD and video records. This factor is the precise value of \( S_{\text{QPD}} \) for that specific trapped organelle. With \( S_{\text{QPD}} \) determined, \( <X^2> \), \( K_{\text{TRAP}} \) and \( F_{\text{MOTORs}} \) can be obtained from equations (4), (3) and (2). \( \text{Figure 1b} \) plots the values of \( S_{\text{QPD}} \) determined in this manner for lipid droplets extracted from rat liver. The \( S_{\text{QPD}} \) values vary widely for lipid droplets because these organelles have variable size, whereas beads of a given diameter show only small variation in \( S_{\text{QPD}} \).

Although a slow (30 frames per s) camera undersamples thermal fluctuations, it precisely determines the horizontal plateau-like stalled position of the organelle (usually lasting >0.5 s; \( \text{Fig. 1a} \)). Further, video tracking algorithms are largely insensitive...
Figure 1 | The VMatch method for quantitative optical trapping of cellular organelles. (a) The displacement ($X$) of a motor-driven cargo against an optical trap (red focused beam) is shown schematically (top left). The trap works as a spring whose stiffness ($K_{TRAP}$) must be determined to measure $F_{MOTOR}$. The plot (top right) shows the stall force record acquired simultaneously with a QPD and a video camera for a plus-moving lipid droplet. The two data sets are then matched to find the calibration factor of the trap ($S_{QPD}$). This value is used to calculate the variance in position ($\langle X^2 \rangle$) of the thermally fluctuating cargo in the trap. $K_{TRAP}$ and $F_{MOTOR}$ are subsequently determined from $\langle X^2 \rangle$. (b) QPD calibration factor ($S_{QPD}$) for beads of different mean diameters and for lipid droplets (LDs) determined using VMatch. (c) Stall force of kinesin-1 coated on 0.5-μm beads (59 stalls and 40 beads) and 1-μm beads (119 stalls and 82 beads). Stall force was measured by the power spectrum (PS) and VMatch (VM) methods. Error bars, s.d. (d) Stall force of single molecules of kinesin-1 on 20 different beads, each measured by both the PS and VM methods.

To organelle size (Supplementary Fig. 2). Therefore, $X_{VIDEO}$ is the correct displacement irrespective of organelle size. We call this the video-matching method (VMatch) for motor force measurement. VMatch can quantify not just force but also step sizes and dynamics (attachment or detachment) of motor complexes on organelles with kilohertz time resolution. However, VMatch cannot calibrate a trap inside a cell, where possible cargo attachment to microtubules or actin would measure a composite stiffness of the trap along with the stiffness of these unknown attachments.

To test VMatch, we measured the force of purified kinesin-1 on beads of known sizes (diameters, 0.49 ± 0.01 μm (mean ± s.d.) and 0.99 ± 0.04 μm) by the power spectrum and variance methods (Fig. 1c). We were able to use the power spectrum method because the bead size was known (this is not known for organelles). We recorded 59 stalls from 40 beads of 0.5-μm diameter and 119 stalls from 82 beads of 1-μm diameter. The stall force on the 0.5-μm beads was $5.67 ± 1.1$ pN (mean ± s.d.) using VMatch and $5.51 ± 1.0$ pN using the power spectrum method. The stall force on the 1-μm beads was $5.74 ± 0.9$ pN using VMatch and $5.68 ± 1.1$ pN using the power spectrum method. One-way analysis of variance showed no significant difference at a 0.05 significance level ($F_{3,35} = 0.84, P = 0.47$) between the values for both methods. This shows that VMatch measures the force for purified kinesin-1 correctly regardless of cargo size. The stall force obtained by the power spectrum and VMatch methods on 20 beads showed point-to-point agreement for every kinesin-1 molecule (Fig. 1d).

We next assayed the motility of lipid droplets extracted from rat liver (Fig. 2a, Supplementary Fig. 3 and Online Methods) on microtubules labeled at the minus end with avidin-coated magnetic beads. We observed robust lipid-droplet motility (75% of the lipid droplets moved when placed on microtubules with the

Figure 2 | Motility and force measurement on lipid droplets extracted from rat liver.

(a) Position of lipid droplets (LDs) along microtubules (MTs) over time from representative video tracks of an in vitro motility assay at 1 mM ATP. Insets, differential interference contrast image of lipid droplets isolated from rat liver (top) and anti-kinesin-1 (kin-1) western blot of liver cytosol (high-speed supernatant, HSS) and proteins extracted from purified LDs (bottom). Scale bar, 3 μm. (b) Distribution of $K_{TRAP}$ for LDs (obtained using VMatch) and 1-μm beads (obtained using the power spectrum method). Images of LDs that yielded specific values of $K_{TRAP}$ are shown (arrows). (c) Histogram of stall force for plus-moving LDs from normally fed (‘normal’) rats using the $K_{TRAP}$ values determined for each LD by VMatch. The black line is a fit to the sum of two unconstrained Gaussians (reduced $\chi^2 = 2.42$). Inset, representative stalls of LDs corresponding to the first and second peaks. (d) Histogram of stall force for plus-moving lipid droplets extracted from the liver of rats after a 16-h fast. The black line is a fit to one Gaussian. (e-g) Plots comparing the fraction of LDs moving on MTs (e), the run length of plus runs for LDs (f) and the velocity of LDs (g) from normal (N) and fasted (F) rats. ***$P < 0.001$, ****$P < 0.0001$, NS, not significant ($P = 0.52$). All error bars are the s.d.
In liver remained unchanged by fasting (Supplementary Fig. 5). As there are more lipid droplets in the liver in the fasted lipogenic state, our results can be interpreted to mean less kinesin-1 is present per lipid droplet in the fasted liver. However, other interpretations are possible, such as inactivation of lipid droplet-associated kinesin-1 by fasting, and these options remain to be investigated.

VMatch has several advantages over other methods of trap calibration for objects of unknown size (Supplementary Note 3). For certain methods, the long calibration time and requirement of relative motion between the trapped object and the trap result in small cytosolic particles of irregular shape getting trapped along with the cargo of interest. This induces errors in trap calibration. Such particles, which are unavoidable in cell extract, can usually be excluded from the trap during the short calibration time for VMatch. Other methods of calibration may require thermal fluctuations using a camera but yield artificially inflated trap stiffness because of slow sampling by the camera. All these methods have used beads of known size to verify trap calibration but have not been used to report motor forces on beads or organelles, as we have demonstrated with VMatch.

To summarize, we have developed a simple method (VMatch) to measure the force exerted by motors on single organelles in cell extract. VMatch extends quantitative optical trapping beyond artificial motor-coated beads to real cellular cargoes.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
R.M. designed the experiments. P.B., A.R., P.R. and R.M. performed the experiments. R.M., P.B. and A.R. analyzed the data and wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Reagents. The Kif5B 914-930 amino acid peptide from rat GHSQAIAKPIRPGQHP was synthesized by BiotechDesk, India. ADRP mouse monoclonal antibody (651102) was purchased from Progen Biotechnik, Germany. KHC rabbit polyclonal antibody was a kind gift from K. Verhey (University of Michigan). S-200 Sephacryl High-Resolution beads were purchased from GE Biosciences. Protease inhibitor cocktail was purchased from Roche. All other chemicals were purchased from Sigma-Aldrich.

Animals. Sprague Dawley rats were bred and maintained by the animal house facility at Tata Institute of Fundamental Research, Mumbai. All animal protocols were approved by the Institutional Animal Ethics Committee (IAEC) formulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Instrumentation and data acquisition. The instrument and detection system has been described earlier17 and has been used by us to quantify motor function in Dictyostelium discoideum endosomes8. Motion was observed at 37 °C in a custom-developed differential interference contrast microscope (Nikon TE2000-U) using a 100×/1.4-numerical aperture (NA) oil objective. The microscope is housed in an acoustically protected room on an optical table with active isolation (Newport). Image frames were acquired at video rate of 30 frames s⁻¹ (no binning) with a Cohu 4910 camera. Each pixel measured 98 nm × 98 nm. Image frames were digitized and saved as AVI files using an image acquisition card (National Instruments). No image-processing hardware or software was used for image enhancement. The positions of lipid droplets and beads were tracked frame by frame using custom-written software in LabVIEW (National Instruments). The tracking algorithm calculates the position of the centroid of a cross-correlation image with subpixel resolution. Subpixel resolution was confirmed by moving a bead stuck on the coverslip in steps of 10 nm using a piezo stage (Physik Instrumente) and tracking its position (Supplementary Fig. 2).

The optical trap and detection setup is also shown schematically (Supplementary Fig. 1a). A single-mode diode laser at 980 nm (Axcel photonics) was used after expansion to fill the back focal plane of the objective2,3. The laser power at the sample plane was typically 50–70 mW. We have earlier shown that there is no optical damage at these powers8,19,20. As confirmed, as lipid droplets that stalled in the trap moved away with normal velocity after being released. The expected heating at the sample plane is negligible (<1 °C) at this power2,3. A QPD was used to obtain stall-force records and thermal fluctuations2,3. For optical trapping with beads of uniform size, S_QPD was obtained from the voltage pattern generated by a bead stuck to a coverslip and moved in known distance increments across the trap with a piezo stage2. For stall-force records, data were anti-alias filtered at 1 kHz and digitized at 2 kHz. For variance measurements, thermal fluctuations were recorded at 40 kHz. For power spectrum measurements, the power in the thermal fluctuations was plotted as a function of frequency2. This curve was fitted to a Lorentzian function (see Supplementary Fig. 1 for an example) to obtain a corner frequency (\(F_c\)), and \(K_{\text{TRAP}}\) was determined using the equation \(K_{\text{TRAP}} = 12\pi^2\eta F_c \times R\). For in vitro assays using motor-coated beads, the uniform bead radius (\(R\)) and viscosity of medium (\(\eta\)) were known, and the same \(R\) was used for all beads in the assay2. The Lorentzian power spectrum of thermal fluctuations (Supplementary Fig. 1b) shows that the optical trap functions as a linear spring for beads and lipid droplets. The range of QPD linearity for 0.5-µm beads was estimated to be ±160 nm (Supplementary Fig. 1b). This range extends further for larger objects (such as large lipid droplets) because of the larger diameter. However, only stalls within ±160 nm were used. The drag- force method2,3 was used to determine that the optical trap was linear out to ±160 nm from the trap center for 0.5-µm beads (and further for larger beads and lipid droplets).

Purification of rat liver lipid droplets for motility. Lipid droplets are a convenient organelle for isolation at a large scale by density-gradient ultracentrifugation because of their low density. Detailed protocols for the isolation of pure lipid droplets from adipose tissue21 and liver22 are available, and several proteomic studies23 that have investigated the lipid-droplet proteome have used these protocols. A brief description of the protocol used by us follows. Male Sprague Dawley rats 2–4 months old were anesthetized using sodium thiopentone. The liver was perfused with ice-cold PBS through the hepatic portal vein and dissected out. Liver was homogenized using a Dounce homogenizer in 1 M MEPS buffer (35 mM PIPES, 5 mM EGTA, 5 mM MgSO₄ and 1 M sucrose, pH 7.2) with 2× protease inhibitor cocktail, 4 mM PMSF, 2 µg/ml of pepstatin A and 4 mM DTT. The liver was homogenized in 1 M MEPS buffer at a ratio of 1:1.5 (wt/v). The homogenate was spun at 1,800g at 4 °C for 10 min to pellet the debris and obtain a post-nuclear supernatant (PNS). Then, 1× protease inhibitor cocktail, 4 mM DTT and 2 µg/ml of pepstatin were added to the PNS before loading on a Sephacryl S-200 column (40-ml bed volume) to separate organelles from the cytosolic protein fraction. The first 4 ml of eluate was collected, supplemented with 1× protease inhibitor cocktail and 4 mM DTT and adjusted to 1.4 M sucrose using 2.5 M MEPS (2.5 M indicates the molarity of sucrose). This eluate was loaded at the bottom of a step gradient consisting of 2 ml each of 1.4 M, 1.2 M and 0.5 M MEPS. The gradient was spun in a SW41 Beckman rotor tube at 120,000g at 4 °C for 1 h. The topmost 0.5 M MEPS fraction contained lipid droplets and was collected and flash frozen in liquid nitrogen as 40-µl aliquots to be used for motility. This gradient largely removes cellular particles (debris), though some small debris remains. We took care to reject stalls in which debris was observed in the trap. We checked this for every droplet before and after the trapping. Purified organelles were identified as lipid droplets on the basis of their high buoyancy, refractile spherical appearance and abundance of ADRP24, a specific marker for lipid droplets.

Motility assays. Tubulin (5–10 mg/ml) was purified from goat brain as described8,17,25. Western blots using MAP antibodies showed no detectable contamination from microtubule-associated proteins after purification. The MAP antibody was purchased from Sigma (M7273), and was used at dilution 1:1,000. Taxol-stabilized microtubules were polymerized in BR880 buffer containing 1 mM GTP and 20 µM taxol for 45 min at 37 °C. For determining the direction of lipid droplet motion, polarity-labeled microtubules were prepared using biotinylated seeds and magnetic avidin-coated beads as described17. Motility was
observed in flow chambers of ~10 μl volume prepared by sticking poly-1-lysine–coated coverslips to a microscope slide using two strips of double-stick tape.

Lipid droplet motility was observed using a motility mix containing 38 μl of the lipid droplet fraction and 2 μl of a 20× ATP regenerating system (20 mM ATP, 20 mM MgCl₂, 40 mM creatine phosphate and 40 U/ml creatine kinase). The final ATP concentration was 1 mM. The motility mixture was introduced in a flow chamber containing microtubules. Each slide was observed for 20 min or less. Extremely robust motion was observed: 75% of the lipid droplets (total investigated, 160 tested from multiple rats) moved when placed on microtubules using an optical trap. To the best of our knowledge, this is the first in vitro reconstitution of lipid-droplet motility on microtubules. We tested the possibility that motors are lost from the lipid droplets or are recruited nonspecifically from the cytosol to lipid droplets during the purification procedure. To do this, we incubated lipid droplets in an ionic buffer and then recovered them by flotation. This treatment had no effect of the motility of the lipid droplets: the frequency and velocity of motion for the treated lipid droplets were statistically identical to those of the untreated lipid droplets. Thus, plus-directed motion seems to be driven by endogenously associated motors on lipid droplets.

Conventional kinesin (kinesin-1) was purified from fresh goat brain through a nucleotide-dependent microtubule affinity procedure. No dynein or dynactin was detected in the purified brain through a nucleotide-dependent microtubule affinity procedure. To do this, we incubated lipid droplets in an ionic buffer and then recovered them by flotation. This treatment had no effect of the motility of the lipid droplets: the frequency and velocity of motion of the treated lipid droplets were statistically identical to those of the untreated lipid droplets. Thus, plus-directed motion seems to be driven by endogenously associated motors on lipid droplets.

Specificity of kinesin-1 attachment to lipid droplets. We tested whether kinesin-1 is lost from lipid droplets during purification or spurious kinesin-1 gets attached nonspecifically to lipid droplets through electrostatic interactions when the droplets come in contact with cytosol. This was done by treating lipid droplets with an ionic buffer. If kinesin-1 is retained after such treatment, it is probably not lost during purification (as this is a harsher treatment than the purification process). Further, this also tests whether the kinesin-1 driving the motion is actually cytosolic contamination, as contaminating kinesin-1 should be lost by incubation in ionic buffer. This approach has been used earlier to remove contaminating proteins in proteomic analysis of lipid droplets. Lipid droplets were incubated in 100 mM sodium carbonate for 30 min on ice, separated by flotation and washed twice in motility buffer. They were then tested for motility, and no difference was found by comparison with the motility of untreated lipid droplets.

Kinesin-1 inhibition studies. For kinesin-1 inhibition assays, lipid droplets were incubated with the rat Kif5B 914–930 amino acid tail-domain peptide (KTD) for 15 min. Motility was observed at the end of the incubation with KTD in the motility buffer. Lipid droplets were trapped and placed on microtubules. The fraction of motile lipid droplets was scored. A dose-dependent reduction in motility with KTD was seen (Supplementary Fig. 4). A similar construct is known to reduce the enzymatic activity of Drosophila mélano gaster kinesin-1 without affecting its microtubule-binding properties. We have separately verified that KTD blocks purified kinesin-1–driven in vitro motion of beads. The KTD peptide did not have any effect on the plus- or minus-directed motion of D. discoideum endosomes, which are driven by cytoplasmic dynein and a different kinesin (DdUnc104). Additional control experiments with nonspecific peptide did not show any effect on the motility of lipid droplets or kinesin-1 beads.

Isolation of lipid droplets for immunodetection of kinesin-1 and ADRP. For western blotting, lipid droplets were isolated as described earlier with some modifications. Liver was lysed, and the PNS was prepared in 1 M MEPS buffer (1 M indicates the sucrose molarity). PNS was adjusted to 1.4 M sucrose-containing MEPS using 2.5 M MEPS. Then, 7 ml of 1.4 M PNS was loaded at the bottom of a sucrose step gradient containing 7 ml each of 1.4 M, 1.2 M and 0.5 M sucrose in MEPS (total volume of 28 ml). The topmost fraction of 0.5 M MEPS (~5 ml) was collected and concentrated to 500 μl for western blotting. This fraction was enriched in lipid droplets, as confirmed by the detection of adipocyte differentiation-related protein (ADRP), a specific marker for lipid droplets in the liver, by western blotting (Supplementary Fig. 3) and also by immunofluorescence on individual lipid droplets. To doubly validate the lipid droplet isolation procedure, a similar experiment using fat pads from rats was also done. In that experiment we detected abundant perilipin (marker for adipose lipid droplets) in the top fraction. HSS to detect cytosolic proteins in western blots was prepared by spinning the PNS for 30 min at 4 °C and 500,000g in a Beckman MLA 130 rotor. The pellet was discarded, and the supernatant (HSS) was collected.

Western blotting. Total protein was stripped from purified lipid droplets using a two-dimensional cleanup kit (ReadyPrep, Bio-Rad). The amount of protein was quantified using a bicinchoninic acid kit (Sigma). For detection of ADRP, 25 μg of total protein from lipid droplets, 40 μg of protein from the endosome fraction and 50 μg of protein from the HSS were loaded in separate lanes and subjected to SDS-PAGE. For kinesin-1 detection, 60 μg of lipid droplet protein and 50 μg of HSS protein were loaded. The protein was transferred to a polyvinylidene fluoride membrane and then blocked in 5% milk. Membranes were probed with ADRP and kinesin-1 antibodies at a 1:1,000 dilution for 1 h. Secondary mouse and rabbit horseradish peroxidase–conjugated antibodies (Santa Cruz Biotechnology) were used at a 1:20,000 dilution. The blots were developed using the Pierce ECL Western Blotting Substrate (Thermo Fisher). Band intensity was quantified using ImageJ.

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