3D tracking of extracellular vesicles by holographic fluorescence imaging

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Fluorescence microscopy is the method of choice in biology for its molecular specificity and super-resolution capabilities. However, it is limited to a narrow z range around one observation plane. Here, we report an imaging approach that recovers the full electric field of fluorescent light with single-molecule sensitivity. We expand the principle of digital holography to fast fluorescent detection by eliminating the need for phase cycling and enable three-dimensional (3D) tracking of individual nanoparticles with an in-plane resolution of 15 nm and a z-range of 8 μm. As a proof-of-concept biological application, we image the 3D motion of extracellular vesicles (EVs) inside live cells. At short time scales (<4 s), we resolve near-isotropic 3D diffusion and directional transport. For longer lag times, we observe a transition toward anisotropic motion with the EVs being transported over long distances in the axial plane while being confined in the horizontal dimension.

INTRODUCTION
Understanding and controlling cargo transport and delivery in vivo is one of the main challenges preventing successful implementations of minimally invasive future nanomedical platforms. Engineered nanoparticles (NPs) and extracellular vesicles (EVs) hold promise as potential candidates for delivery vehicles, but a precise understanding of their journey through and interaction with complex, living tissue is still lacking (1–4). Furthermore, EVs are suspect to being important mediators of metastasis via intercellular communication pathways, and unraveling the mechanistic details of this spatial information transport is of utmost importance (5–7). One of the main obstacles for overcoming these challenges is the lack of widefield three-dimensional (3D) single-particle imaging methodologies that can track many individual particles simultaneously as they travel to their intended destination.

One of the most promising approaches to 3D tracking is a digital implementation of holography (8), which is markedly changing the field of optics, mainly as a result of the rapid increase in computational processing power and the emergence of advanced image-analysis algorithms. Holography is now routinely used in applications as diverse as x-ray imaging (9), super-resolution methodologies without lenses (10), 3D tracking (11), optical tomography of biological specimens (12, 13), and even label-free single protein detection (14–16). Its implementation relies on simultaneously measuring both intensity and phase of an object, in other words, the full electric field. Such a measurement is made possible by interfering the image with a known reference wave (17).

Despite its great potential, holographic approaches to fluorescence microscopy remained challenging (18–22). This unexpected shortcoming is due to the incoherence of fluorescent light, which is generated by the intrinsically stochastic process of spontaneous emission. Individual molecules emit at most one fluorescent photon every few nanoseconds, and these photons have very short temporal coherence lengths of approximately 50 fs. Even worse, these emitters act as light sources with random phase. As expected, it is extremely challenging to generate a suitable reference wave for such light sources and initial implementations therefore relied on phase-cycling schemes, which require the acquisition of multiple images (19). This approach is ill-suited for live-cell or single-molecule work as moving particles or the blinking and photobleaching of single emitters prevent acquiring the multiple, quasi-identical, images necessary for phase recovery.

To enable the necessary single-shot recording of dynamic processes, shearing interferometry could be a potentially promising route (23–27). The basic idea behind shearing interferometry is self-interference under a slight spatial offset, which allows accessing the phase gradients, down to the single-photon level (note S1). Initial attempts have already demonstrated single-molecule z localization over a z range of ±350 nm (22), an improvement over more traditional approaches such as astigmatism–based z localization (28), and extending these initial demonstrations toward full-scale amplitude and phase recovery of fluorescent images should, in principle, enable single-shot fluorescence holography.

Here, we demonstrate fluorescence holography–based 3D particle localization over a z range of 8 μm. We use this technology to track EVs inside live cells and observe often strongly confined EVs with periods of active transport serving as the main mode of intracellular translocation over micrometer length scales.

RESULTS
Imaging principle
We extend a widefield fluorescence microscope with a wavefront shearing sensor as shown in Fig. 1. The sensor is composed of a relay imaging system equipped with an appropriately chosen 2D 0–π phase grating (see note S2 for a detailed discussion of the grating design), which is slightly offset from the conjugate image plane, formed by the imaging lens. This geometry ensures that nonzero phase gradients are measured (see notes S3 and S4 for detailed
are the strong intensity modulations, in both x out-of-focus beads. A direct consequence of the grating-induced shear and Methods). Figure 2A shows a representative image obtained for et al. 6: eabc2508 4 November 2020. SCIENCE ADVANCES | RESEARCH ARTICLE

...otherwise identical copies. This system allows single-photon self-image on the camera, which is composed of four slightly offset but second lens then recombines the four orders to generate the final image on the camera, which is composed of four slightly offset but otherwise identical copies. This system allows single-photon self-interference across the entire image alongside background and twin image free phase-gradient isolation and offers the additional advantage of being intrinsically phase-locked (25, 29).

System validation
To validate the steps necessary for a phase measurement, we image fluorescent polystyrene beads (d = 200 nm, λ em = 645 nm; Materials and Methods). Figure 2A shows a representative image obtained for out-of-focus beads. A direct consequence of the grating-induced shear are the strong intensity modulations, in both x and y dimensions, visible across the point spread functions (PSFs). This modulation enables us to isolate the interference terms by Fourier transformation of the raw image into k-space (Fig. 2B), as described above. The phase information of interest is contained in the frequency-shifted terms (note S3). The k-space position of each individual interference term indicates the direction of the phase gradient measurement. For simplicity, we concentrate on the purely x- or y-shifted terms but remark that all other terms, or sums thereof, can be decomposed into x/y components by simple projections (25, 29). Hard aperture selection followed by demodulation and inverse Fourier transformation yields the desired phase gradients Δθ x and Δθ y, shown in Fig. 2C (see notes S3 and S4 for additional details). As information in both dimensions is available, it is possible to perform a 2D integration with a Poisson solver to unwrap the phase (Fig. 2D) (30–32). The raw phase data are further refined in the following way (Fig. 2E and notes S4 and S5): (i) scaling to account for the exact grating to conjugate image distance and (ii) linear fitting to Zernike polynomials to remove minor phase distortions due to 2D integration–induced artifacts. The intensity information is extracted as the argument modulus of the filtered images used for phase gradient extraction.

3D particle tracking
Once the full electric field is obtained, we use Fourier optics to perform correction of complex scattering-induced aberrations or to construct images at any plane of choice (33–35). In this work, we concentrate on 3D localization experiments, which require the recovery of the precise position of an emitter of interest in all dimensions, including z. We rely on the angular spectrum method where we convolve the image of the complex electric field with a propagation kernel. This operation analytically propagates the images over a known distance corresponding to a different focal plane (Materials and Methods) (35). Figure 3A shows an example of such a computational refocusing operation where we experimentally position a 200-nm fluorescent bead 4.4 μm above the focal plane using a closed-loop piezo. After recording an image and processing it as described earlier, we propagate the image back into the focus.
Comparing the computationally obtained and an experimentally recorded in-focus image shows excellent agreement in both $x/y$ positions and the shape and width of the PSF (Fig. 3A and inset). This encouraging result suggests that computational focusing can be applied to determine the 3D position of multiple freely diffusing fluorescent particles. To test such reasoning, we generate a 3D trajectory, spanning a total $z$ range of 9 μm, with a closed-loop piezo. We move a sample composed of immobilized fluorescent beads and record images along the path (movie S1). As previously, we recover the phase and amplitude information and then determine the 3D positions of the individual particles by means of numerical propagation. Figure 3B shows the 3D trajectories for individual particles (Materials and Methods) along with the ground-truth piezo position. Overall, particle and stage positions agree with each other. Some deviations at large vertical displacements are observed, which can be attributed to a reduction of the collection numerical aperture (NA) for strongly diverging out-of-focus light that might be cropped by the imaging lens. To further quantify the accessible $z$ range, we experimentally defocus individual particles and then computationally refocus the image. On the basis of these calibrations, we conclude that artifact-free measurements are possible over a $z$ range of approximately 8 μm (note S6).

To image diffusing nanoscale particles, it is important to ensure accurate and precise nanometer localizations over micrometer length scales in 3D. Fluorescence holography meets these requirements as demonstrated by an experiment with sub-diffraction–limited displacements (Fig. 3C). We simultaneously image 17 particles positioned 900 nm above their focal plane and follow their complex movement within a $400 \times 300 \times 200 \text{ nm}^3$ volume. The measured particle trajectories closely follow the ground truth (stage movement) with a mean deviation of $\sqrt{\sigma_x^2 + \sigma_y^2 + \sigma_z^2} = 30 \text{ nm}$ with the standard deviations in $x$, $y$, and $z$ being 15, 15, and 21.5 nm, respectively (see note S7 for histogram representations of all deviations from ground truth). Importantly, each individual letter of the input word “holography” measures less than 50 nm in width, and the word is well resolved even though the particles are considerably defocused, confirming the super-resolution performance of fluorescence holography.

### Single-molecule imaging

To demonstrate that fluorescence holography is capable of operating under the biologically important super-resolution conditions, we measure a sample composed of individual ATTO647N molecules 650 nm above the focal plane. The out-of-focus PSFs are reminiscent of randomly oriented dipolar emission patterns (Fig. 3D) (36). Despite the markedly reduced fluorescence intensities, computational focusing to the diffraction limit is achieved even for photon levels as low as $10^4$ photons. Even the weakly emitting molecule on the bottom right corner is successfully resolved in the focused image. We observe single-step photobleaching of almost all emission sites, which further supports the notion that phase and amplitude of single ATTO647N molecules are measured (Fig. 3E).

### Monitoring cellular uptake of NPs

As proof-of-concept biological application of the developed system, we set out to visualize intracellular trafficking of inorganic NPs (37–39) and EVs (4, 40, 41). Understanding the internalization processes of such particles and identifying their accumulation sites within cells could help developing rational strategies to deliver nanodrugs to a specific location of interest (38). As a model system, we first use fluorescently labeled gold nanorods ($44 \times 11 \times 11 \text{ nm}^3$). These inert particles do not noticeably interfere with the cells’ function and accumulate in the cytoplasm (Fig. 4A and Materials and Methods) as both the particle size and the surface functionalization prevent active internalization via the nuclear pore complex. Figure 4B verifies the accumulation of particles in the cytoplasm with the majority of particles being present outside of the nucleus, a notion that we verified by acquiring dark-field images and widefield $z$ scans to capture the entire volume of the nucleus.

To follow the particles’ trajectories (Fig. 4B), we record time-lapse fluorescence images and extract the phase and amplitude terms (Fig. 4C and Materials and Methods). We observe widely varying PSFs, which indicate that nanorods are present at different $z$ positions.
with respect to the focal plane. This notion is further supported by the phase image. Regions of positive (black) and negative (white) curvature correspond to converging and diverging spherical waves (Fig. 4C), respectively. Computational propagation of the image by an arbitrarily chosen distance of ±2 μm qualitatively refocuses some of the measured PSFs, while others remain defocused (Fig. 4D). We perform 3D localization of each individual nanorod within the cell and reconstruct the particles’ trajectories over the 100 frames of observation. We obtain a total of 26 particle trajectories within a z range of 6.6 μm, which is comparable to the typical heights of adherent cells and within our artifact-free propagation window of 8 μm (note S6) (42, 43). Figure 4E shows six representative trajectories. The particles’ motion could be grouped into three broad categories: Some particles are immobile during the 200 s of observation time, others freely diffuse across several micrometers, and the rest shows both bound and diffusing states. Particles showing low mobility as expected for bound particles, thus making it very difficult to select suitable imaging parameters for automated trajectory linking.

Figure 5A shows the overlay of time-lapse amplitude projections of fluorescent holograms with simultaneously recorded bright-field images of the individual cells (field of view, 83 × 83 μm²). Most EVs are localized at the edges of adherent cells. Comparison with reconstructed EV trajectories (Fig. 5B), however, reveals the dynamic nature of EV transport even at the flat cell edges, and we obtain several tens to hundreds of 3D trajectories per HeLa cell. For further analysis, we calculate the EVs’ net displacement (δ = √(Δx² + Δy² + Δz²)) between frames and classify their motion into two categories: active transport (δ > 100 nm) or random, near stationary (δ ≤ 100 nm). The cutoff (100 nm) is chosen based on visual inspection of the EV trajectories.

On the basis of this initial classification, we attempt further EV motion analysis, on both the active and the passive fraction, by computing mean square displacements (45) [MSD(t)] as a function of the lag time t:

$$\text{MSD}(t) = \frac{1}{ND} \sum_{t=0}^{t_{\text{max}}} \sum_{n=1}^{n_{\text{max}}} \| \vec{a}_n(t) - \vec{a}_n(t + \Delta t) \|^2$$

where \(\vec{a}_n(t)\) is the position of particle \(n\) at time \(t\), \(D\) is the dimensionality of \(\vec{a}_n\), and \(N\) is the total number of summed squared differences. Figure 5C shows the analysis result in the typical log-log representation, along with power-law fits MSD ∝ \(\Delta t^{2\alpha}\) for the first 28 s of lag time. The exponent \(\alpha\) classifying the underlying diffusion model with \(\alpha = 1\) corresponding to Brownian diffusion, \(\alpha < 1\) to sub-diffusion, and \(\alpha > 1\) to super-diffusion (46). In the xy plane, we observe super-diffusivity for the actively transported EVs and near-Brownian motion otherwise. The behavior in the z dimension, however, is confined irrespective of the classification. The step-size distributions for \(x\), \(y\), and \(z\) movements are essentially identical (Fig. 5D), which suggests that the space inside cells can be regarded as isotropic on short time scales.

To understand the mechanistic origin of this discrepancy, we analyze the time delay–dependent turning angles between trajectory steps

$$\theta_{\Delta t} = \text{ArcCos} \left( \frac{\vec{v}_t \cdot \vec{v}_{t+\Delta t}}{\| \vec{v}_t \| \| \vec{v}_{t+\Delta t} \|} \right)$$

with \(\vec{v}_t\) and \(\vec{v}_{t+\Delta t}\) being vectors between subsequent EV positions at \(t\) and \(t + \Delta t\). In this angle representation, \(\theta_{\Delta t} = 0°\) corresponds to EVs continuing to move into the same direction and \(\theta_{\Delta t} = 180°\) to reversing the step. Figure 5E shows that the active fraction exhibits not only a larger probability of continuing to move into the same direction but also a considerably increased chance of reverse motion. In general, the motion exhibits directionality memory with the probability of moving forward, or backward, being considerably larger than intermediate angles [±(65° to 105°)], even for time
delays between steps as large as 60 s (Fig. 5F). The random trajectories show a markedly different behavior (Fig. 5E) with near-isotropic angle distributions albeit a slightly increased probability of turning back at short time scales (Fig. 5F). These observations suggest that the EVs are trapped inside a potential, which confines their motion to a certain volume, with the most likely candidates being the cellular cytoskeleton.

DISCUSSION
We devised large field-of-view (>80 × 80 μm²) single-shot fluorescence holography that allows 3D single-particle tracking over a z range of approximately 8 μm at frame rates that are solely limited by the brightness of the label under observation. To prove the concept, we implemented an easy-to-use experimental setup with an optimized photon throughput of >60% (grating plus relay imaging system). We eliminated phase cycling, which allowed us to image dynamic scenes, such as diffusion or transport phenomena in live cells. Our advances hold great potential for digital aberration correction analogous to complex experimental wavefront-shaping schemes that are increasingly being used in advanced microscopy (47). Taken together, these features make fluorescence holography an ideal approach for studying particle trafficking in real time. Compared to other self-interference–based localization microscopies (22), which used look-up table–based particle localization, our approach relies on phase retrieval of the fluorescence signal in combination with digital image propagation, and we envision that a combination of both localization techniques will ultimately yield the best possible z estimates.

Applying the developed platform, we demonstrated 3D single-particle tracking with precisions comparable to those achieved in established super-resolution techniques. For potential biological applications, we tracked the motion of nanoscale objects introduced into live cells: fluorescently labeled gold nanorods and EVs. For gold nanorods, we observed both static and freely diffusing particles and were able to verify that the internalization into the nucleus is severely hindered, as expected for the particles chosen. In the case of EVs, we showed that those vesicles accumulate at the edges of adherent cells, which results in considerable crowding. The EVs showed strong 3D confinement within the cells and were near stationary unless active trafficking occurred. We observed tug-of-war–like behaviors where individual EVs seemingly turned around between individual steps, thus resulting in the typical back-and-forth motion...
illuminated area with a flat illumination profile. A 490 nm long-pass microscope under a magnification of 1/6 resulting in an 83.3 clean-up filter (Chroma) and coupled into a 500 \( \mu \)m multimode

The EV experiments are performed with a 470 nm light-emitting diodes and 647LP Razoredge (Semrock) for the fluorescent beads. Semrock) for the ATTO647N and fluorescence-detected cell experiments (22) and should be equally well suited for other volumetric imaging challenges such as tracking in tissue or calcium imaging.

Grating fabrication

The 2D periodic chessboard phase grating with a pitch of 19.1 \( \mu \)m and a duty cycle of 0.5 was patterned first by exposing optical photore sist (AZ5214, MicroChemicals) by laser lithography at 405 nm wavelength and a dose of 220 ml/cm\(^2\) (LaserWriter LW405B, Microtech) followed by a reactive ion etching (Oxford Plasma Technology Plasmalab system 100) under the following conditions: pressure of 30 mtorr, fluxes of 40 sccm (standard cubic centimeters per minute) for Ar and 5 sccm for CHF\(_3\), and radio frequency power of 300 W, leading to a DC bias of 850 V to etch 670 nm of material. Last, the etch mask was removed with acetone.

Optical setup

Samples of interest are mounted on a home-built inverted microscope equipped with an Olympus UPlanS APO 60x, NA = 1.2 water immersion objective, and widefield illuminated by a 633 nm HeNe laser equipped with an FLH633-5 (Thorlabs) clean-up filter. Sample illumination and fluorescence collection are achieved by means of an F48-643 dichroic laser beam splitter (AHF Analysentechnik) with further fluorescence filters mounted between the objective and the imaging lens: 635LP EdgeBasic and 720SP Brightline (both from Semrock) for the ATTO647N and fluorescence-detected cell experiments and 647LP Razoredge (Semrock) for the fluorescent beads. The EV experiments are performed with a 470 nm light-emitting diode (LED; M470F3, Thorlabs) equipped with an HQ460/40M clean-up filter (Chroma) and coupled into a 500 \( \mu \)m multimode fiber. The output is then relay-imaged into the image plane of the microscope under a magnification of 1/6 resulting in an 83.3 \( \mu \)m illuminated area with a flat illumination profile. A 490 nm long-pass filter (DMLP490L, Thorlabs) separates excitation and fluorescent light, and a 488LP RazorEdge (Semrock) blocks residual excitation light. A 500 mm lens then generates the conjugate image at the entrance of the wavefront sensor. The 0-\( \pi \) phase grating is placed 500 \( \mu \)m offset with respect to this image plane, and the diffracted images are relay-imaged onto an ORCA Flash 4.0 sCMOS camera (C11440-22CU, Hamamatsu) by means of a 100 mm F/2.8 macro lens (Canon EF 100MM F/2.8 Macro USM) equipped with a hard aperture mask that removes all but the four first diffraction orders. The overall magnification is such that one camera pixel corresponds to 48.7 nm. A PXY 16 CAP 2D closed-loop piezo equipped with ENV40CAP amplifiers (Piezosystem Jena) is used for nanometric xy movement, whereas macroscopic displacements are realized with a manual translation stage. The z focus is controlled by a MIPOS 100UD SG M 25 closed-loop piezo equipped with an ENV40SG amplifier (Piezosystem Jena). The piezo movement is controlled by an NI-9263 (National Instruments) voltage source. The bright-field images are obtained by top illuminating the sample with the collimated output of a fiber-coupled LED equipped with a 710/40 BrightLine HC (AHF Analysentechnik) filter while using the same imaging system as for all other experiments.

Optical imaging

The fluence is adjusted to 2 kW/cm\(^2\) (experiments with fluorescent beads), 4.7 kW/cm\(^2\) (experiments with ATTO647N), 1 kW/cm\(^2\) (experiments with live cells and functionalized Au-NPs), and 50 W/cm\(^2\) (experiments with EVs). The proof-of-concept fluorescent bead experiments (Figs. 2 and 3) are performed at 10 frames per second (FPS) with five frames being averaged per image, the single-molecule samples are recorded at 10 FPS until full photobleaching, and the entire dataset is then averaged into one image. To allow long-term observation in the live-cell tracking experiments, we use integration times of 100 ms and imaging duty cycles of 1/20 (Au-NPs) and 1/40 (EVs), respectively. The sample is only illuminated during the acquisition period of each frame with the laser otherwise being blocked by a mechanical shutter (YS25 Uniblitz, Vincent Associates). As the EV experiments are performed with an LED, a simple electronic trigger is used instead of a mechanical shutter.

Proof-of-principle samples

Unless stated differently, all experiments are performed with \#1.5 borosilicate cover glass (VWR), which is cleaned by sonication in acetone followed by Milli-Q water (10 min each), dried under a stream of N\(_2\) followed by 20 min of ultraviolet-ozone cleaning (Bioforce ProCleaner-Plus). Single-molecule samples are prepared by spin coating a poly(methyl methacrylate)@toluene (2%, w/w) solution doped with ATTO647N (Atto-Tec) molecules, at sufficiently low concentration to allow for single-molecule observations, onto a freshly cleaned cover glass. Bead samples are obtained by spin coating an aqueous solution of polyvinyl alcohol (2%, w/w) doped with 200 nm fluorescent beads [FluoSpheres Carboxylate-Modified, 0.2 \( \mu \)m, crimson fluorescent (625/645), Molecular Probes] onto a freshly cleaned cover glass. Samples obtained by spin coating undoped solutions are used to check for possible contaminations. The EV experiments are performed while maintaining the sample and microscope objective temperature at 37°C using a TC-144 dual temperature controller (Warner Instruments).

EV preparation from cell culture

OVCA (ovarian cancer cells) cells are cultured in RPMI-1640 medium (Cellgro) with 10% fetal bovine serum (FBS) at 37°C in a humidified
atmosphere with 5% CO₂. Before EV collection, cells are grown in RPMI with 5% exosome-depleted FBS (Thermo Fisher Scientific) for 48 hours. Supernatants from cell culture media are centrifuged at 300g for 5 min to remove cell debris. Supernatant is filtered through a 0.2 μm membrane filter (Millipore). Filtered medium is concentrated at 100,000g for 70 min. After the supernatant has been removed, the EV pellet is resuspended in PBS and then centrifuged at 100,000g for 70 min. The EV pellet is resuspended in PBS and stored at -80°C. EV concentration is determined by Nano-particle Tracking Analysis (NanoSight, Malvern). The EVs’ mean particle size is determined to be 140.9 nm (see also note S8).

### Image propagation and 3D fitting

The aberration-corrected fluorescence holograms were propagated along the optical axis (z) according to the angular spectrum method. Briefly, the processed N x N holograms were convolved with a propagation kernel of the form

\[ K(x, y, z) = \exp(iz \sqrt{k_{\text{mp}}^2 - k_x^2 - k_y^2}) \]

where \( k_{\text{mp}} = 2\pi n/\lambda_s \), with \( n \) being the refractive index of medium through which the light is propagated through, in this case, water. The discretized spatial frequencies are \( (k_x, k_y) = 2\pi n/\Delta x(x, y) \) for \( -N/2 < x, y < N/2 \), with \( \Delta x \) representing the magnified pixel size of the imaging system.

For 3D localization, each hologram was first propagated from -5 to +5 μm with a spacing between different z planes (dz) of 100 nm. The resulting 3D fluorescence intensity map was then used to find local maxima. To achieve sub-pixel localization in the x, y coordinates, particles that were in focus at a specific z plane were fitted by a 2D Gaussian. For the z coordinate, sub-dz sampling localization was first calculated by finding the Tamura values \( T(z) = \sqrt{\sigma(z)/\text{mean}(z^2)} \) for a region of interest of \((=2 \times 2 \mu m)\) centered about the intensity maxima for each z plane and then fitting a parabola using the two most adjacent pixel values along the maximum.

### Supplementary Materials

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/45/eabc2508/DC1

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