A lateral nanoflow assay reveals nanoplastic fluorescence heterogeneity
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ABSTRACT: Colloidal nanoplastics present technological opportunities, environmental concerns, and measurement challenges. To meet these challenges, we develop a lateral nanoflow assay which integrates complex nanofluidic replicas, super-resolution optical microscopy, and comprehensive statistical analyses. We apply this sample-in-answer-out system to measure polystyrene nanoparticles which sorb and carry hydrophobic fluorophores. An elegant scaling of surface forces within our silicone devices hydrodynamically automates the advection and dominates the diffusion of the nanoparticles. Through steric interaction with the replica structure, the particle size distribution reciprocally probes the unknown limits of replica function. Multiple innovations in the integration and calibration of device and microscope improve the accuracy of identifying single nanoparticles and quantifying their steric diameters and fluorescence intensities. A statistical model of the measurement approaches the lateral information limit of the system, discriminates size exclusion from surface adsorption, and reduces nonideal data to return the particle size distribution with nanometer resolution. A Bayesian statistical analysis of the dimensional and optical properties of single nanoparticles reveals their fundamental structure-property relationship. Fluorescence intensity shows a super-volumetric dependence, scaling with nanoparticle diameter to nearly the fourth power and confounding basic concepts of chemical sorption. Distributions of fluorescence—an intrinsic property that we isolate and define as the product of the number density, absorption cross section, and quantum yield of an ensemble of fluorophores—are ultrabroad and asymmetric, limiting ensemble analysis and dimensional or chemical inference from fluorescence intensity. These results reset expectations for optimizing nanoplastic products, understanding nanoplastic byproducts, and applying nanoplastic standards.

KEYWORDS: nanofluidics, nanoplastics, nanoparticles, fluorescence

Colloidal nanoparticles of polymeric materials and organic chemicals are at the vanguard of commercial nanotechnology, with diverse applications ranging from environmental remediation to therapeutic delivery. At the same time, environmental contamination from both nanoparticle products and nanoplastic byproducts is of grave concern, with potential hazards of chemical sorption and tissue penetration. Quantifying the heterogeneous structures and properties of nanoparticles is fundamental both to optimizing product quality and to understanding byproduct hazards. However, measuring and correlating the property distributions of colloidal nanoparticles remains challenging. Precise and accurate measurements of dimensional and chemical properties, often by optical proxies, can be inefficient or impractical using conventional methods. For example, the combination of multiple methods of microscopy encounters the limits of preparing colloidal samples and sampling many single nanoparticles, while incurring the issue of calibrating and correlating the disparate image data. In contrast, rapid and economical measurements of nanoparticle ensembles in colloidal suspensions, such as by dynamic light scattering or fluorescence correlation spectroscopy, yield little information about property distributions. The combination of chromatographic methods with ensemble measurements provides access to more distributional information but still obscures population heterogeneity from variation among single nanoparticles and introduces challenges of surface interactions. In this way, conventional methods of nanoparticle characterization tend to achieve either quality or efficiency. For this reason, new methods are emerging to achieve both quality and efficiency, often by combining optical microscopy, nanoparticle tracking, and fluidic devices, with any method exhibiting certain capabilities and limitations. In the present study, we develop a nanofluidic and microscopic analogue of a lateral flow assay, achieving a new combination of quality and efficiency in colloidal metrology. We apply this assay to reveal that a common nanoparticle system—which is simultaneously a commercial nanotechnology, model nanoplastic, and unofficial standard—is hiding surprising optical properties in plain sight, with important implications.

In previous studies, we fabricated the first nanofluidic devices with complex three-dimensional structures in the form of confining staircases, separated colloidal polystyrene nanoparticles at staircase step-edges by size exclusion from shallower steps, and referred nanoparticle positions to step depths to measure particle size distributions with subnanometer accuracy and mean fluorescence intensity per step. In this way, our devices function as separation matrices and dimensional standards for use with an optical microscope. However, three main issues limit the practical application of our method, extending across as many domains of nanoscale science and technology. First, our devices require a large input of fabrication and characterization but have a short lifetime, as nanofluidic size-exclusion involves forcing nanoparticles into
confinement at the steric limit. This motivates the efficient replication of disposable devices in a laboratory context, but polymeric nanostructures, even in a production context, can be mechanically and chemically unstable.\(^2^9\)\(^3^0\) In particular, the elastic deformation of silicone can limit control of the structure and function of nanofluidic devices.\(^3^1\)\(^3^2\) Second, a high throughput of single nanoparticles across a wide field is necessary to sample a heterogeneous population. However, accurate measurements of position and intensity are uncommon in super-resolution microscopy,\(^3^3\) as errors result from nonuniform illumination, magnification, and various aberration effects. Third, nanoparticles can adsorb nonspecifically to device surfaces,\(^2^6\)\(^2^8\) resulting in spurious data that obscure the steric interaction of size exclusion, which is the basis of the measurement. Surface interactions are generally challenging for controlling the transport of nanoparticles through fluidic and chromatographic systems\(^3^4\) and could be limiting for nanoplastics with unknown surface properties.

We address these issues, developing our lateral nanofluidic assay from sample-in to answer-out, and enabling its practical application to a fundamental study of nanoplastic fluorescence heterogeneity. We advance the replication\(^3^5\)\(^-^3^7\) and characterization of complex nanofluidics near the atomic scale. An elegant scaling of surface forces within our silicone devices hydrodynamically automates the advection and dominates the diffusion of model nanoplastics. Through steric interaction with the replica structure, the particle size distribution reciprocal probes the unknown limits of replica function. Multiple innovations in transient confinement of nanofluidic device and optical microscope encompass the complete measurement, improving the accuracy of quantifying nanoparticles sizes and signal intensities. A statistical model of size exclusion accounts for nearly all dimensional parameters to approach the lateral information limit of the system, discriminates size exclusion from surface adsorption, reduces nonideal data to return the particle size distribution with nanometer resolution, and further elucidates hydrodynamic interactions in complex nanofluidics. On the basis of new precision, accuracy, and throughput, a Bayesian statistical analysis of the dimensional and optical properties of a model nanoplasic reveals the fundamental structure-property relationship of this common, if unofficial, standard. The fluorescence intensity of this sample shows a super-volumetric dependence, scaling with nanoparticle diameter to nearly the fourth power and confounding basic concepts of chemical sorption. Distributions of fluorescency—an intrinsic optical property that we isolate and define as the product of the number density, absorption cross section, and quantum yield of an ensemble of fluorophores that can interact within the bounding surface and dielectric volume of a nanoparticle—are ultrabroad and asymmetric, limiting common practices of ensemble analysis and inference of dimensional or chemical properties from fluorescence intensity. These results reset expectations for optimizing nanoparticle products, understanding nanoplasic byproducts, and applying nanoplasic standards.

**RESULTS AND DISCUSSION**

**Complex nanofluidic replicas.** Control of vertical dimensions is essential to our method, as step depths limit the separation resolution. Therefore, we begin by testing the fidelity of our replication process (Figure 1a-d).\(^3^8\) We machine complex molds in silica using a focused ion beam (Table S1) and we measure surface topography by atomic force microscopy.\(^2^9\) Both systems can resolve vertical dimensions at the atomic scale. In an initial test of pattern transfer, staircase structures with subnanometer steps form inverse replicas in silicone with a fidelity of approximately 0.1 nm (Figure S1).\(^1^6\) However, deposition of a fluoroisilane release-agent can increase surface roughness by up to 1 nm (Figure S2). In our devices, staircase structures in a parallel array decrease in both depth and width to maintain an aspect ratio that prevents channel collapse.\(^3^1\)\(^3^9\) Device patterns transfer (Table S2, Figure S3) first from a silica mold (Figure 1a, e) to an inverse replica in silicone (Figure 1b) and then to a silicone replica (Figure 1c, f). In this process, step depths of 1.8 nm ± 0.5 nm decrease on average by 0.3 nm ± 0.2 nm, and surface roughness increases from 0.65 nm ± 0.07 nm to 0.74 nm ± 0.07 nm (Table S3). We report uncertainties as 95 % coverage intervals. In a last test of pattern transfer, we form complex surfaces in nanoscale films of silicone (Figure S4). These results are necessary to quantify the subnanometer fidelity of pattern transfer, and enable future thin-film devices, but are still insufficient for our application. Accordingly, in an operando test, nanofluidic size-exclusion probes the unknown limit of replica structure and function through the steric interaction of colloidal nanoparticles with a reference size distribution. Silica coverslips enclose silicone replicas (Figure 1c-d), forming nanofluidic devices, which automate transport and separation of colloidal nanoparticles (Figure 1g-i, Scheme S1). Brief exposure of the coverslips and replicas to oxygen plasma\(^4^0\) hydroxylates their surfaces to promote bonding and to increase hydrophilicity, induce capillarity, and repulse nanoparticles with anionic surfaces, which are common in colloidal interfaces.

**Nanofluidic size exclusion.** An aqueous suspension of spherical polystyrene nanoparticles (Table 1), carrying boron-dipyrromethene fluorophores and having surfaces terminating in carboxylic acids, fills the complex devices. This sample-in process is effortless from the practitioner perspective and results in an innovative transport effect. We elucidate the theoretical hydrodynamics of the system, which exploits surface forces to selectively transport the suspension. While the device surfaces remain hydrophilic and are wetting, a capillary pressure drives fluid flow. As the staircase structures decrease in depth and width, the flow speed of the dispersion medium and resulting advection of nanoparticles increase, while hydrodynamic interactions hinder the diffusion of nanoparticles. The effect is increasing Brenner number\(^2^6\) of order 10^11 to 10^13 during size exclusion, despite the increasing diffusivity of nanoparticles of decreasing size (Figure 1g-h, Table S4, Figure S5). This unusual scaling suppresses Brownian noise, elegantly solving a problem of many schemes of colloidal separation. In a further test of the system hydrodynamics, we study size exclusion in channel arrays with variable depths. Hydraulic resistance and nanoparticle sampling are inversely proportional (Figure S6), informing the design of devices for measurements of nanoparticles with complex size distributions.

**Measurement system calibrations.** After size separation, brightfield microscopy forms images of fiducials (Figures 1f, S7), and fluorescence microscopy forms images of nanoparticles (Figure 1i). These optical micrographs are a rich source of data but are also rife with sources of error, including microscope illumination nonuniformity, nanoparticle image proximity, widefield imaging aberrations, and thin-film interference. We develop four calibrations to correct the resulting errors (Figure 2), integrating the nanofluidic device and optical microscope into a measurement system that achieves a high level of metrological reliability.

The first calibration is a flatfield correction. A featureless and autofluorescent film, with a nanoscale thickness just exceeding that of the deepest steps of the staircase structures, serves as a reference object (Figure 2b). Imaging this reference object forms a focal volume that closely resembles the interaction of the nanofluidic device and widefield irradiance for the different imaging modes. The resulting images manifest the illumination nonuniformities of brightfield and fluorescence microscopy. Analysis of the resulting signal intensities yields accurate flatfield corrections, which differ significantly due to the differences of illumination optics. The flatfield corrections significantly affect the results of localization and intensity analyses (Figure 2c, Figure 8).\(^3^1\)\(^4^1\) The second calibration is a point-spread-function filter. Device fiducials and sparse nanoparticles from control experiments provide reference images for both microscopy modes. Symmetric Gaussian models\(^4^2\) approximate the images of fiducials that begin to resolve and saturate in brightfield micrographs (Figure S9, Table S5)\(^4^5\) and subresolution nanoparticles in fluorescence micrographs (Figure S10).\(^3^2\) Beyond demonstrating the counterintuitive concept of localization above the saturation limit with subnanometer accuracy, Gaussian fitting enables filtration of localization data to preclude analysis of nanoparticles in the aperture limitation of the measurement system, which is an advantage over direct summation. Analysis of the standard deviations of sparse nanoparticle images, which resemble the point spread function with varying defocus, yields a threshold value of 175 nm at 95 % confidence to
discriminate between the images of sparse and proximate nanoparticles (Figure 2d-e, Figure S11). This maximizes sampling and minimizes errors from image overlap. We confirm that Gaussian integration underestimates signal intensity and establish a reliable proportionality between Gaussian integration and direct summation (Figure S12).

The third calibration is a position correction. An aperture array provides reference positions to correct aberration effects including distortion and defocus in both microscopy modes, improving localization accuracy by orders of magnitude across our ultrawide field. Analysis of apparent aperture positions for the different imaging wavelengths yields position corrections that differ significantly for fiducials and nanoparticles (Figure 2f-g, Figure S13). Depending on field position, the correction magnitude ranges from 10 nm to 120 nm. After correction, analysis of fiducial positions establishes the lateral placement accuracy of our focused ion-beam and quantifies localization errors with a root-mean-square value of 6 nm. This result is an important advance for the focused-ion-beam machining of complex devices with reference dimensions.

In comparison, nanoparticle images have smaller values of theoretical localization precision, decreasing from 10 nm to 1 nm as fluorescence intensity increases (Figure S13), emphasizing the importance of the position correction.

The fourth calibration is an intensity correction. A fluorophore solution, with an emission spectrum closely resembling that of our nanoparticles, fills the nanofluidic staircases to reveal interference effects in complex films of dielectric materials. The resulting relationship between nanofluidic depth and fluorescence intensity allows linearization of fluorescence intensity in replicas through an intensity correction of appreciable effect (Figure 2h-i, Figure S14). Replica autofluorescence is uniformly low, facilitating this calibration and enabling future detection of faint signals from colloidal nanoplastics and molecular adsorbates.

Statistical measurement model. With all calibrations complete, we are able to accurately refer nanoparticle positions to device depths to measure apparent diameters (Figure 3a-d). Step depths define the separation resolution and bin widths of diameter histograms (Figure 3c-d), exceeding Nyquist sampling in the discretization of the size distribution. In a statistical model of size exclusion, Monte-Carlo simulations account for many dimensional parameters of devices and nanoparticles, and their uncertainties (Figure 3a-b, Table S6, Figure S15). The resulting distributions
define the dimensions of size-exclusion regions and positions of nanoparticles, which reduce to 95% coverage intervals to reject outlying nanoparticles (Figure 3c). The combination of position and diameter data then allows rejection of pairs of nanoparticles with apparent distances between of less than the sum of the apparent radii in each size-exclusion region (Figure S16, Table S7). Approximately 40% of nanoparticles are in size-exclusion regions and 60% are in outlying regions during the measurement (Table S8). This analytical yield quantifies the hindrance by surface adsorption of nanoparticle advection toward size-exclusion regions. Results from comparable experiments (Figure S17) are robust to variation of oxygen plasma exposure and resulting surface properties, with a mean analytical yield of 35% ± 7% and apparent stability for up to 100 h, (Video S1), which is near the end of the duration of device capillarity. Smoother surfaces, as well as faster flow, may decrease adsorption and increase yield. At least as importantly, analytical discrimination of size exclusion from surface adsorption enables the robust analysis of colloidal nanoparticles with surface properties that are unknown. The measurement throughput is scalable through both focused-ion-beam machining and optical microscopy across wide fields. The result of thousands of nanoparticle images per optical micrograph, along with comprehensive calibrations, provides ample statistics to support our rigorous approach to data reduction.

**Particle size distribution.** Pooling size exclusion data from four comparable experiments gives a histogram of apparent diameters (Figure 4a). To analyze these data, we consider the manufacturer specification of nanoparticle diameter, which defines a 99.7% coverage interval, assuming a normal distribution. In a first analysis of the histogram, we use only the prior information of an upper bound of particle size. An estimate of this value is a prerequisite for device design and sample preparation, such as by filtration of larger particles. This minimal information must be available to future practitioners of our method. We interpret the 14% of apparent diameters that exceed this upper bound as nanoparticles that are spuriously in size-exclusion regions without the possibility of size exclusion. Interestingly, we find only a fraction of the nanoparticles that would result from a uniform probability of attractive interactions with device surfaces in these size-exclusion regions. Previous simulations support this result, indicating that local decreases of channel depth increase flow speed to yield repulsive hydrodynamic interactions and intrinsic robustness against attractive surface interactions near step edges. Quantification of the spurious analytical yield allows estimation of a noise floor and uniform correction factor for particle sizing (Figure 4b, Tables 1, S8), which is accurate to within a root-mean-square error of 2.9 nm. Making use of more prior information to test the limit of accuracy of our method at present, we analyze apparent diameters only within the 99.7% coverage interval of the manufacturer specification. Even without correcting for spurious size-exclusion within this range, we achieve accuracy (Tables 1, S8) to within a root-mean-square error of 1.9 nm, emphasizing the ability to accurately size nanoparticles, even from nonideal data.

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**Figure 2. Measurement system calibrations.** (a) Schematic showing a calibration sample for optical microscopy. Four embodiments follow. (b) Schematic showing a fluorescent film with a thickness just exceeding the device depth to form a focal volume for flatfield correction. (c) Plot showing the flatfield correction for epi-illumination fluorescence micrographs. (d) Schematic showing a control sample of sparse nanoparticles to quantify limits of the standard deviation of the point spread function (PSF). (e) Histogram showing standard deviations from (green) control measurements and (grayscale) experimental results in nanofluidic devices. (Dash green lines) A 95% coverage interval of the standard deviation of sparse nanoparticles, ranging from 102 nm to 175 nm, filters emitter images with larger standard deviations due to proximate nanoparticles in nanofluidic devices. (Gray tick mark) The theoretical value of the standard deviation is approximately 92 nm (Figure S11). Experimental values exceed 92 nm due to field curvature and defocus. (f) Schematic of an aperture array and fluorophore solution to provide reference positions for fluorescence micrographs. (g) Vector plot on a linear scale and color map on a logarithmic scale showing the position corrections of nanoparticles. The field dependence results from distortion among other aberrations. (h) Schematic of a nanofluidic staircase array and fluorophore solution to quantify interference effects in dielectric films. (i) Plot showing intensity correction for fluorescence intensity as a function of nanofluidic depth. Normalization is with respect to the intensity at the reference depth (black circle) of 106 nm ± 3 nm. Horizontal and vertical bars are increments of nanofluidic depth and 95% coverage intervals of the intensity correction.
Bayesian statistical analysis. Building on our sizing accuracy, we correlate the fluorescence intensities of single nanoparticles. This answer-out provides a key data set for Bayesian statistical analysis (Figure 5a-c). Surprisingly, the fluorescence intensity of single nanoparticles varies by almost two orders of magnitude around the mean diameter. Analysis of nanoparticles in isolation confirms this range (Figure S12), which greatly exceeds the variation of 1% that is attributable to occupancy statistics of 10^4 fluorophores per nanoparticle, according to ensemble measurements.28 This extreme heterogeneity motivates an analysis of the variation of intensity by two hierarchical models,52 which we term the power-law model and the mean values model. The former constrains the latter on the basis of the expectation that intensity is proportional to volume for these particles.28, 43 Our analysis takes inspiration from the definition of R^2 for Bayesian regression and, for the first time, propagates uncertainty completely to quantify the contribution of each source of variability to the total variability that we measure. The power-law model attributes variation of intensity to three sources—measurement uncertainty of intensity and diameter, a power-law dependence of intensity on diameter, and variation of fluorescivity. This model estimates a power of 3.7 ± 0.6 (Fig. 5b), which is consistent with our previous finding of a super-volumetric intensity of smaller nanoparticles,28 but reveals that variation of fluorescivity dominates variation of intensity, with attributions ranging from 0.67 to 0.85 (Figure 5d, Figure S18). Building on this finding, the mean-values model allows each diameter bin to have its own value of mean intensity without constraint. This model also determines that variation of fluorescivity causes most of the variation of intensity, with attributions ranging from 0.40 to 0.72 (Figure 5d, Figure S18). The mean-values model fits slightly better than the power-law model per Bayesian leave-one-out cross-validation and the widely applicable information criterion.54 Both models assume lognormal distributions of intensities for each diameter bin, which is consistent with the experimental data, indicating a multiplicative product of random processes of sorption of varying numbers of fluorophores to nanoparticles, with varying absorption of fluorescence excitation and yield of fluorescence emission due to heterogeneous molecular environments.

Nanoplastic fluorescence heterogeneity. Our deeper understanding of the fluorescence heterogeneity of a model nanoplastic has important implications. For example, previous studies have reported lognormal distributions of fluorescence

| Table 1. Nanoparticle diameters                        | mean value | error  | standard deviation | error |
|--------------------------------------------------------|------------|--------|--------------------|-------|
| manufacturer specification defining lower and upper bounds | 99.0 nm ± 0.6 nm | —      | 7.8 nm ± 0.6 nm    | —     |
| measurements including spurious results above upper bound| 105.0 nm ± 1.2 nm | 6.0 nm | 18.1 nm ± 0.8 nm   | 10.3 nm |
| measurements with uniform correction from upper bound   | 102.3 nm ± 0.9 nm | 3.3 nm | 10.2 nm ± 0.6 nm   | 2.4 nm |
| measurements within upper and lower bounds              | 99.8 nm ± 0.8 nm | 0.8 nm | 10.4 nm ± 0.6 nm   | 2.6 nm |

Manufacturer specification is by transmission electron microscopy.
intensity for polymeric nanoparticles,\textsuperscript{55} but the causes of such heterogeneity are unknown without correlative measurements of the sizes of single nanoparticles and statistical analysis of the structure–property relationship. As well, a super-volumetric intensity raises questions about the proportionality of fluorophore number to surface area or volume,\textsuperscript{26,28} while ultrabroad and asymmetric distributions of fluoroscivity can invalidate ensemble correlations to infer the sizes of single nanoparticles from fluorescence intensity.\textsuperscript{37} Moreover, heterogeneous distributions of fluorophores doping the surfaces and volumes of polymeric nanoparticles can affect the sorption and detection of toxic chemicals by ensemble analysis of fluorescence intensity.\textsuperscript{58} Similarly, heterogeneous properties of nanoparticles can confound understanding of ensemble analysis of the sorption of toxic chemicals to polystyrene nanoparticles with surfaces terminating in carboxylic acids.\textsuperscript{5,7} In these ways, from the sorption of toxic chemicals to polystyrene nanoparticles\textsuperscript{55} but the causes of such heterogeneity are unknown without correlative measurements of the sizes of single nanoparticles and statistical analysis of the structure–property relationship. 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The atomic-force microscopy.\textsuperscript{28} We characterize the thickness of the silica film, completing a device etching. We dice the substrates into chips that contain a microfluidic separation. We approach the lateral information limit of our microscopy measurement by precise and accurate identification, localization, and integration of nanoparticle signals in reference to replica topography across an ultrawide field to achieve high throughput. This enables a comprehensive analysis of size exclusion that is both intrinsically and analytically robust to spurious data from surface adsorption, which is important for future applications to measure nanoparticles with unknown surface properties. Defying the general expectation of dimensional instability for silicone nanofluidics,\textsuperscript{31,32} our replica stability supports a sizing accuracy approaching that of electron microscopy.\textsuperscript{59} In contrast, our measurement occurs in aqueous media and yields the optical properties of colloidal nanoparticles with high efficiency. In comparison to other emerging methods,\textsuperscript{22,23} including those that involve tracking single nanoparticles in confinement,\textsuperscript{19-21,60} our method enables record precision, accuracy, and throughput, in a readily deployable and inherently scalable device that obviates the need for thermometry or viscometry. Application of our lateral nanoflow assay to a ubiquitous nanoplastic sample results in a surprising data set. Bayesian statistical analysis confirms a super-volumetric dependence of the fluorescence intensity of molecular adsorbates in polystyrene nanoparticles, confounding the basic concepts of surface adsorption or volume absorption. Moreover, this analysis attributes most of the intensity heterogeneity to intrinsic fluoroscivity, which we define and isolate from nanoparticle size. Our finding indicates a multiplicative cascade of random processes from fluorophore sorption to fluorescence detection, permitting inferences from optical intensity, particularly in ensemble analyses. In these ways, our study deepens understanding of the fluorescence heterogeneity of what are among the most important nanoparticles in optical microscopy and flow cytometry, with many uses as probes, tracers, and fiducials. This unexpected characterization of common nanoparticles shows the utility of our method to understand and optimize structure–property relationships, even after decades of development and application of nanoparticle products. Moreover, our finding indicates the unofficial standard that we test is a model of nanoplastic byproducts that sorb toxic fluorophores, such as nonylphenol, phenanthrene, and pyrene. The composition, size, and surfaces of environmental nanoplastics can be only more heterogeneous than that of model nanoplastics, informing future studies of both types of samples. Other topics of interest include engineering device geometries and surface properties for sample preparation, including filtration and concentration, prior to analytical separation of nanoplastic mixtures, integration of thin films and fiducial arrays into fluidic devices to enable microscope calibration without extrinsic reference materials, and application of nanofluidic replicas to control and measure biomolecules.\textsuperscript{51,63}

**EXPERIMENTAL METHODS**

**Device fabrication.** We fabricate device molds on silicon substrates. We form arrays of microfluidic channels by photolithography and etching. We dice the substrates into chips that contain a microfluidic inlet and outlet and form thin films of silicon dioxide by thermal oxidation of the silicon chips. We machine arrays of nanofluidic staircase structures that connect the microfluidic inlets and outlets by focused-ionic-beam milling\textsuperscript{58} of the silica film, completing a device mold. We replicate device molds by soft lithography\textsuperscript{28} in two steps, first forming inverse replicas and then forming replicas in polydimethylsiloxane. We bond the silicone replicas to coverslips by bringing the surfaces into contact after brief exposure to oxygen plasma. We form submicrometer films of photoresist for optical microscopy and flatfield correction by spin-coating photoresist onto a coverslip and baking the film. We form submicrometer films of hard silicone by dilution and compression during the curing process.

**Device characterization.** We characterize the thickness of the silica film by ellipsometry. We characterize the surface topography of the silica molds, silicone inverse replicas, and silicone replicas by atomic-force microscopy.\textsuperscript{56} We characterize the thickness of photoresist films for flatfield correction by surface profilometry.

**Fluorescent nanoparticles.** We probe fluorescent polystyrene nanoparticles having carboxylate surface functionalization. The

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**Figure 5.** Dimensional and optical heterogeneity. (a) Marginal histogram showing the apparent diameters of all nanoparticles in size-exclusion regions. Red data is outside of the 99.7% coverage interval bounding a normal approximation of the diameter specification. (b) Two-dimensional histogram showing the fluorescence intensity of nanoparticles as a function of apparent diameter. Green circles are the mean-values model. The blue line is the power-law model. Bounds are 95% prediction intervals. (c) Marginal histogram showing the fluorescence intensity of all nanoparticles in size-exclusion regions. Lone bars in each plot are 95% coverage intervals near the limits of either distribution. (d) Plot showing the fractions of intensity variation that are attributable to diameter and fluoroscivity variation. Data markers are the mean values of posterior distributions. Solid lines are the major axes, or approximately 95%, of these distributions. The minor axes are comparable to line widths. 726 nanoparticles collapse to 14 bins due to similar uncertainties for each bin. The dash gray line indicates sum to unity, with a slight distance to the data markers being attributable to measurement uncertainty. For either model, fluoroscivity and diameter attributions are independent of nanoparticle diameter.

**CONCLUSIONS**

We develop a disposable nanofluidic device that upgrades an ordinary optical microscope into an extraordinary metrology system. Our sample-in-answer-out methodology enables integrative yet independent measurements of the steric diameter and fluorescence intensity of colloidal nanoparticles with high throughput. We study suspension hydrodynamics in complex confinement, elucidating a lateral nanoflow assay in which an elegant scaling of surface forces automates advection and dominates diffusion to achieve analytical separation. We approach the lateral information limit of our microscopy measurement by precise and accurate identification, localization, and integration of nanoparticle signals in reference to replica topography across an ultrawide field to achieve high throughput. This enables a comprehensive analysis of size exclusion that is both intrinsically and analytically robust to spurious data from surface adsorption, which is important for future applications to measure nanoparticles with unknown surface properties. Defying the general expectation of dimensional instability for silicone nanofluidics,\textsuperscript{31,32} our replica stability supports a sizing accuracy approaching that of electron microscopy.\textsuperscript{59} In contrast, our measurement occurs in aqueous media and yields the optical properties of colloidal nanoparticles with high efficiency. In comparison to other emerging methods,\textsuperscript{22,23} including those that involve tracking single nanoparticles in confinement,\textsuperscript{19-21,60} our method enables record precision, accuracy, and throughput, in a readily deployable and inherently scalable device that obviates the need for thermometry or viscometry. Application of our lateral nanoflow assay to a ubiquitous nanoplastic sample results in a surprising data set. Bayesian statistical analysis confirms a super-volumetric dependence of the fluorescence intensity of molecular adsorbates in polystyrene nanoparticles, confounding the basic concepts of surface adsorption or volume absorption. Moreover, this analysis attributes most of the intensity heterogeneity to intrinsic fluoroscivity, which we define and isolate from nanoparticle size. Our finding indicates a multiplicative cascade of random processes from fluorophore sorption to fluorescence detection, permitting inferences from optical intensity, particularly in ensemble analyses. In these ways, our study deepens understanding of the fluorescence heterogeneity of what are among the most important nanoparticles in optical microscopy and flow cytometry, with many uses as probes, tracers, and fiducials. This unexpected characterization of common nanoparticles shows the utility of our method to understand and optimize structure–property relationships, even after decades of development and application of nanoparticle products. Moreover, our finding indicates the unofficial standard that we test is a model of nanoplastic byproducts that sorb toxic fluorophores, such as nonylphenol, phenanthrene, and pyrene. The composition, size, and surfaces of environmental nanoplastics can be only more heterogeneous than that of model nanoplastics, informing future studies of both types of samples. Other topics of interest include engineering device geometries and surface properties for sample preparation, including filtration and concentration, prior to analytical separation of nanoplastic mixtures, integration of thin films and fiducial arrays into fluidic devices to enable microscope calibration without extrinsic reference materials, and application of nanofluidic replicas to control and measure biomolecules.\textsuperscript{51,63}
manufacturer sizes the dry nanoparticles by transmission electron microscopy, specifying a mean diameter of 99.0 nm ± 0.6 nm and a standard deviation of 7.8 nm ± 0.6 nm. We prepare a buffer system of \(0.1\times\) phosphate-buffered saline containing phosphate at a concentration of 1 mmol L\(^{-1}\) and sodium chloride at a concentration of 15 mmol L\(^{-1}\). We adjust the pH of the buffer to approximately 7.0 by adding hydrochloric acid and then we add nonylphenyl-polyethylene glycol at a volume fraction of 0.5%. We disperse the nanoparticles into this buffer at a number concentration of \(10^7\) mL\(^{-1}\) to \(10^9\) mL\(^{-1}\). We prepare a sparse array of fluorescent nanoparticles on a silica coverslip. To promote adsorption, we functionalize the coverslip surface with amino groups by vapor deposition of \((3\text{-}\text{aminopropyl) triethoxysilane.}\)

**Optical microscopy.** We record brightfield micrographs of silica molds (Figure 1a) and silicone replicas to show qualitative variation of vertical dimensions (Figure 1b, Figure S4). We record brightfield and fluorescence micrographs of various samples for quantitative analysis. We correct micrographs that we analyze quantitatively for errors that result from nonuniform intensity of illumination and nonuniform response of the imaging sensor. Subtraction of pixel value offsets from each micrograph and normalization of the resulting pixel values by the maximum values determines a flatfield correction factor for each pixel for each micrograph type (Figure S8). We perform localization analysis of images of device fiducials and nanoparticles by open-source software.\(^{44}\) We correct errors in position measurements that result from nonuniform magnification, among other optical aberrations, using an aperture array.\(^{33}\) We correct errors in intensity measurements that result from optical interference in nanofluidic replicas, which causes fluorescence emission intensity to vary non-linearly with device depth.\(^{28}\)

**Nanoparticle size analysis.** We infer the diameters of nanoparticles from their positions with respect to regions of size exclusion within device replicas. To compute size-exclusion regions for each step in a nanofluidic staircase, a Monte Carlo simulation accounts for statistical variance of device dimensions, as well as fiducial and nanoparticle locations (Table S6). We filter localization data of nanoparticles by excluding localization results of nanoparticles outside of regions of size exclusion and by excluding localization results of any nanoparticle pairs with positions that yield distances between nanoparticles less than the sum their radii. We establish a correction factor for diameter histograms by counting nanoparticles in deep regions of replicas where size exclusion should not occur.

**Nanoparticle intensity analysis.** We analyze the fluorescence intensity of nanoparticles sufficiently close to size-exclusion regions and of a diameter within a 99.7% coverage interval of the manufacturer specification. We assume that fluorescence intensities follow photon statistics from shot noise and construct Poisson distributions of intensity for each nanoparticle.\(^{46}\) We calibrate nanoparticle intensities for interference effects after background subtraction and flatfield correction and normalize the resulting quantities by their mean value.\(^{28}\)

**Bayesian statistical analysis.** We develop a Bayesian statistical analysis using two hierarchical models,\(^{32}\) which we refer to as the power-law model and the mean-values model. Hierarchical models allow for explicit incorporation of multiple sources of variability. We apply noninformative improper priors to express a state of ignorance about the model parameters before observing the data. We evaluate each model using open-source software,\(^{65}\) to attribute intensity variation to three fractional sources – measurement uncertainty of nanoparticle diameter and intensity, variation of diameter, and variation of fluorescence.

**ASSOCIATED CONTENT**

**Supporting information**

The Supporting Information includes additional details of experimental methods and supporting results, including theoretical and statistical models.

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K.-T.L. and A.C.M contributed equally. S.M.S conceived and supervised the study. S.M.S. designed the study with contributions from K.-T.L., A.C.M., C.R.C., and A.L.P. K.-T.L. and B.R.I. fabricated the devices. K.-T.L. and C.R.C. performed experiments with contributions from A.C.M. A.C.M. and C.R.C. performed analysis of data with contributions from S.M.S., except Bayesian statistical analysis, which A.L.P. performed solely. S.M.S. and A.C.M. prepared the manuscript with contributions from all authors.

**Notes**

The authors declare no competing interests, financial or otherwise.

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Supporting Information for
A lateral nanoflow assay reveals
nanoplastic fluorescence heterogeneity

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Experimental Methods

Device fabrication

Silicon substrates
We use prime-grade, p-type silicon (100) substrates, with a resistivity from 10 Ω cm to 20 Ω cm, a thickness of 525 µm, and a diameter of 100 mm.

Silicon chips
Our device layout for each chip consists of a single inlet and microfluidic channel to transport buffer solution and nanoparticle suspension into the deep side of nano-fluidic staircase structures, and a single micro-fluidic channel and outlet to extract buffer solution from the shallow side. We pattern an array of micro-fluidic features into a silicon substrate by photolithography and inductively-coupled plasma etching. We spin-coat photoresist onto the substrate to protect it during subsequent dicing into chips with lateral dimensions of 20 mm by 20 mm for thermal oxidation.

Silica film
We grow a silica film on silicon chips by thermal oxidation in a furnace at 1,100 °C and atmospheric pressure, with an oxygen flow rate of approximately 50 L min⁻¹ evacuates the bell jar for a duration of 45 s, vaporizing the TFOCS. Further details are in our previous study.2

Focused-ion-beam patterning
We mill patterns in silicon substrates and in silica films using a focused beam of gallium ions. Our focused-ion-beam system is from a commercial manufacturer, and we operate the system under typical conditions and using bitmap images for pattern control.1 The pattern parameters are in Table S1. We mill devices with ion-beam currents ranging from approximately 40 pA to 800 pA across pattern areas ranging from approximately 500 µm² to 6,400 µm² within 12 h. The resulting beam profile forms step edges with submicrometer widths (Figure S15). Lower currents can form narrower step edges but require longer milling times, which can result in defective nanostructures due to drift of the focused-ion-beam system.

| figure | dose (pC µm²) | ion-beam current (pC s⁻¹) | number of pixels | number of passes | pattern area (µm²) |
|--------|--------------|----------------|-----------------|-----------------|------------------|
| 1 (base) | 3.8 x 10³ | 8.3 x 10⁹ | 9.2 x 10⁹ | 2,850 | 5.7 x 10³ |
| 1 (steps) | 7.5 x 10³ | 8.7 x 10⁹ | 9.2 x 10⁹ | 5,320 | 5.7 x 10³ |
| 1 (fiducials) | 8.4 x 10³ | 8.7 x 10⁹ | 9.2 x 10⁹ | 6,000 | 5.7 x 10³ |
| S1a | 9.2 x 10³ | 4.3 x 10⁵ | 1.5 x 10⁵ | 90 | 6.3 x 10³ |
| S1b | 1.9 x 10³ | 1.0 x 10⁵ | 1.5 x 10⁵ | 75 | 6.3 x 10³ |
| S2 | 7.5 x 10³ | 1.0 x 10⁵ | 1.5 x 10⁵ | 30 | 6.3 x 10³ |
| S3a (base) | 2.1 x 10³ | 4.9 x 10⁵ | 1.6 x 10⁵ | 175 | 6.4 x 10³ |
| S3a (steps) | 1.1 x 10³ | 4.9 x 10⁵ | 1.6 x 10⁵ | 910 | 6.4 x 10³ |
| S3a (fiducials) | 2.4 x 10³ | 4.9 x 10⁵ | 1.2 x 10⁵ | 2,000 | 4.9 x 10³ |
| S4 | 7.5 x 10³ | 7.8 x 10⁵ | 1.6 x 10⁵ | 350 | 5.7 x 10³ |
| S6a (base)-top | 5.6 x 10³ | 9.3 x 10⁵ | 8.4 x 10⁵ | 3,790 | 5.3 x 10³ |
| S6a (steps)-top | 1.2 x 10³ | 9.1 x 10⁵ | 8.4 x 10⁵ | 7,937 | 5.3 x 10³ |
| S6a (base)-middle | 5.0 x 10³ | 9.2 x 10⁵ | 8.4 x 10⁵ | 3,374 | 5.3 x 10³ |
| S6a (steps)-middle | 1.2 x 10³ | 9.3 x 10⁵ | 8.4 x 10⁵ | 8,166 | 5.3 x 10³ |
| S6a (base)-bottom | 6.3 x 10³ | 9.5 x 10⁵ | 8.4 x 10⁵ | 4,174 | 5.3 x 10³ |
| S6a (steps)-bottom | 1.1 x 10³ | 9.6 x 10⁵ | 8.4 x 10⁵ | 7,937 | 5.3 x 10³ |
| S6a (fiducials) | 9.2 x 10³ | 9.6 x 10⁵ | 1.3 x 10⁶ | 6,000 | 7.9 x 10³ |

Dwell time per pixel is 1 μs for all cases.

Table S1. Pattern parameters

Pattern parameters for Figure 1 also correspond to Figures S7, S9, S10, S12, S13a-d, S14, S15, S17a-d, and Video S1.

Staircase structures
Our device design consists of an array of 20 staircase structures with a pitch in the y direction of 7.5 µm. Each structure has an inlet width of 2.5 µm and an outlet width of 0.5 µm, and a linear taper between the two widths along the structure length of 229.6 µm. Four bitmaps, including a base that underlies staircase steps, an inlet channel, and an outlet channel, form the pattern (Table S1).

Fluorsilanization of silica molds
We silanize silica molds with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (TFOCS) prior to each replication to ensure reliable release of silicone inverse replicas. We place the molds in a vacuum bell jar with a volume of approximately 3.6 L and deposit approximately 0.5 µL of TFOCS (Table S2) into a container adjacent to the molds. A mechanical vacuum pump with a free air displacement of approximately 50 L min⁻¹ evacuates the bell jar for a duration of 45 s, vaporizing the TFOCS. Further details are in our previous study.2
Table S2. Replication materials

| purpose          | product name     | chemical name                                    | quantity |
|------------------|------------------|--------------------------------------------------|----------|
| hard silicone    | Gelest VDT-731   | (7.0 % to 8.0 % vinylmethylsiloxane)-dimethylsiloxane copolymer, trimethylsiloxy terminated | 3.4 g    |
|                  | Sigma-Aldrich    | 2,4,6,8-tetramethylcyclotetrasiloxane             | 50 µL    |
|                  | Gelest platinum catalyst | platinum-divinyltetramethyldisiloxane | 18 µL    |
|                  | Gelest HMS-301   | (25 % to 35 % methylhydrosiloxane)-dimethylsiloxane copolymer, trimethylsiloxy terminated | 2 mL    |
| soft silicone    | Dow Corning Sylgard 184 | pre-polymer                                    | 15 g     |
|                  |                  | curing agent                                    | 1.5 g    |
| silanization     | United Chem. Tech. TFOCS | tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane | 0.5 µL  |

Typical recipe for silicone unless we note otherwise
We store HMS-301 at 4 °C
The quantity of Sylgard 184 is variable while the ratio of pre-polymer and curing agent is constant

Silicone inverse replicas
We modify our process of forming a bilayer of hard and soft silicone. Briefly, this process yields a microscale film of hard silicone under a milliscale film of soft silicone. To prepare the hard silicone, we mix 3.4 g of VDT-731 with 50 µL of modulator and 18 µL of platinum catalyst (Table S2). We degas the mixture in a vacuum bell jar using a mechanical vacuum pump, add 2.0 mL of HMS-301, and gently stir. We degas the mixture again, deposit it on a silica mold with TFOCS coating, and spin-coat the mixture onto the mold at 104.7 rad s⁻¹ (1,000 RPM) for 45 s. After curing in an oven at 80 °C for 5 min, we place the silica mold in a petri dish with a diameter of 60 mm and pour a soft silicone mixture over the silica mold. We cure the soft silicone in an oven at 80 °C for at least 4 h and peel off the inverse silicone replica.

Fluorosilanization of silicone inverse replicas
We apply the same process of silanizing silica molds with TFOCS to silicone inverse replicas for mold release. However, a single process of TFOCS silanization suffices for several tens of uses.

Silicone replicas
We prepare silicone replicas by a process that is similar to the molding of silicone inverse replicas. One important difference is that we apply three films of hard silicone to the silicone inverse replicas, curing in an oven at 80 °C for 5 min between subsequent films, before adding soft silicone, which we cure for 4 h in an 80 °C oven. Informal tests indicate that the trilayer of hard silicone increases mechanical rigidity and decreases elastic deformation of nanofluidic structures (not shown).

Submicrometer silicone films
We form a submicrometer film of hard silicone by three thinning processes. First, we dilute the hard silicone with an organic solvent to reduce its viscosity. We add a mixture of 3.4 g of VDT-731, 50 µL of modulator, and 18 µL of platinum catalyst to 2.25 g of hexane and mix by vortexing. We add 1.5 mL of HMS-301 to the mixture and vortex again. After degassing the dilute mixture in a vacuum bell jar with a mechanical vacuum pump, we sieve the mixture through a filter with a pore size of 0.2 µm. Second, we deposit the dilute mixture on a silicon staircase mold after TFOCS fluorosilanization and spin-coat at a frequency of 1,047 rad s⁻¹ (10,000 RPM) for 5 min. Third, we press the hard silicone film by a silicon substrate on which we place a mass of 2 kg, applying a constant pressure of approximately 125 Pa during curing for at room temperature for approximately 12 h. We remove the cover wafer, transferring the submicrometer film from the substrate mold to the cover wafer and obtaining a submicrometer film of hard silicone with an inverse staircase structure (Figure S4).

Silica coverslips
We use microscope coverslips of ultraviolet-grade fused silica with a thickness of approximately 170 µm, a root-mean-square surface roughness of less than 0.8 nm, and a surface quality with a scratch/dig specification of 20/10.

Device bonding
We punch holes through silicone replicas to make inlet and outlet reservoirs at the microchannel termini. We clean a silicone replica with methanol and a fused silica coverslip with piranha solution. Exposure of silicone replicas and silica coverslips to an oxygen plasma at a pressure of 27 Pa (0.2 Torr) and a power of 18 W for less than 30 s terminates both surfaces with silanol groups. We bring the two surfaces into contact at room temperature to bond them by condensation of the silanol groups.

Aminosilanization of silica coverslips
We functionalize silica coverslips with amino groups by vapor deposition of (3-aminopropyl) triethoxysilane (APTES) to promote adsorption of nanoparticles to coverslip surfaces for a control measurement. During the deposition of APTES, a nozzle with a head temperature of 150 °C
injects 2 mL of water with flow rate of 0.4 mL min\(^{-1}\) in discrete pulses with volumes of 0.1 mL per pulse over 300 s at a frequency of 66.7 mHz into a vacuum chamber at 100 °C and at a base pressure of approximately 133 Pa (1 Torr). After one purge cycle, the chamber pressure reduces to approximately 93 Pa (0.7 Torr), and a nozzle injects 0.3 mL of APTES at a rate of 0.1 mL per pulse over 900 s (0.02 mL min\(^{-1}\)) into the chamber.

**Photoresist film**

We form submicrometer photoresist films for optical microscopy and flatfield correction. We spin-coat polydimethylglutarimide photoresist onto a coverslip at 104.7 rad s\(^{-1}\) (1,000 RPM) for 60 s. We bake the coverslip and photoresist film first in an oven with a nitrogen flow rate of 50 mL min\(^{-1}\) (50 sccm) at 180 °C for 5 min, and then in a vacuum oven at 90 °C for 3 h.

**Device characterization**

We characterize the silica film into which we pattern molds by ellipsometry. The film thickness is 488 nm ± 2 nm and the index of refraction of the film is approximately 1.46 at a wavelength of 632.8 nm.

We characterize trilayer films of hard silicone after curing by surface profilometry. The thickness of each film is 22.6 µm ± 0.9 µm.

We characterize the surface topography of critical features of the silica mold, silicone inverse replica, and the silicon replica with the same atomic-force microscope and measurement parameters as in our previous study.\(^1\) However, we image silica surfaces at a line scan rate of 1.0 Hz, whereas we image silicone surfaces at a line scan rate of 1.5 Hz. Microfluidic channel depths are 0.48 µm ± 0.02 µm. We characterize the fidelity of pattern transfer from molds to inverse replicas and then to replicas, as well as the effects of fluorosilanization on replica surface roughness (Figures S1, S2, and S3). Table S3 presents results for test devices that share replication parameters with the microfluidic devices but differ in channel width and step depth to facilitate access of the probe tip of the atomic-force microscope. Nanofluidic structures have lateral dimensions that exceed the lateral range of our atomic-force microscope, so we measure the surface topography of four nanofluidic channels in regions of 36 µm by 36 µm throughout the 200 µm lateral extent of the staircase structure, as well as the fused silica coverslips that seal the devices after bonding. From the micrographs, we measure nanofluidic depth, root-mean-square surface roughness, and step-edge width. The diameter of the probe tip sets a lower bound of surface roughness\(^1\) through a steric interaction at a higher spatial frequency than that of a colloidal nanoparticle interacting with a device surface. We use these microscopy results to define statistical variables that propagate through our measurement model.

We characterize the thickness of photoresist films for flatfield correction by surface profilometry. The thickness is 210 nm ± 12 nm.

**Fluorescent nanoparticles**

**Manufacturer specifications**

We use polystyrene nanoparticles that are commercially available. The manufacturer synthesizes the nanoparticles by an emulsion-polymerization process, resulting in approximately spherical particles of amorphous polystyrene. The manufacturer measures the diameters of dry nanoparticles by transmission electron-microscopy, specifying a mean diameter of 99.0 nm ± 0.6 nm and a standard deviation of 7.8 nm ± 0.6 nm. After synthesis, the manufacturer disperses the nanoparticles into an organic solvent to sorb hydrophobic borondipyrrromethene molecules, resulting in fluorescent nanoparticles with a peak excitation wavelength of 505 nm and a peak emission wavelength of 515 nm. The manufacturer functionalizes the fluorescent nanoparticles with carboxylate groups at a surface density of 0.07 nm\(^{-2}\) ± 0.02 nm\(^{-2}\). Our analysis of ensemble measurements by the nanoparticle manufacturer implies that fluorescence intensity scales volumetrically with particle diameter.\(^1\)

**Nanoparticle suspension**

We prepare a buffer system of 0.1× phosphate-buffered saline containing phosphate at a concentration of 1 mmol L\(^{-1}\) and sodium chloride at a concentration of 15 mmol L\(^{-1}\). We adjust the pH of the buffer to approximately 7.0 by adding hydrochloric acid and then we add nonylphenyl-polyethylene glycol at a volume fraction of 0.5 %. The resulting buffer has an electrostatic screening distance of approximately 3 nm. We disperse the nanoparticles into this buffer at a number concentration of 10\(^6\) mL\(^{-1}\) to 10\(^7\) mL\(^{-1}\). We analyze the shape of pendant drops of the nanoparticle suspension to measure the interfacial tension of the nanoparticle suspension, and we analyze the shape of sessile drops of the nanoparticle suspension to measure the contact angle of the nanoparticle suspension on fused silica coverslips and planar pieces of hard silicone less than 0.5 h after exposure to oxygen plasma.

**Sparse array**

We prepare a sparse array of fluorescent nanoparticles on a coverslip. We disperse the nanoparticles into pure water at a number concentration of approximately 10\(^6\) mL\(^{-1}\) to 10\(^7\) mL\(^{-1}\) and sonicate the suspension with an input power of approximately 50 W for approximately 8 h. We deposit the suspension onto a coverslip with APTES functionalization. After particle adsorption to the coverslip surface, we enclose the suspension on the first coverslip with a second microscope coverslip for imaging.
Hydrodynamic transport

 Soon after bonding, we pipette the nanoparticle suspension into the device inlet. The device primarily exploits a capillary force, and to a much lesser extent a hydrostatic force, to induce flow of the suspension and advect nanoparticles into the array of staircase structures (Figure S1). We estimate the resulting rates of advective and diffusive transport. Relevant hydrodynamic variables are in Table S4. For each experiment, we wait at least 6 h before illuminating the sample and recording one fluorescence micrograph of nanoparticles in the staircase structures.

Scheme S1. Device schematic. Schematic showing capillary and hydrostatic forces, which advect a suspension of fluorescent nanoparticles into a nanofluidic staircase, where size exclusion occurs. Both the width and depth of the staircase decrease from right to left in the schematic.

Adective transport

We estimate the magnitude of the flow speed in the staircase. We calculate the pressure difference across the entire device, $\Delta p$, due to a capillary pressure difference from the air-suspension interface, $\Delta p_c$, which advances across the device as it fills, as well as a hydrostatic pressure difference, $\Delta p_g$, from the suspension column of the inlet of the device. As the device fills, the pressure difference from the capillary force exceeds that of the gravity force by a factor of $10^2$. We compute the volumetric flow rate for the entire device, $Q$, treating the microfluidic channels and the nanofluidic staircase array as a series of hydraulic resistances,

$\Delta p = \Delta p_c + \Delta p_g = \gamma \left( \frac{\cos \theta_l + \cos \theta_r}{w_{mc}} + \frac{\cos \theta_t + \cos \theta_b}{d_{mc}} \right) + \rho g h_{inlet} = QR = Q(R_{mc} + R_{sa})$  

(S1)

where, $\gamma$ is the surface tension of the suspension, $\theta_l = \theta_r = \theta_t = \theta_{silicone}$ are the contact angles of the suspension on the hard silicone walls at the left, right, and top of the channel of the device, $\theta_b = \theta_{silica}$ is the contact angle on the fused silica coverslip at the bottom of the device, $w_{mc}$ is the width of the microchannel, $d_{mc}$ is the depth of the microchannel, $\rho$ is the density of the suspension, $g$ is the acceleration of gravity, $h_{inlet}$ is the height of the fluid column above the microchannel, $R_{mc}$ is the hydraulic resistance of the microchannels, and $R_{sa}$ is the hydraulic resistance of the parallel array of staircase structures. Equation (S2) gives an estimate of the hydraulic resistance of fluidic channels with rectangular geometry,\(^5\)

$$R_i = \frac{12 \eta l_i}{w_i d_i^2 \left( 1 - 0.630 \frac{d_i}{w_i} \right)}$$  \hspace{1cm} (S2)

where $\eta$ is the dynamic viscosity of the suspension, $l_i$ is the length, corresponding to the $x$ direction of the $i^{th}$ segment of the channel, $w_i$ is the width corresponding to the $y$ direction of the $i^{th}$ segment of the channel, and $d_i$ is the depth, corresponding to the $z$ direction of the $i^{th}$ segment of the channel. The microfluidic channels are approximately rectangular, so we calculate $R_{mc}$ directly from equation (S2). In contrast, we calculate $R_{sa}$ as the equivalent hydraulic resistance of the parallel array of 20 staircases. In turn, we calculate the resistance of each staircase in the array, $R_s$, as a series of hydraulic resistances of each of the 36 steps,

$$R_{sa} = \left[ \sum_{i=1}^{N_s} \frac{1}{R_s} \right]^{-1} = \frac{1}{N_s} \sum_{i=1}^{N_s} R_s = \frac{1}{N_s} \sum_{i=1}^{N_s} \frac{12 \eta l_i}{w_i d_i^2 \left( 1 - 0.630 \frac{d_i}{w_i} \right)}$$  \hspace{1cm} (S3)
where $N_i$ is the number of staircases in the array and $N_r$ is the number of steps in each staircase. After solving equation (S1) for $Q$, we calculate the pressure difference across the $i^{th}$ step of the staircase, $\Delta p_i$, in terms of the hydraulic resistance at each step.

$$\Delta p_i = \frac{Q}{N_r} R_i \quad (S4)$$

Estimation of the volumetric flow rate and the total volume of the device allows us to approximate a lower bound on the time necessary to fill the entire device, $t_{fill}$. Estimation of the pressure difference across the device allows us to approximate the magnitude of the flow speed in the $x$ direction at each step, $u_{x,i}$, which we calculate using the analytical solution to the Navier-Stokes equation for laminar flow in a rectangular channel at small scales,$^6$

$$u_{x,i}(y, z) = \frac{4d_i \Delta p_i}{\pi^2 \eta L_i} \sum_{n=1,3,5,\ldots}^{\infty} \frac{1}{n^2} \left[ 1 - \frac{\cosh \left( \frac{n\pi}{2} \frac{y}{d_i} \right)}{\cosh \left( \frac{n\pi}{2} \frac{w_i}{2d_i} \right)} \right] \sin \left( n\pi \frac{z}{d_i} \right). \quad (S5)$$

**Diffusive transport**

We estimate the lateral diffusivity of nanoparticles near regions of size exclusion. We begin by calculating the diffusion coefficient of a spherical nanoparticle in free solution, far from any confining surfaces, using the Stokes-Einstein relation,$^7$

$$D_0 = \frac{k_B T}{6\pi \eta a} \quad (S6)$$

where $k_B$ is the Boltzmann constant, $T$ is absolute temperature, and $a$ is the radius of the nanoparticle. Hydrodynamic interactions between the nanoparticle and the floor and ceiling of our device hinder the diffusion of nanoparticles near regions of size exclusion. To conservatively estimate an upper bound diffusion of single nanoparticles in the device, we ignore hydrodynamic interactions from the Poiseuille flow, which can only reduce diffusive transport.$^8$-11 For simplicity, we also ignore hydrodynamic interactions between nanoparticles. A nanoparticle suspension with number concentration of $10^8 \text{ mL}^{-1}$ to $10^9 \text{ mL}^{-1}$ has a mean interparticle distance of approximately 10 µm. This value is a factor of 10$^2$ larger than the mean diameter of the nanoparticles, allowing us to reasonably neglect hydrodynamic interactions between diffusing nanoparticles. As the nanoparticles reach their terminal positions and concentrate near size-exclusion regions, we expect the mean interparticle distance to decrease. Accordingly, we expect hydrodynamic interactions between particles to increase in magnitude and further reduce diffusivity. Considering only the hydrodynamic interactions between nanoparticles and the surfaces of the device, the diffusivity of a nanoparticle in uniaxial confinement is,

$$D_{2w||i}(z, a, d_i) = D_0 f_{2w||i}(z, a, d_i), \quad (S7)$$

where $f_{2w||i}(z, a, d_i)$ is the correction term from the linear superposition approximation of the diffusivity in free solution for a single nanoparticle in uniaxial confinement between two parallel planar surfaces.$^{12}$ This correction term ranges from 0 to 1 and is, itself, a function of the correction terms for a single nanoparticle near a single planar surface, $f_{1w||i}(z, a)$,

$$f_{2w||i}(z, a, d_i) = \left[ \frac{1}{f_{1w||i}(z, a)} + \frac{1}{f_{1w}(d_i - z, a)} - 1 \right]^{-1} \quad (S8)$$

$$f_{1w||i}(z, a) = 1 - \frac{9}{16\omega} + \frac{1}{8\omega^2} - f(\omega) \quad (S9)$$

where $\omega = z/a$ and,

$$f(\omega) = \begin{cases} 
\frac{15}{8} \ln(\omega - 1) - \frac{9}{16} \frac{1}{\omega} + \frac{1}{8} \frac{1}{\omega^3} + \exp[1.80359(\omega - 1)] + 0.319037(\omega - 1)^{0.2592} & \omega \leq 1.1 \\
\frac{45}{256} \frac{1}{\omega^4} + \frac{1}{16} \frac{1}{\omega^5} - \frac{0.220205}{\omega^6} + \frac{0.205216}{\omega^7} & \omega > 1.1
\end{cases} \quad (S10)$$

**Brenner number**

We compare magnitudes of advective transport to diffusive transport by calculating the Brenner number, $Br$, for nanoparticles near reagions of size exclusion at each step of the staircase.$^{13}$ We compute the mean magnitude of the flow speed at the $i^{th}$ step of the staircase, $u_{x,i}$, which we estimate theoretically, a characteristic length scale equal to the mean width of the size exclusion regions ($w_{SER,i}$), and the lateral diffusivity resulting from the uniaxial confinement of nanoparticles in the staircase.

$$Br_i(z, a, d_i) = \frac{u_{x,i}(w_{SER,i})}{D_{2w||i}(z, a, d_i)} \quad (S11)$$
Device aging
After exposure to oxygen plasma, we expect the hydrophilicity of the silicone and silica surfaces to decrease,\textsuperscript{14, 15} reducing the magnitude of the capillary pressure difference across the air-suspension interface and slowing advection. We estimate that the contact angles of the nanoparticle suspension on either silica or hard silicone increase from approximately 0.25 rad (14°) soon before the experiment to approximately 1.2 rad (70°) after 6 h, when we record fluorescence micrographs of the nanoparticles in confinement. This aging effect would reduce the advection rate and Brenner numbers by a factor of approximately 3. After approximately 10^2 h, as the hydrophobicity of the silicone replicas recovers, we expect capillarity to approach the end of its useful duration to drive hydrodynamic transport in the device.

Reference materials
Photoresist film
We use a photoresist film on a coverslip to develop flatfield corrections. The flatness, thickness, transparency, and autofluorescence of the photoresist film enable its use as a universal artifact for each microscopy mode in this study.

Aperture array
We use an aperture array with a mean pitch of 5,000 nm ± 1 nm.\textsuperscript{16} This uncertainty of 0.02 % is an estimate of the effect of placement accuracy in the fabrication of the aperture array by electron-beam lithography. Moreover, reconfiguration of the microscope system can affect the apparent value of mean pitch by up to 0.07 %.\textsuperscript{16} Considering these sources of error and estimates of uncertainty, we take a value of 0.1 % with a uniform coverage interval as an uncertainty estimate for the aperture array pitch in this study.

Optical microscopy
Micrograph acquisition
We record brightfield micrographs of molds in silica (Figure 1a) and replicas in silicone to show qualitative variation of vertical dimensions (Figure 1b, Figure S4). For these inspection micrographs, a light-emitting diode illuminates the samples with a wavelength range of 370 nm to 700 nm. An objective lens with a nominal magnification of 50×, a numerical aperture of 0.95, and corrections for chromatic and flatfield aberrations focuses illumination and collects reflection through air immersion. A tube lens projects images onto a color charge-coupled device (CCD) camera with an on-chip pixel size of 4.54 µm.

We record brightfield and fluorescence micrographs of a variety of samples for quantitative analysis. For both types of micrographs, a light-emitting diode illuminates the samples at a peak wavelength of approximately 460 nm with a full width at half maximum of less than 27 nm. For brightfield micrographs, a beamsplitter with 50 % reflection and 50 % transmission directs light within the microscope system. For brightfield micrographs of photoresist films, a neutral-density filter attenuates the illumination intensity. For brightfield micrographs of aperture arrays, we transilluminate an empty aperture array. For fluorescence micrographs, an excitation filter with a bandpass from 430 nm to 475 nm, a dichroic beamsplitter with a transition at 495 nm, and an emission filter with a bandpass from 503 nm to 548 nm discriminate between fluorescence excitation and emission. For fluorescence micrographs of aperture arrays, we fill the aperture array with a solution of fluorescent molecules with a concentration of approximately 100 µmol L\textsuperscript{-1} and an emission spectrum closely resembling that of the fluorescent nanoparticles. For fluorescence micrographs of nanoparticles, an exposure time of 100 ms results in sufficiently high signals for precise localization and integration. For both types of optical micrographs, an objective lens with a nominal magnification of 63×, a numerical aperture of 1.4, and corrections for chromatic and flatfield aberrations focuses illumination and collects emission, reflection, or transmission through oil immersion. A tube lens projects the image onto a complementary metal–oxide–semiconductor (CMOS) camera with 2,048 pixels by 2,048 pixels, each with an on-chip size of 6.5 µm by 6.5 µm. A mean factor of 2.0 converts from photoelectrons to analog-to-digital units, per the specification of the camera manufacturer. We operate the camera at a sensor temperature of -10 °C by thermoelectric and water cooling, without on-board correction of pixel noise, and in fast-scan mode, and we calibrate the imaging system for these parameters. For experiments in which we localize device fiducials and fluorescent nanoparticles, we record brightfield optical micrographs of device fiducials for registration immediately before we record fluorescence micrographs of nanoparticles.

The microscope systems equilibrate for at least 1 h before we record optical micrographs near the z position of best focus.

We show all optical micrographs after flatfield correction and background subtraction.

Flatfield corrections
To reduce errors in measurements of intensity and position that would otherwise result from nonuniform intensity of illumination and nonuniform response of the imaging sensor, we develop three independent flatfield corrections for the three imaging modes of transillumination brightfield microscopy of aperture arrays, epi-illumination brightfield microscopy of device fiducials, and epi-illumination fluorescence microscopy of aperture arrays and nanoparticles. In a previous study, we showed that the flatfield correction for our imaging sensor is approximately independent of signal intensity.\textsuperscript{16} In this study, we record optical micrographs of photoresist films with intensity values near the middle of the sensor range. We record and average 1,000 transillumination brightfield micrographs at a single region of the film, and
approximately 100 epi-illumination brightfield micrographs and approximately 100 epi-illumination fluorescence micrographs each at different regions of the films. We filter the epi-illumination brightfield micrographs and epi-illumination fluorescence micrographs by inspection to reject any micrographs with photoresist defects. Subtraction of pixel value offsets and normalization of the resulting pixel values by the maximum values determines a flatfield correction factor for each pixel for each micrograph type (Figure S8). The selection of maximum value, rather than mean value, for normalization is arbitrary and enables flatfield correction of micrographs with pixel value saturation. Flatfield correction converts pixel values from analog-to-digital units to arbitrary units with a maximum value of 65,535.

**Localization analysis**

For pixel values without saturation, subtraction of pixel value offsets and division of the resulting pixel values by the corresponding flatfield correction factors prepares images for localization analysis by open-source software.17 We do not modify pixel values with saturation. Input settings for our CMOS camera include a root-mean-square readout noise of 1.9 electrons, a mean value of conversion factor of 2.0 analog-to-digital units per photoelectron, and an image pixel size of 100.05 nm for brightfield micrographs and 100.63 nm for fluorescence micrographs after position correction, as we describe below. We neglect the effect of flatfield correction on shot noise in maximum-likelihood estimation. Approximation of the variable readout-noise of individual pixels by their root-mean-square readout noise causes a negligible error (Figure S13). A wavelet transform approximates initial locations of fiducials and nanoparticles by applying a threshold filter with a basis spline order of three and scale of 2.0. Peak intensity thresholds of the standard deviation of the first wavelet level of each input image correspond to local maxima for neighborhoods of eight pixels. A multiple-emitter algorithm localizes nanoparticles that can be in proximity near the limit of imaging resolution. The algorithm fits symmetric Gaussian approximations of the point spread function of the microscope system, PSF\(_{G}(x, y)\), to each emitter image by maximum-likelihood estimation, yielding measurements of the signal intensity, \(I_j\), standard deviation of the Gaussian model, \(\sigma_{ij}\), x position, \(x_0\), y position, \(y_0\), and background signal level, \(b_{Gj}\), of the of the \(j\)th nanoparticle.18

\[
\text{PSF}_{Gj}(x, y) = \frac{I_j}{2\pi\sigma_{ij}^2} \exp\left\{-\frac{(x-x_0)^2 + (y-y_0)^2}{2\sigma_{ij}^2}\right\} + b_{Gj} \tag{S12}
\]

Various fitting parameters constrain the analysis to yield reproducible results, including a fitting radius of 13 pixels, an initial standard deviation of the Gaussian model of 3.0 pixels, a maximum number of emitters per region of interest of 5, and a model selection threshold of \(1 \times 10^{-6}\). The same parameters apply to all micrographs. Localization results include x and y positions, standard deviations of Gaussian models, total signal intensity, offset, and a standard deviation of the background signal for each region of interest.

**Fiducial images**

In fiducial images, some pixel values saturate due to high intensities of brightfield micrographs. We test the effect of pixel value saturation on localization accuracy by simulating and localizing symmetric Gaussian approximations of fiducial images with signal intensities that, after pixelation, approach and then exceed 65,535 arbitrary units in some pixels. We limit these pixel values to a maximum of 65,535 arbitrary units, reproducing the saturation limit of our 16-bit imaging sensor (Figure S9). We simulate 5,000 images that closely resemble the experimental images, with corresponding values of image pixel size, Gaussian standard deviation, signal-to-noise ratio without and with saturation, number of pixels that saturate, and relevant parameters of the CMOS imaging sensor including variable offset, total noise, and response.16 We localize synthetic images of fiducials without and with saturation to assess localization accuracy (Table S5).

**Nanoparticle images**

For nanoparticle images, we reject any localization result with an intensity less than 5,000 arbitrary units and a theoretical localization precision\(^19\) of more than 10 nm. We also reject any replicate results that coincide in position within a factor of 10 of the theoretical localization precision of single nanoparticles, retaining the result with the lowest uncertainty in the group (Table S7, Figure S10). We then filter the localization data of nanoparticles in replicas to preclude potential analysis of apparent agglomerates, which have standard deviations of the symmetric Gaussian approximation of the point spread function in excess of those of single nanoparticles (Figure S11). In a control experiment, we record epi-fluorescence micrographs of a sparse array of nanoparticles on a microscope coverslip. We fit symmetric Gaussian functions to the nanoparticle images as we describe above, compute a histogram of standard deviations, and identify the lower and upper bounds of the 95 % coverage interval of the distribution of standard deviations. This provides a statistical characterization of the point spread function of single nanoparticles across the imaging field, including effects of optical aberrations. We filter nanoparticles with standard deviations outside of this range, omitting 1,686 nanoparticles or approximately 35 % of the emitters that pass the intensity and replicate filters, which we apply prior to the analysis of size exclusion (Table S7).

**Position calibration**

We correct errors in position measurements that result from nonuniform magnification, among other optical aberrations, using an aperture array. For both brightfield and fluorescence microscopy, we record a series of micrographs of the aperture array through focus in axial increments of 10 nm, and localize each aperture in each micrograph. A similarity transformation registers this set of aperture positions to those in an ideal array with a pitch of 5,000 nm, with the transformation scale factors providing mean values of image pixel size. The effects of optical aberrations, placement precision from the nanofabrication process, and fitting errors produce registration errors that have both random and systematic components, the latter showing a complex dependence on position in the imaging field. A linear combination of Zernike polynomials
models these systematic errors, providing a function to correct positions. This correction function has an axial dependence which requires that the z position of reference micrographs match those of the nanoparticle and fiducial position data from the nanofluidic device. For brightfield micrographs, we accomplish this by selecting from the micrograph series the correction function that, upon application to position data for device fiducials, best returns the fiducial positions in a row. For fluorescence micrographs, we accomplish this by selecting from the series the micrograph at best focus. The results of these position corrections are in Figure S13.

Intensity calibration
We correct errors in intensity measurements that result from optical interference in nanofluidic replicas, which causes fluorescence emission intensity to vary non-linearly with device depth. We correct this interference effect by filling the nanofluidic staircase with an aqueous solution of a fluorophore that has an emission spectrum closely resembling that of the fluorescent nanoparticles and that also has carboxylate terminal groups. Phosphate buffered saline with an ionic strength of 0.1 mmol L\(^{-1}\) phosphate and 1 mol L\(^{-1}\) sodium chloride reduces the electrostatic screening distance to approximately 0.3 nm\(^2\) and minimizes any effects of electrostatic repulsion on filling uniformity. A fluorophore concentration of 4 mmol L\(^{-1}\) results in high values of emission intensity in fluorescence micrographs. We measure the mean emission intensity of fluorophore solution filling each step of the staircase from regions with widths of at least 2 \(\mu\)m and lengths of at least 5 \(\mu\)m. We measure corresponding values of mean emission intensity from background just outside of the nanofluidic staircase and analyze their spatial variation due to nonuniform excitation intensity. Normalization of this function by its mean value yields a multiplicative correction for the fluorescence intensities of nanoparticles in the staircase. Subtraction of background emission intensity from fluorophore emission intensity, and division of the resulting values by the multiplicative correction, results in fluorescence intensity in arbitrary units as a function of nanofluidic depth (Figure S14). We normalize fluorescence intensity and nanofluidic depth by dividing them by their values in the center of the device, which corresponds to a nanofluidic depth of 106 nm ± 3 nm. We calculate a ratio of fluorescence intensity after normalization to nanofluidic depth after normalization as a calibration function for the subsequent analysis of single nanoparticles.

Nanoparticle size analysis
Reference analysis
We refer nanoparticles in size-exclusion regions to nanofluidic depths. Our device design includes fiducials at step edges of staircase structures as position references. Correction of fiducial and nanoparticle positions (Figure S13) reduces root-mean-square position errors in the x direction to less than 6 nm. Fitting correct y positions of fiducials to correct x positions of fiducials models the positions o tests f step edges in image space, \(r = (x, y)\). Uncertainties in the linear fit-parameters propagate into the vector, \(e_i = (e_{ix}, e_{iy})\) and offset, \(o_i\), of the hyperplanes, \(H_i; c \cdot r + o_i = 0\), that define the centers of each size-exclusion region. We constrain \(c_{ix}\) to unity, which yields \(c_{ix}\) as the slope of the line corresponding to fiducials of the \(i\text{th}\) step in the replica. To compute size-exclusion regions for each step, a Monte-Carlo simulation accounts for statistical variance of device dimensions, as well as fiducial and nanoparticle locations (Table S6). Summation of the nanofluidic depth, which we approximate as a uniform distribution between steps \(i\) and \(i+1\), with uncertainties from the root-mean-square surface roughness of the replica and the coverslip and relevant uncertainties from atomic force microscopy, yields the diameter, \(2a_i\), of nanoparticles near the \(i\text{th}\) step edge.

\[
d_i = 2a_i = \mathcal{U}(d_i, d_{i+1}) + \mathcal{N}(0, R_{ix}^2) + \mathcal{N}(0, R_{iy}^2) + \mathcal{N}(0, \sigma_{\text{calibration}}^2) + \mathcal{N}(0, \sigma_{\text{roughness}}^2) + \mathcal{N}(0, \sigma_{\text{flatness}}^2),
\]

where \(\mathcal{U}\) and \(\mathcal{N}\) denote uniform and normal distributions, \(d_i\) and \(d_{i+1}\) denote device depth from atomic force microscopy, \(R_{ix}\) and \(R_{iy}\) denote root-mean-square surface roughness of the replica and coverslip, respectively, and \(e_{\text{calibration}} = \mathcal{N}(0, \sigma_{\text{calibration}}^2)\) denotes a relative uncertainty of 0.5% from calibration of the atomic-force microscope, \(e_{\text{roughness}} = \mathcal{N}(0, \sigma_{\text{roughness}}^2)\) is the uncertainty from the configuration of scan rate, scan resolution, and probe-tip radius, and \(e_{\text{flatness}} = \mathcal{N}(0, \sigma_{\text{flatness}}^2)\), accounts for lateral flatness errors.

Step edges, \(\xi_i\), also broaden size-exclusion regions. Atomic-force micrographs show step edges with profiles that we approximate by error functions. The widths of these error functions follow a lognormal distribution (Figure S15). These widths propagate into a horizontal offset, \(x_{\text{offset}, i}\), into lines of best fit of fiducial locations, \(y_{\text{FER}, i}\), which define the centers of the size-exclusion regions:

\[
x_{\text{offset}, i} = \frac{d_i}{2} + \frac{\mathcal{N}(0, \ln(\sigma_{\text{calibration}} \cdot \mu_{\text{calibration}} \cdot s_{\text{calibration}}))}{\cos(\arctan(-\frac{1}{c_{ix}}))},
\]

\[
y_{\text{FER}, i} = c_{ix}(x - x_{\text{offset}, i}) + o_i
\]

where \(\mathcal{LN}\) denotes the lognormal distribution with shape, location, and scale parameters, \(\sigma_{\text{calibration}}, \mu_{\text{calibration}},\) and \(s_{\text{calibration}},\) respectively, and the bracket operator, \(\langle ... \rangle\), denotes the expectation value of the distribution. As such, the distribution that we derive for \(x_{\text{offset}, i}\) incorporates uncertainties from a complete set of measurements of nanofluidic depth and the theoretical localization precision of fiducials, \(\sigma_{\xi_i}\).

Iterating through step edges, we refer nanoparticle locations, \(r_j = (x_j, y_j)\) to nanofluidic depths, \(d_i = 2a_i\), by computing stochastically the Euclidian distance, \(D_{ij}\) between the center of the \(i\text{th}\) size-exclusion regions and the \(j\text{th}\) nanoparticle location.
The field of radiometry provides a framework for our measurements of fluorescence intensity. Quantitative radiometry also presents issues of nomenclature\textsuperscript{21} which motivate a clear definition of our measurands and their units. The fluorescence intensity of a nanoparticle is the radiant power emission from the nanoparticle per unit solid angle, and is equivalent to the photon flux emission from the nanoparticle per unit solid angle. Fluorescence intensity is a quantity that results from spatial and spectral integration of the fluorescence spectral radiance of the nanoparticle, $L_{ij}(\lambda)$, which is the radiant power emission from the nanoparticle, per unit wavelength, per unit solid angle, per unit area of a projection onto the imaging sensor, and is equivalent to the photon flux emission from the nanoparticle, per unit wavelength, per unit solid angle, per unit area of projection onto the imaging sensor. The fluorescent spectral radiance of the $j$\textsuperscript{th} nanoparticle relates the fluorescence intensity of the nanoparticle, $I_j$, to three intrinsic optical properties — the number density of fluorophores, $N_j V_j^{-1}$, the absorption cross section of the ensemble of these fluorophores, $\sigma_j(\lambda)$, and the spectral quantum yield of the ensemble of fluorophores, $\phi_j(\lambda)$,

$$I_j = \iint_S \int_{\Delta \lambda} L_{ij}(\lambda) \, d\lambda \, dx \, dy = \iint_S \int_{\Delta \lambda} I_0 \frac{N_j}{V_j} \sigma_j(\lambda) \phi_j(\lambda) \, d\lambda \, dx \, dy,$$

(S17)

where $I_0$ is the incident power of the excitation, $\Omega$ is a geometric factor of the optical system, $V_j$ is the volume of the $j$\textsuperscript{th} nanoparticle, $\Delta \lambda$ is the spectral bandwidth of the emission, and $S$ is the spatial extent of the region of interest for each nanoparticle image.\textsuperscript{22}

We account for spatial nonuniformity of $I_0$, as well as variable responses of individual pixels, by our flatfield correction. We then measure a signal that is proportional to fluorescence intensity, $I_j$ of single nanoparticles. We temporally integrate over the exposure time, $\Delta t_{\text{expo}}$, of each micrograph, spectrally integrate over the bandpass of our emission filter, and spatially integrate over the imaging region, the fluorescent spectral radiance of the images of single nanoparticles, which we model as symmetric Gaussian functions.
\[ I_j \propto \int_{S_{xy}} \int_{S_{z}} \int_{S_{\lambda}} N_j \sigma_j(\lambda) \phi_j(\lambda) \, d\lambda \, dx \, dy. \]  

(S18)

We define the fluorescivity of the ensemble of \( L \) fluorophores within the \( j \)th nanoparticle,

\[ \bar{y}_j = \frac{N_j}{\sigma_j} \sigma_j(\lambda) \phi_j(\lambda) = \frac{1}{\sigma_j} \sum_{j=1}^{N_j} \sigma_j(\lambda) \phi_j(\lambda). \]  

(S19)

In general, fluorescivity is the product of the number density, absorption cross section, and quantum of an ensemble of fluorophores. For nanoplastics, fluorescivity quantifies how a fluorophore ensemble, which can interact within the bounding surface and dielectric volume of a nanoscale particle, absorbs fluorescence excitation and yields fluorescence emission.

**Gaussian integral reliability**

We compare measurements of fluorescence intensity by the two methods of Gaussian integration of nanoparticle images and direct summation of signal intensity. Both methods account for mean values of background noise to isolate signal intensity. Gaussian integration accounts for background noise with a fit parameter for a constant offset. Direct summation accounts for background noise by analysis of background noise around the perimeter of a region of interest. We fit a power-law model to the data with non-linear least-squares estimation with uniform weighting using the trust-region reflective algorithm and a smooth approximation of the absolute value of the fit residual, \( \rho(z) = 2(\sqrt{1+z} - 1) \), as a loss function to establish robustness against outliers\(^{25}\) (Figure S12).

**Nanoparticle fluorescence intensity**

We analyze the fluorescence intensity of nanoparticles sufficiently close to size-exclusion regions and of a diameter within a 99.7% coverage interval of the manufacturer specification. We assume that fluorescence intensities follow photon statistics from shot noise and construct Poisson distributions of intensity for each nanoparticle.\(^{19}\) Uncertainties from background subtraction propagate into the following calculation for fluorescence intensity, \( I_j \), after normalization:

\[ I_j = \frac{\langle P(l_j) \rangle}{\int_{S_{\lambda}} \langle P(l_j) \rangle} \left( \frac{1}{N_{\text{calibration}, i}} \right), \]  

(S20)

where \( \langle P(l_j) \rangle \) is the expectation value of the Poisson distribution of the fluorescence intensity of the \( j \)th of \( M \) nanoparticles and \( I_{\text{calibration}, i} \) is the intensity after normalization of the interference calibration for the nanofluidic depth, \( \delta_i \), at the nanoparticle location,

\[ I_{\text{calibration}, i} = \frac{N(l_{i, i}, \sigma_{l, i})}{N(l_{i}, \sigma_{l, N})} \left( \frac{d\lambda}{d\lambda} \right). \]  

(S21)

where \( l_{i, i} \) and \( l_{i, N} \) are respectively the mean intensities of the fluorophore solution at the \( i \)th and \( N \)th steps of the staircase, after background subtraction and flatfield correction, \( \sigma_{l, i} \) and \( \sigma_{l, N} \) are respectively the standard uncertainties of the mean intensities of the fluorophore solution at the \( i \)th and \( N \)th steps of the staircase, and \( d\lambda_i \) and \( d\lambda_N \) are respectively the nanofluidic depths at the \( i \)th and \( N \)th steps of the staircase, where \( N=19 \), the index of the central step in the staircase, which has a nanofluidic depth of 106 nm ± 3 nm.

**Bayesian statistical analysis**

We develop a Bayesian statistical analysis using two hierarchical models,\(^{24}\) which we refer to as the power-law model and the mean-values model. We evaluate each model using open-source software for statistical analysis.\(^{25,26}\) Hierarchical models allow for explicit incorporation of multiple sources of variability. We apply noninformative improper priors to express a state of ignorance about the model parameters before observing the data. Values of nanoparticle diameters and values of fluorescence intensity are measurements with uncertainties. Nanoparticles of similar diameters yield heterogeneous intensities with different diameters yielding different mean values of fluorescence intensity. The two models allow different attributions of intensity variation to three fractional sources − measurement uncertainty of nanoparticle diameter and intensity, variation of diameter, and variation of fluorescivity. The power-law model nests within the mean-values model, constraining the mean value of intensity to follow a power-law relationship with nanoparticle diameter. In contrast, the mean-values model does not explicitly include the diameter measurements, only the 14 diameter bins.

The power-law model is,

\[ \log(y_{ij}) \sim N(y_{ij}, u_{ij}) \]  

(S22)

\[ \log(x_{ij}) \sim N(x_{ij}, v_{ij}) \]  

(S23)

\[ y_{ij} \sim N(\alpha + \beta x_{ij}, \sigma_{ij}^2). \]  

(S24)

In equations (S22–S24), \( y_{ij} \) is the value that we measure of the intensity of nanoparticle \( j \) in diameter bin \( i \), \( y_{ij} \) is the true but unknown value of the intensity of nanoparticle \( j \) in diameter bin \( i \), \( u_{ij} \) is what we assume to be the true value of the measurement uncertainty of \( y_{ij} \), \( x_{ij} \) is the
diameter of nanoparticle $j$ in bin $i$, $v_{ij}$ are the measurement uncertainties of nanoparticle diameters, $\alpha$ is the intercept and $\beta$ is the slope of the power-law model, and $\sigma_i$ is an estimate of the intensity variation that is attributable to variation of fluorescivity of nanoparticles in bin $i$. The fractions of intensity variation that are attributable to variations of nanoparticle diameter and fluorescivity follow from the definition of $R^2$ for Bayesian regression models. We calculate the posterior distributions for the unknown parameters, $y_{ij}$, $x_{ij}$, $\alpha$, $\beta$, and $\sigma_i$. In the power-law model, $V = [\beta^2/(N - 1)]\sum_i (x_{ij} - \bar{x})^2$ where $\bar{x}$ is the sample mean of the $x_{ij}$, and $N=726$ is the total number of nanoparticles. $V$ is the product of $\beta^2$ and the sample variance of $x_{ij}$. By the power-law model, the fraction of intensity variation that is attributable to diameter variation is $V/[V + \sigma_i^2 + u_{ij}^2]$, and the fraction of intensity variation that is attributable to fluorescivity variation is $\sigma_i^2/[V + \sigma_i^2 + u_{ij}^2]$. The mean-values model is,

$$\log[y_{ij}] \sim N(y_{ij}, u_{ij}^2)$$  \hspace{1cm} (S25)

$$y_{ij} \sim N(\mu_i, \sigma_i^2)$$  \hspace{1cm} (S26)

$$\mu_i \sim N(\mu, \sigma_\mu^2).$$  \hspace{1cm} (S27)

In equations (S25–S27), $\mu_i$ is the mean intensity of nanoparticles in bin $i$, $\mu$ is the mean intensity of all nanoparticles, and $\sigma_\mu$ is an estimate of the intensity variation that is attributable to diameter variation. We calculate the posterior distributions for the unknown parameters, $y_{ij}$, $\mu_i$, $\mu$, and $\sigma_\mu$. By the mean-values model, the variance of $y_{ij}$ is $\sigma^2 + \sigma^2 + u_i^2$. For nanoparticle $j$ in diameter bin $i$, the fraction of intensity variation that is attributable to diameter variation is $\sigma^2/[\sigma_\mu^2 + \sigma^2 + u_i^2]$, and the fraction of intensity variation that is attributable to fluorescivity variation is $\sigma_i^2/[\sigma_\mu^2 + \sigma_i^2 + u_{ij}^2]$. 
Supporting Results

Figure S1. Subnanometer steps. (a-b), Atomic-force micrographs and sections showing the subnanometer fidelity of pattern transfer. The silicon mold in (a) has a mean step depth of 0.43 nm ± 0.13 nm and a surface roughness of 0.26 nm ± 0.01 nm. The inverse silicone replica in (a) has a mean step depth of 0.40 nm ± 0.14 nm and a surface roughness of 0.59 nm ± 0.06 nm. The silicon mold in (b) has a mean step depth of 0.86 nm ± 0.08 nm with a surface roughness of 0.17 nm ± 0.01 nm. The inverse silicone replica in (b) has a mean step depth increment of 0.92 nm ± 0.05 nm with a surface roughness of 0.35 nm ± 0.05 nm. Characterization of the native oxide surface of the silicon mold is prior to functionalization with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (TFOCS) for mold release. In this case, the effects of mold release are evident as inverse patches in the silicone inverse replica. The TFOCS patches do not persist through a subsequent stage of pattern transfer, nor do the TFOCS patches appear on silica substrates.

Figure S2. Fluorosilanization effects. (a), Atomic-force micrograph showing a staircase structure in silicon before fluorosilanization with TFOCS. (b), Root-mean-square surface roughness increases consistently with depth for staircase structures with different step depths. The gray box corresponds to the shallowest structure in (a). (c), Atomic-force micrograph showing a staircase structure in silicon after fluorosilanization with TFOCS. Patchy nanostructures form on the native oxide surface, depending on and increasing the surface roughness of the staircase structures. (d), Root-mean-square surface roughness from the TFOCS increases and then saturates at approximately 1 nm. These results elucidate a practical aspect of silicone nanomanufacturing by replica molding.
Figure S3. Pattern transfer. Atomic-force micrographs showing (a-b), a silica mold, (c-d), a silicone inverse replica, and (e-f), a silicone replica. To characterize replication fidelity, we use a channel width of 4.0 µm to facilitate access to the topography of all critical surfaces. We mill the staircase in silica using a focused ion beam (Table S1). We form inverse silicone replicas from silica molds, and we form silicone replicas from inverse silicone replicas.

Table S3. Replication fidelity

|                        | silica mold       | silicone replica  |
|------------------------|-------------------|-------------------|
| mean increment of step depth | 1.80 nm ± 0.04 nm | 1.50 nm ± 0.10 nm |
| standard deviation of increment of step depth | 0.25 nm ± 0.06 nm | 0.65 nm ± 0.16 nm |
| surface roughness       | 0.65 nm ± 0.07 nm | 0.74 nm ± 0.07 nm |

Measurements correspond to test devices that share replication parameters but differ in channel and step geometry from the experimental devices for nanofluidic size exclusion.

Figure S4. Submicrometer film. Brightfield optical micrograph showing structural colors from a film of hard silicone with a thickness of approximately 250 nm. A staircase structure rises from the zero plane of the film surface up to a height of approximately 156 nm. The root-mean-square surface roughness of the inverse silicone replica is 0.45 nm ± 0.07 nm, in comparison to the root-mean-square surface roughness of the mold of 0.44 nm ± 0.07 nm.
Table S4. Hydrodynamic parameters

| parameter                                      | symbol | value  | units |
|------------------------------------------------|--------|--------|-------|
| height of inlet and outlet                     | ℎ_{inlet} | 1      | cm    |
| radius of inlet and outlet                     | ℏ_{inlet} | 250    | µm    |
| length of microfluidic channel                 | ℓ_{mc}  | 12.7   | mm    |
| width of microfluidic channel                  | ℓ_{mc}  | 350    | µm    |
| depth of microfluidic channel                  | ℓ_{mc}  | 0.48   | µm    |
| length of the entire staircase                 | ℓ_{l}   | 230    | µm    |
| length of each step of the staircase           | ℓ_{l}   | 5.36   | µm    |
| minimum width of the staircase                 | ℓ_{i,min} | 0.5    | µm    |
| maximum width of the staircase                 | ℓ_{i,max} | 2.5    | µm    |
| minimum depth of the staircase                 | ℓ_{i,min} | 45     | nm    |
| maximum depth of the staircase                 | ℓ_{i,max} | 165    | nm    |
| number of staircases in the array              | N_a     | 20     | –     |
| number of steps in the staircase               | N_s     | 36     | –     |
| volume of entire device                        | V_{device} | 2×10^6 | µm^3  |
| nanoparticle radius                            | a       | 45 to 165 | nm |
| surface-to-surface separation                  | s_s     | 0.1 to 10 | nm |
| surface tension of nanoparticle suspension     | γ       | 37     | mN m^{-1} |
| contact angle of nanoparticle suspension on fused silica | θ_{silica} | 0.22 (13) | rad (°) |
| contact angle of nanoparticle suspension on hard silicone | θ_{silicone} | 0.25 (14) | rad (°) |
| density of nanoparticle suspension             | ρ       | 990    | kg m^{-3} |
| dynamic viscosity of nanoparticle suspension   | η       | 0.89   | mPa s |
| absolute temperature                           | T       | 300    | K     |
| pressure difference across staircase           | Δp      | 1.5×10^3 | kPa |
| pressure difference due to capillarity         | Δp_c   | 1.5×10^2 | kPa |
| pressure difference due to fluid column       | Δp_g   | 1×10^1 | kPa |
| pressure difference across single step         | Δp_l   | 0.135 to 33.8 | kPa |
| volumetric flow rate                           | Q       | 540    | µm^3 s^{-1} |
| hydraulic resistance of staircase              | R       | 2.8×10^4 | kg µm^4 s^{-3} |
| hydraulic resistance of single step            | R_l    | 5.0×10^4 to 1.2×10^5 | kg µm^4 s^{-3} |
| lower bound of filling time of the device      | τ_{fill} | 100    | h     |
| mean magnitude of flow speed in x direction    | ⟨u_{x,i}⟩ | 0.07 to 1.2 | mm s^{-1} |
| mean width of size exclusion regions           | ⟨w_{SER,i}⟩ | 560    | nm |
| lateral diffusivity of nanoparticles           | D_{2i,∥,i} | 0.6 to 3.5 | µm^2 s^{-1} |
| Brenner number                                 | Br      | 60 to 200 | – |

We report ranges of diffusivity and Brenner number assuming a separation of 1 nm. More detail is in Figure S5. Quantities are approximate.
Figure S5. Device hydrodynamics. Plots showing theoretical values of (a) pressure difference, (b) mean magnitude of flow speed in the x direction, (c) nanoparticle diffusivity, and (d) Brenner number as a function of x position and nanofluidic depth across the nanofluidic staircase less than 0.5 h after exposure to oxygen plasma. The curves in (a-c) are upper bounds at the onset of the experiment. The curves in (c) and (d) correspond to different values of separation, $s_n$, between nanoparticles and the top and bottom of the device as we indicate in the inset of (c). Table S4 summarizes the hydrodynamic parameters that we use to estimate the rates of advective and diffusive transport of nanoparticles in the staircase.
Figure S6. Hydraulic resistance. (a-c) False color fluorescence micrograph showing the size exclusion of fluorescent nanoparticles in three parallel arrays of nanofluidic staircase structures with variable depth ranges. (a) The top channels (blue) range in depth from 77 nm ± 3 nm to 234 nm ± 4 nm, (b), The middle channels (cyan) range in depth from 72 nm ± 3 nm to 242 nm ± 3 nm, and (c), the bottom channels (green) range in depth from 92 nm ± 2 nm to 244 nm ± 2 nm. The values on the left and right sides of (a-c) correspond with the minimum and maximum depths of each array. These arrays serve in initial tests of replica stability and suspension flow. In a rough reduction of data for only these initial results, we omit the subsequent corrections of position and apparent diameter. (d) Histogram showing apparent diameters of nanoparticles in the top (blue), middle (cyan), and bottom (green) arrays. (e) Plot showing nanoparticle count increasing with the inverse hydraulic resistance of the arrays. We normalize particle count by the number of channels in each array. We use equation (S3) to calculate hydraulic resistance, inputting a dynamic viscosity of 0.89 mPa s ± 0.09 mPa s and values of the channel geometry at the \( i \)th step in the staircase device — width, \( w_i \), length, \( L_i \), each with uncertainties of 25 nm and depth, \( d_i \), with uncertainties that range from 2 nm to 4 nm as we indicate above.\(^1\),\(^2\) Black bars are 95% coverage intervals. The dash line is a best fit to the data, resulting in a reduced chi-square statistic, \( \chi^2 \), of 2.0.

Figure S7. Device fiducials. Brightfield optical micrograph showing a nanofluidic device under epi-illumination after filling. Three rows of circular features are evident. The first column of device fiducials at the left edge of the micrograph marks the beginning of the microfluidic channels. All other fiducials, toward the right of the micrograph, mark the 36 step edges of staircase structures. In each row, the Euclidean distance between fiducials is approximately 5.36 \( \mu \)m. We revisit this quantity after correcting the apparent positions of fiducials in Figure S13.
Figure S8. Flatfield corrections. (a, b, c) Plots showing flatfield corrections for (a) transillumination brightfield, (b) epi-illumination brightfield, and (c) and epi-illumination fluorescence micrographs. (d, e, f) Plots showing standard uncertainties of mean values in (a), (b), and (c), respectively. We neglect these small uncertainties. The fluorescence intensity of the photoresist film is stable within uncertainty during the measurement (not shown). The thickness of the photoresist film that we image to develop flatfield corrections just exceeds the deepest regions of the nanofluidic devices and is also within the nominal depth of field of the imaging system under any illumination condition. In this way, the photoresist film forms a confocal image under widefield illumination that is comparable to the interaction of the nanofluidic devices with the varying focal volume of the microscope system. Inappropriately thicker films interact with more of the microscope focal volume, resulting in different flatfield corrections which would be inaccurate for our measurement system (not shown).
Figure S9. Fiducial localization. (a), (b) Surface plots showing representative brightfield micrographs of device fiducials. The signal intensity varies with illumination intensity across the imaging field. As a result, after pixelation, pixel values can be (a) below or (b) above the saturation limit of the 16-bit imaging sensor of 65,535 arbitrary units after flatfield correction. (c), (d) Plots showing synthetic images corresponding to (a), (b). In this test, pixel value saturation does not appreciably degrade localization accuracy, as Table S5 shows, by a comparison of the mean value of position estimates and the true position. These results demonstrate a new and counterintuitive capability to perform localization microscopy above the saturation limit of an imaging sensor.

Table S5. Pixel saturation

| maximum intensity (arb.) | mean error in x-position (nm) | standard deviation of error in x-position (nm) | mean error in y-position (nm) | standard deviation of error in y-position (nm) |
|--------------------------|-------------------------------|-----------------------------------------------|-------------------------------|-----------------------------------------------|
| < 65,535                 | -0.38 ± 0.01                 | 0.39 ± 0.01                                   | 0.53 ± 0.01                   | 0.39 ± 0.01                                   |
| 65,535                   | -0.66 ± 0.01                 | 0.31 ± 0.01                                   | 0.80 ± 0.01                   | 0.31 ± 0.01                                   |

Figure S10. Nanoparticle localization. (a-b), Fluorescence micrographs showing representative images of nanoparticles (a), before and (b), after applying a filter to reject replicate localization results within a factor of 10 of the theoretical localization precision of single nanoparticles. (a), (red crosses) Localization of what we assume is a single nanoparticle on the left and two nanoparticles in proximity on the right yields six total positions, which reduce after filtering to (b), (blue roundels) three positions. This filter retains the localization result with the smallest localization uncertainty and rejects other localization results within each group of replicates.
Figure S11. Point-spread-function filter. (a), Fluorescence micrograph showing representative images of sparse nanoparticles on a microscope coverslip in a control measurement. We determine the standard deviations of a symmetric Gaussian approximation of the point spread function of nanoparticles between the white dash lines to match the imaging field of experiments in nanofluidic devices. (b), Histogram showing standard deviations from (white) the sum of eight control measurements such as in (a). (Gray region) A 95 % coverage interval of the standard deviation of single nanoparticles, extending from 102 nm to 175 nm, filters emitters with larger standard deviations in nanofluidic devices corresponding to multiple nanoparticles in proximity. (Gray tick mark) The theoretical value of the standard deviation of a symmetric Gaussian approximation of the point spread function at the peak emission wavelength is approximately 92 nm. Experimental values of standard deviation exceed the theoretical value due to field curvature and deviations from best focus. (c) Histogram showing standard deviations from four comparable experiments in nanofluidic devices.

Figure S12. Intensity measurements. Scatter plot showing the reliable proportionality of Gaussian integration and direct summation of signal intensity in nanoparticle images. For this analysis, we select what appear to be single nanoparticles in isolation within a region of interest of 10 µm by 10 µm, avoiding any potential errors from nanoparticle images in close proximity. Fitting a (gray dash line) power-law model to the data results in a coefficient of 0.7 arb. ± 0.1 arb. and an exponent of 0.98 ± 0.01, corresponding to a mean slope of 0.5 ± 0.1 with a reduced chi-square statistic, $\chi^2_r$, of 1.7. The resulting variation is consistent with the heterogeneity of intensity values in Figure 2 of the main text. Insets: (top left) Residuals from a Gaussian model fit to a nanoparticle image and (bottom right) signal intensity of the same image.
Figure S13. Localization microscopy. (a) (False green) Fluorescence micrograph showing an aperture array containing an aqueous solution of fluorophores. The emission spectrum of the fluorophore solution closely resembles the emission spectrum of the fluorescent nanoparticles that we measure. Imaging the aperture array through focus at wavelengths matching those of the fiducials and nanoparticles allows calculation of independent correction functions at different focal positions for fiducials and nanoparticles. (b-c) Vector plots on a linear scale and color maps on a logarithmic scale showing independent corrections to positions of (b), fiducials and (c), nanoparticles. The field dependence results from distortion among other optical aberrations at the different imaging wavelengths and positions of best focus. (d) Composite (false blue) brightfield and (false green) fluorescence micrograph showing device fiducials and fluorescent nanoparticles. (e-g) Scatter plots showing distance errors perpendicular to the row of fiducials resulting from linear fits of the three rows of fiducial positions before (white circles) and after (black circles) correction. Non-zero distances indicate apparent non-linearity of the rows due to aberrations of the optical microscope system and actual non-linearity of the rows due to aberrations of the focused-ion-beam system (not shown). Selection of the focal position in (a) that minimizes these errors matches the focal positions of the reference data and experimental data. After position correction, the three rows of 36 fiducials, totalling 108 fiducials, have a mean pitch of 5,364 nm ± 1 nm. In comparison, the nominal value of fiducial pitch is 5,360 nm. This is the first use of a reference material that we fabricated by electron-beam lithography to test the placement accuracy of a reference material that we fabricate by focused-ion-beam milling. The root-mean-square values of position errors of 6.5 nm ± 0.9 nm in (e-g) are consistent with a root-mean-square value of position errors parallel to the rows of fiducials of 5.8 nm ± 0.8 nm, which we calculate as the standard deviation of the Euclidean distance between neighboring fiducials in each row. This consistency builds confidence in our correction of position errors. (h) Two-dimensional histogram showing theoretical localization precision of nanoparticles as a function of signal intensity before flatfield correction. We test nanoparticles in isolation (Figure S11) to determine that our approximation of the readout noise of individual pixels by their root-mean-square value results in localization errors with a root-mean-square value of 0.03 nm, which is negligible in comparison to the smallest values of localization precision.
**Figure S14. Intensity calibration.** Plot showing nonmonotonic variation of fluorescence intensity due to optical interference as a function of nanofluidic replica depth. Normalization of intensity values is with respect to the intensity at the (black circle) 19th step of the device, which has a depth of 106 nm ± 3 nm. Horizontal bars are increments of nanofluidic depth. 95 % coverage intervals of mean intensity are comparable in size to the data markers.

### Table S6. Statistical variables

| variable                                      | type      | symbol   | distribution       | parameters                                                                 |
|-----------------------------------------------|-----------|----------|--------------------|-----------------------------------------------------------------------------|
| nanofluidic depth                            | dimension | d_i     | uniform            | lower and upper bounds: d_i and d_{i+1}                                    |
| root-mean-square surface roughness of replicas| dimension | R_{k,r} | normal             | mean: 0 nm, s.d.: 0.74 nm                                                  |
| root-mean-square surface roughness of coverslips | dimension | R_{k,c} | normal             | mean: 0 nm, s.d.: 0.77 nm                                                  |
| calibration error of atomic-force microscope  | uncertainty | ε_{calibration,i} | normal             | mean: 0 nm, s.d.: 0.0025 - d_i                                          |
| roughness errors of atomic-force microscope   | uncertainty | ε_{roughness} | normal             | mean: 0 nm, s.d.: 0.030 nm                                                |
| flatness errors of atomic-force microscope    | uncertainty | ε_{flatness} | normal             | mean: 0 nm, s.d.: 0.065 nm                                                |
| nanoparticle radii                           | dimension | a_i     | empirical          | d_f, R_{k,r} - R_{k,c} - ε_{calibration,i} - ε_{flatness}                |
| shape parameter of lognormal distribution of step-edge width | uncertainty | s_{shape} | normal             | mean: 0.72, s.d.: 0.035                                                    |
| scale parameter of lognormal distribution of step-edge width | uncertainty | s_{scale} | normal             | mean: 432 nm, s.d.: 21 nm                                                  |
| step-edge widths                             | dimension | s_{se}  | lognormal          | shape: s_{shape}, location: 0, scale: s_{scale}                           |
| step edges                                    | dimension | ξ_i     | normal             | mean: 0 nm, s.d.: s_{se}                                                   |
| brightfield image pixel size (peak wavelength 460 nm) | dimension | a_{460 nm} | uniform            | range: 99.95 nm to 100.15 nm                                              |
| fluorescence image pixel size (peak wavelength 515 nm) | dimension | a_{515 nm} | uniform            | range: 100.53 nm to 100.74 nm                                             |
| slope of line of best fit to fiducials        | dimension | c_{L,i} | student t          | degrees of freedom: 3                                                      |
| offset of line of best fit to fiducials       | dimension | o_i     | student t          | degrees of freedom: 3                                                      |
| positions of fiducials                        | dimension | r_{c,i} | normal             | mean: (x_{i} , y_{i}), s.d.: s_{c,i}                                      |
| theoretical localization precision of fiducials | uncertainty | σ_{c,i} | normal             | mean: r_{L,i}, s.d.: σ_{c,i}                                               |
| positions of nanoparticles                    | dimension | r_{j}   | normal             | mean: (x_{j} , y_{j}), s.d.: σ_{j}                                        |
| theoretical localization precision of nanoparticles | uncertainty | σ_{n,j} | normal             | mean: r_{j}, s.d.: σ_{j}                                                   |
| horizontal offset of size-exclusion regions   | dimension | x_{offset,i} | empirical          | R_{f,i}, ξ_{i}, c_{Li}                                                    |
| proximity of nanoparticle to centers of size-exclusion regions | dimension | D_{ij}  | empirical          | σ_{Li}, x_{offset,i}, R_{j,i}, o_{i}                                      |
| fluorescence intensity of single nanoparticles | dimension | I_{j}   | poisson            | variance: I_{j}                                                          |
| background signal in fluorescence micrographs  | dimension | I_{bkg} | poisson            | variance: I_{bkg}                                                        |
| fluorescence intensity of fluorophore solution in the staircase | dimension | I_{o}   | normal             | mean: I_{fs}, s.d.: σ_{fs,o}                                               |
| fluorescence intensity of the interference calibration | dimension | I_{calibration,i} | empirical          | I_{fs}, d_i                                                               |

We abbreviate standard deviation in this table as s.d.

We apply mean values of slopes and offsets of lines of best fit to fiducials to Student t-distributions by summation.

We use Student t distributions if the number of degrees of freedom is less than 30.

r_{c,i} = (x_{c,i}, y_{c,i}) denotes x and y positions of fiducials corresponding to the i^{th} step.

r_{j} = (x_{j}, y_{j}) denotes the position of the j^{th} nanoparticle.

σ_{c,i} denotes Cramér-Rao lower bounds of the localization precision of fiducials.σ_{c,i}

σ_{c,i} denotes the Cramér-Rao lower bounds of the localization precision of nanoparticles.29
Figure S15. Step-edge widths. (a), Atomic-force micrograph section showing a representative step edge of a silicone replica (black). Fitting an error function (blue) to the profile, \( z_{i+1} = z_i + \Delta d / 2 \cdot \text{erf} \left( (x - x_0) / (\sqrt{2} \sigma_{se,i}) \right) \), where \( \Delta d \) is the step-depth increment, \( x_0 \) is the x position of the step, and \( \sigma_{se,i} \) is the standard deviation of the Gaussian function corresponding to the error function, determines \( 4\sigma_{se,i} \) as a 95% coverage interval for the width of the \( i \)th step edge. Analysis of this representative step edge results in a width of 595 nm ± 96 nm. (b), Histogram showing step-edge widths throughout the replica. Fitting a lognormal distribution (black dash line) with location 0 to the histogram yields a shape parameter of 0.72 ± 0.07 and a scale parameter of 432 nm ± 42 nm. These uncertainties are sampling errors. The location, shape, and scale parameters define the distribution of step-edge width, \( \sigma_{se,i} \), for the entire device.

Figure S16. Steric filter. Histogram showing the ratio of distances between nanoparticles to the sum of the radii of unique nanoparticle pairs in size-exclusion regions. A steric filter rejects nanoparticle pairs with positions that yield (red bars) distances between nanoparticles that are less than the sum of the nanoparticle radii. We retain nanoparticles with positions that yield ratios of distances between nanoparticles to the sum of the radii that are (blue bars) greater or equal to unity.

| Table S7. Filter results |
|-------------------------|
| filter | emitter count | emitters filtered | fraction of emitters filtered | fraction of single nanoparticles filtered |
| none | 10,072 | 0 | 0 | – |
| low intensity | 6,996 | 3,076 | 0.305 | – |
| replicate | 4,791 | 2,205 | 0.219 | – |
| point spread function | 3,105 | 1,686 | 0.167 | – |
| steric | 2,575 | 1,718 | 0.667 | 0.206 |
| size exclusion | 857 | 131 | 0.013 | 0.051 |
| manufacturer specification | 726 | | | |

Emitter counts are from all four comparable experiments in Figures S17.
We consider single nanoparticles (white region of table) to be those emitters that pass the low intensity, replicate, and point spread function filters.

Table S8. Analytical yield

| experiment | nanoparticle count | nanoparticles in size-exclusion regions | spurious yield | analytical yield |
|------------|-------------------|----------------------------------------|----------------|-----------------|
| 1 | 475 | 179 | 62.3 % | 37.7 % |
| 2 | 431 | 150 | 65.2 % | 34.8 % |
| 3 | 522 | 189 | 63.8 % | 36.2 % |
| 4 | 1,147 | 339 | 70.4 % | 29.6 % |

| | mean | 65.4 % | 34.6 % |
| | standard deviation | 3.5 % | 3.5 % |
Figure S17. Comparable experiments. (a-d) Fluorescence micrographs showing the size separation of nanoparticles in silicone devices. (e-h) Plots showing nanoparticle and fiducial positions and size-exclusion regions. (i-l) Histograms showing diameters apparent from device depths for nanoparticles (gray) outside of and (white) inside of size-exclusion regions. Columns show the results of experiments that differ by both exposure time to oxygen plasma prior to bonding of silicone replicas to silica coverslips and by the time between device wetting and fluorescence microscopy: (a) exposure to oxygen plasma for 30 s and microscopy approximately 6 h after wetting, (b) exposure to oxygen plasma for 15 s and microscopy approximately 6 h after wetting, (c) exposure to oxygen plasma for 5 s and microscopy 6 h after wetting, and (d) exposure to oxygen plasma for 5 s and microscopy approximately 10 h after wetting.

Video S1. Surface stability. A time series of fluorescence micrographs showing the Brownian motion in real time of some nanoparticles in deeper steps of staircase structures. This motion occurs 100 h after wetting the disposable device, which is near the end of the useful duration for capillarity to drive hydrodynamic transport. The presence of Brownian motion indicates the ongoing mitigation of attractive interactions between nanoparticles and confining surfaces, demonstrating the stability of the system at that time scale. These data are for the longest exposure to oxygen plasma of 30 s. The yellow region indicates the common inlet of the device.
Figure S18. Bayesian statistical analysis. Plot showing the fractions of intensity variation that are attributable to diameter and fluorescivity variation. Green circles are the mean-values model. Blue triangles are the power-law model. Data markers are the medians of posterior distributions. Solid lines are the major axes, or approximately 95 %, of these distributions of representative points. The minor axes are comparable to line widths. 726 nanoparticles collapse to 14 bins due to similar uncertainties for each bin. The dash gray line indicates sum to unity, with a slight distance to the data markers being attributable to measurement uncertainty. For either model, fluorescivity and diameter attributions are independent of nanoparticle diameter.

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