Supplementary Information: Description of tracking algorithm and software

The tracking algorithm is composed of four calculation steps adapted from the work of Fields and Cohen[1] and which are performed in real-time by the FPGA in order to estimate the particle's position relative to the center of the focus of the objective lens. First, the particle's position ($\hat{x}_k$) is updated based on the previous position ($\hat{x}_{k-1}$), the laser beam covariance ($w$), the previous variance of the position estimate ($\hat{\sigma}_{k-1}^2$), the current laser position ($c_k$) and the number of photons collected at the current laser position ($n_k$) via equation (1):

$$\hat{x}_{k|k} = \frac{\hat{x}_{k|k-1} w^2 + c_k n_k \hat{\sigma}_{k|k-1}^2}{w^2 + n_k \hat{\sigma}_{k|k-1}^2} \quad (1)$$

The updated variance is calculated using equation (2):

$$\hat{\sigma}_{k|k}^2 = \frac{\hat{\sigma}_{k|k-1}^2 w^2}{w^2 + n_k \hat{\sigma}_{k|k-1}^2} \quad (2)$$

Equations (3) and (4) provide predictions for the position and variance, respectively:

$$\hat{x}_{k|k-1} = \hat{x}_{k-1|k-1} \quad (3)$$

$$\hat{\sigma}_{k|k-1}^2 = \hat{\sigma}_{k-1|k-1}^2 + 2D\tau \quad (4)$$

Where $D$ is the expected diffusion coefficient and $\tau$ is the time between calculations (the bin time). **Figure S1** shows the process flow for the FPGA position estimation. For XY position estimation, the FPGA cycles through coordinates of the knight's tour (**Figure 2**) and scales and outputs the voltage to deflect the laser with the 2D-EOD. The loop then counts photons for a designated bin time (here: 20 µsec). Then equations (1) and (2) are used to calculate the position and variance in the XY plane which are output for use in the position feedback loop. Equations (3) and (4) are used to "predict" new values for the position and variance.

The Z position estimation loop operates differently due to the fact that the TAG lens operates at a fixed frequency and therefore binning is not possible. Instead, the FPGA waits for a rising edge of a photon pulse from the APD, at which point it uses the current TAG lens position along with equations (1) and (2) to calculate the particle position and variance in Z, respectively. As with XY, the Z position is output for use in the feedback loop. Equations (3) and (4) are used to "predict" new values for the position and variance. This process is outlined in **Figure S2.**

For this protocol, two separate software programs are required and are outlined in **Figure S3**. First, a program is needed to raster scan fluorescent particles using the piezoelectric stage. The resulting 3D scans are required to fully align and calibrate the system. The second program is the tracking program. The ideal tracking program is comprised of a graphical user interface which allows control of the integral feedback control constants, bin time, voltage of EODs, intensity threshold of tracking, XYZ scaling and TAG phase offset. The voltage of the EODs is used to determine the size of the scanning square in XY. The TAG phase offset is used define the TAG phase that corresponds to the objective focal plane, while the XYZ integral constants are used to control the movement of piezo nanopositioner.

The tracking feedback loop (**Figure S3**) uses the position estimates for XYZ in an integral controller to achieve positional feedback. First, the position estimates are summed (one sum for each direction XYZ) and scaled by the integral constant, $K_I$ (each direction will have a different constant, though magnitude should be the same for X and Y). The output is then scaled to an appropriate voltage to move the piezo nano-positioner to drive the position estimate to zero. The loop then checks the intensity level to make sure that tracking is interrupted when the photon counts fall below a certain threshold value. Specifically, this threshold value usually be set as twice of the photon counts of background.

1. Fields, A.P., Cohen, A.E. Optimal tracking of a Brownian particle. *Opt. Express*. **20** (20), 22585-22601, doi:10.1364/OE.20.022585 (2012).

Supplementary Figure Captions

**Figure S1.** Position estimation flow chart. (**a**) The top diagram shows the method for XY position estimation. First, the coordinates of the knight's tour for the current step are extracted. Next, the coordinates are scaled to a voltage and output to the 2D-EOD. The voltage scaling factor can be calibrated by checking the size of a particle raster scan (see **Figure 4a**) and choosing the scaling factor which makes the square 1 µm x 1 µm. Next, photons are binned for a predetermined bin time (typically 20 µs). After binning, the number of collected photons, the current laser position and the previous position estimate are used to calculate the new position estimate using equations 1 and 2. The new position estimate is output to the position feedback loop and updated by equations 3 and 4 before the next step in the knight's tour begins. (**b**) The bottom diagram shows the position estimation in Z. Since the TAG lens is constantly oscillating the focal point, no binning is applied. Instead, the FPGA is programmed to loop until a photon is detected. Once a photon is detected the TAG lens phase is extracted to find the current laser focal position. Then the position estimations are updated and exported to the position feedback loop similar to the XY case in (**a**).

**Figure S2**. Process for extracting Z laser position from the TAG lens synchronization signal. (**a**) The TAG lens controlled can provide 3 output trigger signals at different phases. Here, phases of 0, 90 and 270 degrees are used, though any three values that effectively sample the phase space can be used. (**b**) Using a look-up table or linear interpolation, the current TAG phases can be determined based on the elapsed time since the last synch trigger received from the TAG controller. (**c**) An onboard sine calculation is used to calculate a focal position from the TAG phase value. (**d**) When a photon is detected, the current TAG position is extracted. (**e**) The current TAG position and the previous Z position estimate are used to update the current Z position estimate.

**Figure S3.** Software flow. For this protocol, two general software implementations are needed. (**a**) First, a stage scanning program which can raster scan a fixed particle through the objective focal volume in XYZ is needed to calibrate and align the tracking system. The stage scanning program first moves to an XYZ position. Then photons are collected for a set pixel dwell time (typically 10 ms) and the position estimates are updated using equations 1-4. This process is repeated until the final XYZ position is sampled. (**b**) The second required software algorithm is for position feedback. First, the position estimates are extracted from the position estimation loops described in **Figures S1** and **S2**. Next, the position estimates are summed over all k for the current trajectory. Each sum (X, Y and Z) are then scaled by the feedback constant $K_I$, which is determined empirically for each direction by starting with a low value and slowly increasing until a value just before oscillations are observed in the stage position readouts. The product of the sums and the $K_I$ are scaled to a voltage and applied to the 2D-EOD. At this point in the algorithm, a check is done to be sure that the photon count rate is above a tracking threshold, usually set two times the background value. If the intensity falls below the threshold, the trajectory is ended. Otherwise tracking continues.