Supplement 1

The time-dependent diffusion coefficient was derived from mean squared displacement (MSD) versus time traces in order to compare the two simple methods used to emulate spatial crowding in the simulation domain. Specifically, the ensemble MSD was calculated using the evolution of particle displacement \([x(t),y(t),z(t)]\) as a function of each time interval \((\Delta t)\) according to;

\[
MSD(n\Delta t) = \frac{\sum_{m=1}^{N-n} [x(m\Delta t + n\Delta t) - x(m\Delta t)]^2 + [y(m\Delta t + n\Delta t) - y(m\Delta t)]^2 + [z(m\Delta t + n\Delta t) - z(m\Delta t)]^2}{N - n}
\]

where \((N)\) is the total number of time samples and \((N-n)\) is the number of interval samples per \(n\Delta t\). Subsequently, the time-dependent diffusion coefficient \(D(\Delta t)\) was estimated using;

\[
D(n\Delta t) = \frac{MSD(n\Delta t)}{6n\Delta t}
\]

The critical input parameters for the study were \(D_0=400 \mu m^2/s\), \(R_H=1nm\), \(R_c=5nm\), \(\Delta s=R_H\), \(S_{max}=150nm\) and \(T=300K\) where \(R_c\) is the spherical crowding obstacle radius, \(\Delta s\) is the spatial domain voxel size, \(S_{max}\) is the edge length of the cubic simulation domain and \(T\) is the solvent temperature. The number of time steps per simulation was set to equal 50 million. The time increment per virtual Brownian particle displacement was \(\Delta t=R_H^2/6D_o=0.417ns\). The volume crowding percentage was set to 23%. Periodic boundary conditions were implemented at the cubic simulation domain boundaries in order to avoid the spatial boundary confinement effect such only particle–obstacle collisions would perturb diffusive transport. Two simulations were conducted for each model of crowding in order to demonstrate the variation in results.
The time-dependent diffusion coefficient derived from ensemble mean squared displacement versus time simulation data sets. The simplest model of crowding (implementing a simple collision probability) homogenizes the influence of crowding over all time scales which roughly emulates the effect of a more viscous solvent. This is seen as the nearly constant $D(\Delta t)$ over all timescales in the figure labeled as (1). The time-independent diffusion coefficient in an obstacle-free, unhindered solution ($D_o$) is also shown superimposed for reference back to the free diffusion case. Simulation accuracy improves with the addition of a random distribution of crowding obstacles in the simulation domain. A diffusion exploration length on the order of the crowding obstacle spacing, i.e., short timescales, roughly <1μs in this data set, suggests solute diffusion that is anomalous as solute–obstacle collisions begin to perturb the diffusive transport. This is seen in the figure as the decrease in the diffusion coefficient with time increment which is labeled as case (2). In both simulation approaches, an effective time–
independent diffusion coefficient \(D_{\text{eff}}\) emerges at longer timescales as solute explores a larger volume of the crowded solution and individual solute–obstacle collisions average out in space and time. The figure shows that the collision probability approach underestimates the effective diffusion coefficient relative to the results derived from the simulation that includes physical obstacles to emulate a crowded solution (compare at \(\Delta t=10\mu s\)). Characteristic diffusion times derived from FCS experiments occur on the order of milliseconds, a timescale much larger than timescale characterizing the anomalous to effective diffusion transition. As a result, the fluorescence correlation signal carries no information regarding the anomalous effects predicted here to occur at sub–microsecond timescales. The finite and relatively large fluorescence probe volume used during FCS experiments ultimately sets this timescale limitation. The results from individual simulations are shown as semi–transparent curves while the average behavior is shown using circular data points with one standard deviation shown using the superimposed error bars. Also, it is important to note that the increase in the standard deviation at large \(\Delta t\) values is due to a finite simulation length in the time coordinate.
Figure S2  Fluorescence intensity as a function of time in increments of $\Delta t_{acq}=204.5\text{ns}$ derived from 4 select particle tracking simulations. A total number of ~36.7 million data points are shown spanning the simulation time range of 0 to 7.5s. The y–axis shows the log(intensity). The binding probability was 0.001 and the mean residence time bound was 100ms where binding was
possible at both the lid and walls. Bound molecules were assumed to fluoresce although only for cases (c) and (d) where the fluorescence excitation beam overlapped the top lid of the bucket. Superimposed black arrows indicate the locations of obvious binding events for these cases where the signature of binding is the condition \( \frac{dI}{dt}=0 \) over a time range of \(~100\text{ms}\) (\textit{the area underneath the plot in these regions has been shaded yellow}). In cases (a) and (b) binding only effected the mobile concentration of fluorescent particles available to diffuse to the excitation laser focus located at the center of the bucket. (a) 0% crowding volume and centered excitation focal volume, (b) 23% crowding volume and centered excitation focal volume, (c) 0% crowding volume and an excitation focal volume located at \((0,0,2.5\mu m)\) and (d) 23% crowding volume and an excitation focal volume located at \((0,0,2.5\mu m)\).
Figure S3  The convolution of the microfabricated bucket and the incident, focused laser probe (*green, blurred ellipsoid*), forms an effective bucket size with double the volume as shown by the basis element of symmetry. The basis element is created by first inverting the bucket–laser probe spatial convolution in the z-direction followed by an addition operation to yield the complete laser probe spatial profile. Solute particles executing a random walk, with reflecting boundaries along the cylindrical walls and the two lids, explore the equivalent semi–infinite, tubular geometry shown in the far right schematic. Importantly, the basis structure has twice the volume as the actual, real bucket.
The total number of particle-wall and particle-lid collisions per $\Delta t_{\text{acq}}$ for the case of a) 0% crowding and b) 23% crowding by volume. Binding was turned off for the simulations shown in (a) and (b). Intensity here represents the number of collisions and not optical fluorescence. Two time samples are shown for each case leading to a total of 1s of data. The 23%
volume crowding data set reveals an anticipated crowding effect whereby long periods of binding interactions, exceeding the mean residence time of 100ms, are observed (regions devoid of collisions, i.e., at, or near, 0 intensity). A particle that diffuses into a wall or lid region tends to linger, experiencing multiple binding events, due to particle-crowding obstacle collisions keeping the particle in close proximity to the wall or lid region. Specifically, this is seen in the data as small, narrow bursts of collision activity sandwiched between binding events, i.e., periods of reduced particle–lid and/or particle–wall collisions in (b). Notably, 8% fewer total wall/lid collisions were observed at 23% crowding relative to 0% crowding yet a 5% increase in mean bound fraction was found for 23% volume crowding relative to 0% crowding. The mean bound fraction was calculated over the complete 30s simulation time period. These results may be interpreted to indicate that although fewer total collisions occur with walls/lids when the solution is crowded, the adsorption efficiency effectively increases because the wall/lid interactions come in bursts where intermittent periods of a large number of wall/lid collisions actually increases the effective binding probability.