Heterogeneity of crowded cellular fluids on the meso- and nanoscale
– Supplementary Information –

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MATERIALS AND METHODS

Polyethylene glycol (PEG, 10 kDa) and trans-4-[4-(Dimethylamino)styrlyl]-1-methylpyridinium iodide (DASPMI) were purchased from Sigma Aldrich, dextran (70 kDa) from AppliChem. Artificial fluids crowded with PEG or dextran (30% weight per volume) were based on MilliQ water or TE-buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). HeLa cells were cultured and transfected with a low-expression plasmid for EGFP via peqFECT (PEQLAB) as described before [1]. For microscopy, cells were grown on ibidi 8-well chambers for 24 h. Prior to microscopy, cells were incubated 100 min. in medium with 10 µM DASPMI. After washing, cells were supplied with imaging medium (MEM without phenol red, 5% FCS, 5% HEPES). Transparent cytosol from Xenopus laevis was purified as described before [2]. The chosen tracers (DASPMI and EGFP, radii ∼ 1.5 nm) are comparable to typical protein sizes: Assuming globular shapes, protein radii only dependent on the third root of the mass, i.e. using EGFP (25 kDa) as a reference yields radii of ≤ 2.2 nm for soluble proteins with a mass of ≤ 75 kDa. Also the literature values of the artificial crowders’ hydrodynamic radii (10 kDa-PEG, 2.8 nm; 70 kDa dextran, 5 nm) are similar.

Fluorescence correlation spectroscopy (FCS) on 100 nM DASPMI in artificial crowded solutions was performed as described previously [3] on a ConfoCor2 (Carl Zeiss, Jena, Germany) at room temperature with an excitation at 488 nm and a 560 nm fluorescence longpass filter for detection. Data acquisition was limited to 60 s (PEG) and 75 s (dextran) with a maximum of eight curves recorded at each position to cover the fluid’s density fluctuations. All FCS data (≥ 80 FCS curves per sample; see Fig. S1 for individual examples) were evaluated with an expression for anomalous diffusion [1, 3]:

\[ C(\tau) = \frac{A}{(1 + (\tau/\tau_D)^3)\sqrt{1 + q(\tau/\tau_D)^3}}. \]  

Here, \( \alpha \) denotes the diffusion anomaly, and \( \tau_D \) is the mean dwell time of a fluorescent particle within the confocal volume. Elongation of the confocal volume along the optical axis was considered via the fixed parameter \( q = 0.04 \). The prefactor \( A \) reports on the inverse number of particles within the confocal volume and on the photophysics of the dye. Data evaluation was restricted to times \( \tau > 30 \mu s \), i.e. contributions of the photophysics were negligible. As reported before [4], extracting the diffusion anomaly \( \alpha \) by fitting an experimental autocorrelation function relies on the temporal window between \( \sim 10 \mu s \) and the halftime (approximately \( \tau_D \)), corresponding to a length scale in the range of some 100 nm. While a slight over- or underfilling of the back aperture plane, a minor difference in the numerical aperture of the objective, or a change in the excitation wavelength is directly observed in the value of \( \tau_D \), the extraction of the anomaly is more robust as it reflects the FCS curve’s power-law decay over 1-2 orders of magnitude in time for which \( \tau_D \) is just a rough boundary value.

FCS on DASPMI in purified cytosol and on EGFP in cells, and all fluorescence lifetime imaging microscopy (FLIM) experiments were performed on a SP5 SMD system (Leica Microsystems Mannheim and Picoquant Berlin) using a 488 nm continuous wave laser (FCS) or a pulsed 470 nm laser with 40 MHz repetition frequency (FLIM). Experiments were routinely performed with a pinhole of one Airy unit using a 63x/1.2NA water immersion objective except for FLIM on 100 nM DASPMI in artificial crowded solutions was performed on a SP5 SMD system (Leica Microsystems Mannheim and Picoquant Berlin) using a 488 nm continuous wave laser (FCS) or a pulsed 470 nm laser with 40 MHz repetition frequency (FLIM). Experiments were routinely performed with a pinhole of one Airy unit using a 63x/1.2NA water immersion objective except for FLIM on cells where a 63x/1.4NA oil objective was used to achieve higher photon counts. No significant differences in the heterogeneity were observed when switching objectives. Experiments on cells, and in part on cytosol, were performed at 37°C, all other measurements were done at room temperature. Control measurements at 37°C (performed for sucrose and cytosol) did not reveal marked changes in the heterogeneities.

For FLIM on 1 µM DASPMI in purified cytosol or artificial crowded fluids, we recorded in total \( 2 \cdot 10^5 \) photons for each sample in the range 647-703 nm (cytosol) or 607-683 nm (artificial fluids). Using 647-703 nm also for artificial fluids resulted in elevated noise levels due to poor photon counts but did not significantly change the heterogeneity \( \eta(\tau) \). FLIM data acquisition in cells (256x256 pixels, pixel size 134 nm, scan frequency 400 Hz, spectral range 647-703 nm) was limited to 7 min. for each cell and 60 min. per well to minimize bleaching and oxidative stress. As a consequence, the typical number of photons per pixel was \( n_p < 50 \). Polygonal cytoplasm and nucleoplasm areas were manually selected via fluorescence intensity images to allow for determining pixel-wise lifetimes in the respective areas. Raw FLIM data were corrected for background noise by thresholding (see Fig. S2 for examples): For each
compartment and specimen a cut-off time $T_c$ was determined at which the composite fluorescence lifetime histogram $p(T)$ ($\sim 10^6$ photons from all pixels, bin size 16 ps, smoothed over nine bins) had decayed to the twofold base level, $p(T > 23 \text{ ns})$. Calculating average lifetimes from raw FLIM data was then restricted to the range $T \leq T_c$.

To consider the varying and often low number of photons per pixel after background correction in cytoplasm and nucleoplasm, we restricted our subsequent analysis to pixels with $15 \leq n_p \leq 35$ photons. Hence, from more than 12,000 pixels per compartment only representative subsets of 5,400 pixels were taken to determine the heterogeneity as seen by FLIM. For each of these pixels, we calculated from the corresponding set of lifetimes, $T_1, \ldots, T_{n_p}$, the mean lifetime, $\tau$, yielding $p(\tau)$ and $\eta(\tau)$ for cytoplasm and nucleoplasm. To obtain an equivalent data set for artificial fluids and purified cytosol, we randomly picked 5,400 mutually exclusive sequences of $n_p = 25$ photons from twelve different photon currents for each specimen. We would like to emphasize that avoiding the outlined selection according to $n_p$ altered the absolute values of $\eta(\tau)$ only to a minor extent (±2%) but did not affect our main conclusion. Using fluorescence photons in the spectral range 500-550 nm (instead of 647-703 nm) also confirmed our conclusions.

Figure S1: Examples for FCS curves (black symbols) and best fits according to Eq. (1) (red lines) for the indicated samples. Please note that EGFP was used as a tracer in cytoplasm and nucleoplasm for improved photon statistics whereas DASPMI was used in all in-vitro samples. Subfigure with label DASPMI/EGFP shows the favorable overlap of an ensemble of noisy DASPMI curves (grey-shaded) and a single EGFP data set (red symbols), both recorded in the cytoplasm of living cells.

Figure S2: Examples for FLIM data (black lines) on DASPMI for the indicated samples. Shown are the probability distributions of fluorescence lifetimes, $p(T)$, as determined from all pixels in the respective sample/compartment. Red dashed lines indicate the twofold background countrate used for background correction (see text for details).