# **Extended Supplementary Information**

## **Experimental Setup**

The custom-made chamber utilised to monitor real-time loss of monomers from BSA-AF488 solution is presented in **Figure S 1**;



Figure S 1 Custom made chamber utilised to assess the real-time loss of monomers in imaging experiments. Please note that all flow components were sealed to prevent loss of sample due to evaporation.

## Assessment of White Noise Contribution for SpIDA analysis

The contribution of citrate-phosphate buffer (i.e. pH 7.0) to background noise was assessed using confocal microscopy. A sample of citrate-phosphate buffer was placed in an eight well chamber slide and representative confocal images of the buffer acquired at the same pixel dwell time (i.e.  $6.4 \ \mu s$ ) and resolution (i.e.  $44 \ nm$ ) as the image time series. Representative images obtained from imaging citrate-phosphate buffer are presented in Table S 1.

Table S 1 White noise values determined from the fluorescence intensity of citrate-phosphate buffer solution acquired with a pixel dwell time of 6.4  $\mu$ s and pixel size of 44 nm for a 1024 x 1024 pixel image.

Gain	White noise
600	160
650	170
700	170

Subsequently citrate-phosphate buffer images were subjected to analysis using Image  $J^{\text{(B)}}$  and the fluorescence intensity determined for the image determined as white noise. This parameter was inputted into the SpIDA user interface for all analysed image time series experiments.

## Quantification of the Quantal Brightness of Monomeric BSA-AF488 for SpIDA Analysis

The direct relationship between the quantal brightness of a monomeric entity and a dimer ( $\varepsilon = 2\varepsilon_0$ ) may be exploited in order to determine the spatiotemporal evolution of subpopulations in samples (i.e. image time series) consistent with aggregation.

When two fluorescent populations with different molecular quantal brightnesses are present within a sample (i.e. a confocal image) and not spatially segregated, or in the presence of autofluorescence, the total histogram becomes a convolution of the two distributions obtained from each species;

$$H(\varepsilon_1, N_1, \varepsilon_2, N_2, A; k) = H(\varepsilon_1, N_1; k) \otimes H(\varepsilon_2, N_2; k)$$

Where  $A_i$  represents the number of pixels,  $N_i$  represents the number of particles and  $\varepsilon_i$  represents the molecular quantal brightness for the *i*<sup>th</sup> population.

Application of a one-population model in a mixed sample will yield a resultant  $\varepsilon$  intermediate between the present species in the sample, whilst following performance of a monomeric  $\varepsilon$  control test it is possible to extract information about the present populations using a two-population (i.e. monomer-oligomer) model. Thereby, through appropriate knowledge of monomeric  $\varepsilon$  it is possible to determine spatiotemporal aggregation profiles from confocal image time series.

Hence, the brightness of monomeric BSA-AF488 obtained from size exclusion chromatography with a final concentration of 1.5 mg/ml (i.e. 22.5  $\mu$ M) was diluted to 1  $\mu$ M and subjected to confocal microscopy. Resultant images were analysed using SpIDA over 100 frames and the population data and corresponding quantal brightness were determined. The determined quantal brightness was applied to all further measurement of aggregation.

The determined molecular quantal brightness for the monomeric form of BSA-Alexa Fluor<sup>®</sup> 488 from an images time series was  $1.4 \pm 0.2$  intensity units (*iu*) per unit of pixel integration time.

#### **Real-time Assessment of Oligomer Formation with SpIDA**

Time-dependent changes in oligomer formation were monitored using confocal microscopy followed by analysis with SpIDA. Plots representing the temporal evolution of BSA-AF488 samples as a function of thermal stress at 50 °C are presented in Figure S 2 as follows;



Figure S 2 Real-time temporal evolution of dimer formation expressed as dimer to monomer ratio for 44 nm pixel sized images of 0.4 (top) and 1 mg/mL (bottom) BSA-AF488 subjected to thermal stress at 50 °C and subsequent analysis with SpIDA at indicated NaCl concentrations.

Data presented in Figure S 2 indicates the reversible formation of dimers under the conditions examined and within the experimental timescale at all ionic strengths. To assess the formation of trimers, further analysis of confocal image time series was performed, the results of which are presented as follows;



Figure S 3 Real-time temporal evolution of trimer formation expressed as trimer to monomer ratio for 44 nm pixel sized images of 0.4 (top) and 1 mg/mL (bottom) BSA-AF488 subjected to thermal stress at 50 °C and subsequent analysis with SpIDA at indicated NaCl concentrations.

Data presented in Figure S 3 indicates minimal and reversible trimer formation in all samples further supporting the proposed reversibility of dimer formation observed previously in Figure 2.

#### Determination of Reaction Order from t<sub>90</sub> slopes

As aforementioned, an adaptation of the model described by Brummit *et al* was applied to the assessment of monomer loss reaction orders in BSA-AF488 samples was estimated through determination of the curve slope obtained from monomer versus  $t/t_{90}$  plots. The resultant fit for a 1 mg/mL BSA-AF488 sample in the presence of 50 mM NaCl is illustrated as an example in Figure S 4 as an example in which the curve slope was determined as -0.5.



Figure S 4 Plot of monomer loss as a function of  $t_{90}$  on a log-log scale for a 1 mg/mL sample in the presence of 50 mM NaCl. The dotted line represents the slope of the monomer loss curve relative to a (solid) line possessing a slope of unity.

## Assessment of Polydispersity of BSA with Dynamic Light Scattering

### Dynamic light scattering (DLS)

The contribution of BSA labelling with Alexa Fluor<sup>®</sup> 488 to the observed behaviour and formation of larger aggregates was assessed through the performance of dynamic light scattering experiments and subsequent comparison of the data obtained from this method with SpIDA and RICS. Diffusion coefficients of resultant samples were measured at room temperature (i.e. 21 °C) at the start of each experiment and profiled up to two hours of exposure to 50 °C thermal stress.

The measured hydrodynamic radii (from Z-average data) of BSA samples were applied to the determination of the diffusion coefficient using the Stokes–Einstein equation for different NaCl and BSA concentrations. Polydispersities and respective diffusion coefficients are presented as follows;



Figure S 5 Representative plots determined from dynamic light scattering of BSA solutions subjected to thermal stress (50 °C). Time-dependent diffusion coefficient x  $10^7$ (cm<sup>2</sup>/s) variation determined from the z-average for 0.4 mg/mL (A) and 1 mg/mL (B) samples, time-dependent diffusion coefficient variation calculated from the intensity distribution of 0.4 mg/mL samples (C) and 1 mg/mL samples (D), and polydispersity indices determined for 0.4 mg/mL (E) and 1 mg/mL samples (F) in the presence of 50 (•) and 500 (•) mM NaCl in citrate-phosphate buffer.

Results presented in Figure S 5 from the measurement of the mass distribution of hydrodynamic radii of BSA samples formulated in the presence of 50 and 500 mM NaCl in citrate-phosphate buffer indicate an increase in the hydrodynamic radius of BSA under these conditions at earlier time points (i.e. 0-30 minutes) with a consistently slower diffusion coefficient observed in 1 mg/mL samples at both NaCl concentrations compared to 0.4 mg/mL BSA samples. Overall, diffusion coefficient data presented above, demonstrates a small contribution from the presence of large aggregates in intensity-based data (c-d) in comparison to that derived from the volume-based distributions (a-b). Additionally, the observed intensity distribution-derived diffusion coefficients are consistent with that of RICS and in the case of volume-based distribution diffusion coefficients the presence of lower values is in agreement with the existence of aggregates in confocal images (and the observed outliers in the RICS data).