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Bayesian Total Internal Reflection Fluorescence Correlation Spectroscopy Reveals hIAPP-Induced Plasma Membrane Domain Organization in Live Cells

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ABSTRACT Amyloid fibril deposition of human islet amyloid polypeptide (hIAPP) in pancreatic islet cells is implicated in the pathogenesis of type II diabetes. A growing number of studies suggest that small peptide aggregates are cytotoxic via their interaction with the plasma membrane, which leads to membrane permeabilization or disruption. A recent study using imaging total internal reflection-fluorescence correlation spectroscopy (ITIR-FCS) showed that monomeric hIAPP induced the formation of cellular plasma membrane microdomains containing dense lipids, in addition to the modulation of membrane fluidity. However, the spatial organization of microdomains and their temporal evolution were only partially characterized due to limitations in the conventional analysis and interpretation of imaging FCS datasets. Here, we apply a previously developed Bayesian analysis procedure to ITIR-FCS data to resolve hIAPP-induced microdomain spatial organization and temporal dynamics. Our analysis enables the visualization of the temporal evolution of multiple diffusing species in the spatially heterogeneous cell membrane, lending support to the carpet model for the association mode of hIAPP aggregates with the plasma membrane. The presented Bayesian analysis procedure provides an automated and general approach to unbiased model-based interpretation of imaging FCS data, with broad applicability to resolving the heterogeneous spatial-temporal organization of biological membrane systems.

INTRODUCTION

Nonspecific peptide-membrane interactions play an important role in numerous amyloid-related pathologies including the β-cell degeneration that is putatively involved in the development of type II diabetes (1,2). In this process, human islet amyloid polypeptide (hIAPP), a 37-residue peptide that is co-secreted by β-cells with insulin, has been suggested to interact with the cell surface membrane in forming aggregates and larger-scale fibrils that induce cytotoxicity (2,3). Although deposition of hIAPP amyloid fibrils in pancreatic islet cells is a common feature of type II diabetes, a number of studies suggest that oligomeric forms of hIAPP are the toxic species that increase membrane permeability or cause membrane disruption, which subsequently leads to cell death (4–10). However, most studies on the molecular mechanism of peptide-membrane interaction are performed on reconstructed model membranes at high peptide concentration (4, 7, 9, 10) without direct relevance to the roles of physiological hIAPP concentration (5–20 pM) (11) and cellular membrane environment on peptide aggregation in vivo.

Fluorescence correlation spectroscopy (FCS) is a powerful approach for probing the molecular dynamics of plasma membranes in live cells with a single-molecule sensitivity and the ability to resolve local molecular concentrations, aggregation states, and transport mechanisms (12–16). However, traditional confocal FCS uses a point detection scheme that only observes a single submicron size spot at a time, whereas membrane processes such as hIAPP aggregation occur over large spatial regions of the cell membrane and in a time-dependent manner. To capture this spatial-temporal heterogeneity, sequential measurements must be performed at distinct locations in the sample. This issue has been partly overcome by the emergence of imaging FCS techniques, which allow for multiplexed measurements. For example, imaging total internal reflection-FCS (ITIR-FCS) (17) and single-plane illumination-FCS (SPIM-FCS) (18–21) use array detectors including electron-multiplying charge-coupled device (EMCCD) and scientific complementary metal-oxide semiconductor cameras (sCMOS), which now enable simultaneous, parallel FCS measurements at hundreds to thousands of contiguous spatial locations with millisecond time resolution. In contrast to other image-based correlation spectroscopy techniques (22–25), spatial averaging is not employed in imaging FCS—making it ideal for resolving spatial heterogeneity in dynamic membrane processes.

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Although imaging FCS is useful for the investigation of such dynamic, heterogeneous membrane processes, the approach typically generates large datasets that contain hundreds to thousands of temporal autocorrelation functions (TACFs) with varying noise and unknown underlying dynamical physical processes, requiring an automated, objective analysis procedure for their proper interpretation. Conventional imaging FCS analysis procedures typically fit a single-component diffusion model to extract an apparent diffusivity at each spatial location interrogated, or select a best-fitting model based on a simple $\chi^2$-squared statistic without proper consideration of the highly correlated noise that is present in a given TAC function (26–28). Further, the FCS diffusion law that employs spatial averaging to quantify the overall extent of membrane heterogeneity and thereby distinguish distinct domain organizations, does not spatially resolve membrane heterogeneity, providing only limited insight into the temporal dynamics and detailed mechanism of peptide-membrane interactions (29). For example, Bag et al. (30) recently used ITIR-FCS to interrogate the spatial-temporal dynamics of hIAPP-membrane interactions. They demonstrated that below the critical concentration for peptide aggregation in solution, hIAPP induced the formation of microdomains on the plasma membrane of neuroblastoma cells, which were used as a model cellular system to probe peptide-membrane interactions. However, the spatial organization of these microdomains as well as the temporal dynamics of microdomain formation remained unclear due to the foregoing limitations of conventional ITIR-FCS analysis.

To overcome these limitations of conventional FCS analysis for the study of spatially and temporally heterogeneous processes in an in vivo setting, we recently developed a Bayesian model selection and parameter estimation approach to analyze confocal FCS data (26,27). Importantly, we demonstrated that a statistical blocking procedure could be used to estimate, in a model-free manner, the longest correlation time present in a given FCS measurement, which is essential to the proper estimation of the correlated noise present in TACF curves that is required for unbiased model selection and associated parameter estimation. Here, we extend our TACF-based Bayesian analysis procedure to ITIR-FCS and demonstrate its utility for resolving membrane heterogeneity in model membranes and neuroblastoma cells treated with hIAPP. Consistent with previous observations in supported lipid bilayer (SLB) model membranes, analysis of live cell data supports the carpet model for the organization of hIAPP-induced domains on the plasma membrane (31,32), which was not resolved previously using conventional FCS analysis (30). Although we focus on the application of the Bayesian procedure to ITIR-FCS measurements in this work, the approach is generally applicable to imaging FCS using different optical configurations such as SPIM-FCS or spinning disk confocal FCS (33,34).

MATERIALS AND METHODS

Two-dimensional compartmentalized diffusion simulations

Compartmentalized diffusion of fluorescent particles is implemented in the software MATLAB (The MathWorks, Natick, MA). One-thousand particles are initially distributed randomly in a $30.7 \times 30.7 \mu m^2$ two-dimensional simulation plane with circular domains placed at the center of the plane. At each time step, the position of each diffusing particle is updated by adding a normally distributed random number with standard deviation $\sqrt{2D\Delta t}$ and zero mean to each coordinate dimension, where $D$ is the particle diffusion coefficient that depends on the particle position ($D_{in}$ and $D_{out}$ for inside and outside microdomains, respectively), and $\Delta t$ is the size of the time-step, which is set to be equal to the imaging time resolution (1 ms). Following Wawrezinieck et al. (29), particles can diffuse freely either into or out of microdomains or cross boundaries with a prescribed crossing probability to simulate confined diffusion. Periodic boundary conditions are employed for boundaries of the simulation plane.

To avoid loss of numerical precision in particle positions and to avoid convolution artifacts, images with $16 \times 16$ pixels and 0.24 $\mu m$ pixel size are simulated by first creating a $64 \times 64$ square grid with 0.12-\(\mu m\) grid size at the center of the simulation box. Particle counts in the grid are stored at each time step. Particle counts are weighted by their brightnesses to obtain photoelectron counts, which are then converted to raw image matrices by applying a normalized Gaussian filter with 0.16 $\mu m$ standard deviation (320 nm $\epsilon^{-2}$ radius). Images with the desired dimensions are obtained using 2 \times 2 binning on the image matrices and cropping the $16 \times 16$ pixel central portion of the image. Unless stated otherwise, particle brightness of 20,000 photoelectrons/particle is employed, which yields the relative noise level of the TACF similar to the RhoPE-labeled SLB data with the same number of frames and diffusion coefficient. The relative noise level of the TACF is defined as the average of the first 16 values of $\sigma G(\tau)/\langle G(\tau) \rangle$, where $\sigma G(\tau)$ is the noise of the TACF $G(\tau)$.

EMCCD noise is simulated with the Poisson-$\gamma$-normal model (35) using camera parameters measured from our imaging system (see Section S1 in the Supporting Material). The maximum multiplication gain of our camera was measured to be 164 (corresponding to a maximum setting on an arbitrary scale of 0–300 from the manufacturer’s software). All parameters were determined at this gain. Specifically, the detected photoelectron count is generated from a Poisson random number generator with the photoelectron count at each pixel of the raw image as the mean. The electron-multplying (EM) noise is simulated by generating a $\gamma$-distributed random number with the EM gain of 164 as the shape parameter and detected photoelectron count as the scale parameter. The readout noise is simulated by adding a normal-distributed random number with zero mean and standard deviation of 54. The resulting electron count at each pixel is then divided by the analog-to-digital gain of 16.92 electrons/image-count, converted to a 16-bit integer, and added with the camera offset of 96 image-counts. Parameters of the noise model are measured using the camera at the same EM gain as in the experiments. The noise distribution generated using this model is in a good agreement with the experimentally measured noise distribution (see Fig. S1 in the Supporting Material).

Lipids and dyes

Lipids and dye used for SLBs were DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine) and DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), which were headgroup-labeled with rhodamine dye RhoPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfo-nyl) (ammonium salt). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Lipid and dye stock solutions were prepared in chloroform. Dil-C18 (1,1’dioctadecyl-3,3,3’,3’-tetramethylindocarbocya-nine perchlorate, C18) was purchased from Invitrogen (Singapore). Stock
Preparation of SLBs

SLBs were prepared by vesicle fusion. Briefly, a specific amount of lipids and RhoPE were mixed in a cleaned round-bottomed flask. The solvent was evaporated by warming a rotary evaporator (Rotavap R-210; Buchi, New Castle, DE) for at least 3 h. The lipid film thus obtained was resuspended in buffer containing 10 nM HEPES and 150 mM NaCl (pH 7.4) and vortexed vigorously. This milky solution was processed in a bath sonicator (Model No. FB15051; Fisher Scientific, Singapore) until a clear solution was obtained in which large unilamellar vesicles had formed. A quantity of 200 μL of the solution was placed on a clean cover glass (24 × 20 × 1 mm2; Fisher Brand Microscope cover glass; Fisher Scientific, Singapore) containing 200 μL of the same buffer. Deposited vesicles were incubated at 65°C for 20 min followed by cooling at room temperature for 20 min to form SLBs. Unfused vesicles were removed by washing 50 times with 100 μL of the same buffer.

Cell culture and Dil staining

Adherent neuroblastoma cells (SH-SY5Y) were cultivated in DMEM (Dulbecco’s Modified Eagle Medium; Invitrogen, Singapore), supplemented with 10% FBS (fetal bovine serum; Invitrogen) and 1% PS (penicillin, and streptomycin; Invitrogen) at 37°C in a 5% (v/v) CO2 humidified environment. For Dil staining, the stock solution was diluted to a final concentration of 50 nM with Phenol Red free DMEM medium. The culture medium (DMEM, 10% FBS, and 1% PS) was first removed from the cover dish (chamber mounted on No. 1.0 borosilicate cover glass, 8 units; Nunc, Roskilde, Denmark and Thermo Scientific, Singapore), which was seeded with cells beforehand. The Dil solution was then added to the cover dish and incubated at 37°C for 30 min. After 30 min, the cover dish was rinsed with Phenol Red free medium (DMEM and 10% FBS) twice before adding the Phenol Red free medium to the cover dish for imaging.

Preparation of hIAPP peptide solution

hIAPP was synthesized following the protocol in Bag et al. (30). A weighed amount of hIAPP (dry powder) was mixed with desired amount of HFP (1,1,1,3,3,3-Hexafluoro-2-propanol). The mixture was then carefully heated at 45°C for 30 min to dissolve hIAPP. The solution was then very slowly filtered by a 0.45-μm Teflon filter (E. I. Du Pont de Nemours, Wilmington, DE) after cooling. The clear solution was able to be stored at 4°C for more than a week without loss of reproducibility. The upper limit of stock concentration without aggregation is 4 mM. The stock solution was diluted in Phenol Red free medium (DMEM and 10% FBS) to a final concentration of 1 μM and incubated at 37°C for 30 min before being added to the sample.

ITIR-FCS measurements

Following Sankaran et al. (36), ITIR-FCS measurements were performed on an inverted epi-fluorescence microscope (Model No. IX-71; Olympus, Singapore) using a high NA refractive index (1.516 at 23°C) oil immersion objective (PlanApo, 100×, NA 1.45; Olympus). The excitation beam from a 532-nm laser (Samba; Cobolt, Solna, Sweden) was introduced into the microscope using two tilting mirrors. The beam was then focused onto the back focal plane of the objective using a dichroic mirror (Cat. No. Z488/532RPC; Semrock, Rochester, NY). The incident angle for total internal reflection was adjusted using the same tilting mirrors. Fluorescence emission from the sample was collected using a back-illuminated EMCCD camera (128 × 128 pixels with 24-μm pixel size. Andor iXON 860; Andor Technology, Belfast, UK) after filtering using an emission filter (Cat. No. Z488/532M; Semrock). The software SOLIS (Ver. 4.18.30004.0; Andor Technology) was used for image acquisition. The standard deviation of the point spread function (PSF) is calculated by σ = λem/NA, where λem is the emission wavelength of the dye, and σ was previously measured to be 0.4 (37). The calculated σ is 161 nm (322 nm e−2 radius) for RhoPE and 156 nm (312 nm e−2 radius) for Dil-C14. In general, a stack of 50,000 frames from a 21 × 21 pixel region of interest (5 × 5 μm2) on the membrane was collected with 1-ms time resolution and saved in a 16-bit tagged image file format. The EM gain of the camera employed was 300 (on a scale of 0–300). The kinetic mode of image acquisition and a baseline clamp were used to minimize baseline fluctuation. All live cell measurements were performed at 37°C and in a 5% CO2 environment.

Data analysis

The first 500 frames of the fluorescence movie are discarded in the calculation of TACFs to avoid the unstable baseline present at the outset of image acquisition. Photobleaching is corrected pixelwise by subtracting a double-exponential fit to the intensity trace and adding a constant to retain the same mean intensity before correction. The background is removed by subtracting the average pixel intensity measured without illumination. The TACF of the corrected intensity trace is computed as a function of lag-time τ at each pixel according to

\[ G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2}, \]  

where \( \delta F(t) = F(t) - \langle F(t) \rangle \) is the fluctuation of the measured fluorescence intensity, and \( \langle \ldots \rangle \) denotes the time-average over the acquisition time \( T_{av} \). TACFs are computed up to and including a lag-time of 5 s using the multi-τ algorithm (Schätzel and Peters (28) and Wohland et al. (18)), which generates TACFs with a quasi-logarithmic timescale increment. Specifically, the increment of the lag-time for the first 32 points in the TACF is equal to the time increment in the raw intensity trace, and the increment is doubled for each of the following 16 points.

Noise and noise covariance matrices in TACFs are estimated from the corrected intensity trace using an automated blocking procedure (see Section S2 in the Supporting Material) (26). The blocking curve at the minimal lag-time \( \tau_1 \) is calculated from the intensity product trace

\[ \{ \delta F(t) \delta F(t + \tau) \}_{\tau = 0}^{T_{av}} \]

for each TACF, from which the minimal block-time (or fixed-point) is determined.

The intensity product trace at \( \tau_1 \) has the strongest correlation in time and therefore also the longest minimal block-time, ensuring that the blocks defined by this block-time are independent for all \( \tau \).

The maximum-likelihood parameter estimation is performed for each TACF using two-dimensional free diffusion models with different numbers of diffusive components derived for camera-based FCS (36),

\[ G(\tau) = \sum_{i=1}^{N_0} \alpha_i \left( \frac{e^{-(p_i(\tau))^2} - 1}{\sqrt{\pi p_i(\tau)}} \right) + G_w, \]  

where \( G(\tau) \) is the TACF with \( N_0 \) diffusive components,

\[ p_i(\tau) = \frac{a}{\sqrt{4D_i \tau + w_i^2}}. \]
and

\[ \alpha_i = \frac{B_i^2 \langle N_i \rangle}{\sum_i (B_i \langle N_i \rangle)^2} \]

is the amplitude of component \( i \). The values \( D_i, \langle N_i \rangle \), and \( B_i \) represent the diffusion coefficient, average number of particles in the detection area, and brightness of component \( i \), respectively. The values \( a \) and \( w_0 \) are the pixel size and the \( e^{-2} \) radius of the PSF. \( G_N \) is the long-time value of \( G(t) \), which should be close to zero as a result of the estimation process. Model probabilities and parameter estimates are calculated using the Bayesian inference procedure described previously (26,27) (Fig. 1).

**RESULTS AND DISCUSSION**

**Automated blocking procedure for correlated noise estimation**

A key initial step in the proposed Bayesian imaging FCS procedure is the use of an automated blocking method to perform unbiased estimation of the highly correlated noise that is present in the hundreds-to-thousands of TACF curves analyzed, as well as to estimate the temporal sampling interval, \( \Delta T \), for the time-dependent FCS measurement (Fig. 1). As previously shown, the correct noise level and correlation in the mean TACF may be computed from multiple independent TACF measurements that interrogate the same stationary physical process, or alternatively from a single raw intensity trace itself using the blocking procedure (26). Because the blocking procedure computes the correlated noise directly from the raw intensity trace without requiring multiple, independent TACFs, it is essential to FCS measurements of time-varying, transient biological processes in which repeated measurements of the same physical-chemical process are not available.

To obtain correct TACF noise estimates from the raw intensity trace, the blocking procedure divides the raw intensity trace into a number of sequential temporal blocks and

![Bayesian ITIR-FCS approach.](https://example.com)
computes the noise estimate (standard error) from local block averages as a function of increasing block-time (or block-size). The block-time corresponding to the plateau region (fixed-point) in the blocking curve represents the time at which samples become independent (or decorrelated), so that TACF noise levels and their correlations may be estimated without bias that is otherwise introduced by correlations in intensity fluctuations. Noise estimations from samples with block-times smaller than this minimal block-time will necessarily be underestimated, likely leading to erroneous model selection and associated parameter estimation (Fig. 2 A, top).

To facilitate the application of the blocking procedure to thousands of pixels simultaneously, we designed an algorithm consisting of a set of heuristic criteria to automatically determine blocking curve plateaus for use in TACF noise estimation (see Section S2 in the Supporting Material). When the plateau is indeterminate due to high noise in the blocking curve at long block-times, or the intensity trace is too short to reach the plateau in the blocking curve, the algorithm cannot resolve the plateau and its associated block-time (Fig. 2 A, bottom). For pixels that do not exhibit plateaus in their blocking curves (i.e., that fail the blocking test), we use the maximum block-time available from the blocking curves (corresponding to 8–15 blocks) to estimate noise in the TACF and distinguish these pixels accordingly. For multiple-component diffusion models evaluated in this work, simulations show that the rate of misclassification is typically <10% when noise is estimated using the maximum block-time (see Fig. S6 and Fig. S7).

**FIGURE 2** Automated blocking yields accurate noise estimates for unbiased multiple hypothesis testing of spatially resolved FCS data. (A) (Top) Schematic showing definitions of block times required for automated application of the blocking procedure. (Middle and bottom) Sample blocking curves that respectively pass and fail the blocking test. (B) Bayesian analysis of simulated compartmentalized two-component, two-dimensional diffusion with respective diffusivities $D_1 = 4 \mu m^2/s$ and $D_2 = 0.4 \mu m^2/s$ outside and inside the domains, respectively, and decreasing noise levels (left to right; number of frames = $10 \times 10^3$, $50 \times 10^3$, $200 \times 10^3$). Automated blocking is performed to identify optimal block times for model selection and parameter estimation. Scale bar: 1 \( \mu \)m. (C) Bayesian model selection and parameter estimation using noise estimates from raw intensity traces without blocking. (Light-blue circles) Domain boundaries and dots indicate pixels where multiple diffusing components ($N_D = 2, 3$) are detected. Corresponding distributions of estimated diffusion coefficients from the inferred models are shown below each image. To see this figure in color, go online.
For dynamic, time-varying cellular processes such as hIAPP aggregation, the blocking procedure provides an estimate of the total acquisition time $T_{aq}$ that each measurement should employ to ensure that it is 1), sufficiently long for proper noise estimation, and 2), adequately short compared with the longest characteristic timescale of the time-varying physical-cellular process, which is essential to minimize spurious correlations that may result from long timescale fluorescence fluctuations (Fig. 1).

To demonstrate that unbiased FCS model inference requires proper TACF noise estimates in the context of two-dimensional membrane systems, we first apply the Bayesian procedure to simulated images of partitioned membrane domains consisting of differential diffusivities across their boundaries (see Materials and Methods and see Section S4 in the Supporting Material). Pure diffusion models with one, two, and three diffusive components are tested and their associated diffusivities are evaluated for distinct noise levels estimated with and without use of the automated blocking procedure (Fig. 2 B).

When the blocking procedure is used, the one-component model is preferred in the entire field of view at high noise, and the two-component diffusion model is increasingly resolved near the domain boundary as the noise level decreases (Fig. 2 B, top row). This is because the PSF averages intensity fluctuations from particles inside and outside the domains with distinct diffusivities near the domain boundary, thereby resulting in two-component TACFs even though the two distinct diffusing species are in fact only present on either side of the domain boundary. The large difference in diffusivities inside and outside the domains results in the large difference in the equilibrium particle concentrations inside and outside the domains, as shown by the ~100 times higher intensity of the microdomains. Two-component diffusion is detected outside the boundary because the two diffusing components are better resolved when their contributions to the TACF are nearly equal ($\alpha_2/\alpha_1 = 1$).

The ability of the Bayesian approach to resolve the two components improves with decreasing noise level, as shown using simulations of confocal FCS in prior work (26,27). Further, two-component diffusion is only detected near the domain boundary for pixels that both pass and fail the blocking test at all noise levels. In contrast, two or more components are detected both in regions far from the boundary as well as near to it when noise is improperly estimated without the blocking procedure (Fig. 2 C, top row). The parameter estimate of the two-component diffusion model has a broader distribution because it is less reliable when model selection is biased (Fig. 2 B and C, bottom row) (26,27). Thus, the automated blocking procedure yields correct noise estimates essential to unbiased model selection and accurate downstream parameter estimation.

When the models being evaluated are nested, e.g., diffusion models with different number of components, model selection may alternatively be performed using conventional frequentist hypothesis testing procedures such as the F-test (see Section S3 in the Supporting Material). However, the F-test exhibits a higher rate of overfitting than the presented Bayesian procedure even when noise is estimated using the proposed blocking procedure (see Fig. S8 and Fig. S9). Indeed, frequentist hypothesis testing procedures are known to be more likely to reject the null hypothesis than Bayesian procedures that appropriately penalize model complexity, empirically leading to higher rates of overfitting (38). Further, note that unlike Bayesian procedures that condition hypothesis testing on models themselves, the F-test and other frequentist tests cannot be used to test nonnested models, such as passive diffusion versus active transport. Nonnested models are of central interest to membrane systems that may contain motor-driven or motor-related activity (39).

In addition to noise level, the effects of domain type, domain size, and fluorophore brightness on the ability of the Bayesian procedure to detect microdomains are also examined using simulations (see Section S4 in the Supporting Material). Interestingly, whereas we focus on membrane microdomains that are larger than the diffraction limit here, simulations indicate that subdiffraction-limited microdomains may be resolved using this approach under certain conditions (see Fig. S10 and Fig. S11).

### Analysis of two-component supported lipid bilayers

To further evaluate the proposed Bayesian procedure on experimental membrane systems, we applied it to two-component SLBs (DLPC/DSPC = 1:1) in which lipid bilayers exhibit phase separation consisting of a DLPC-enriched fluid-phase with high diffusivity and DSPC-enriched gel-phase with low diffusivity (37). The gel-phase is visible as micron-sized domains with higher intensity in TIRF images because the dye used to stain the lipid bilayers (RhoPE) partitions differentially into the two phases (Fig. 3 A).

Similar model selection patterns to those found in simulations are resolved when a comparable number of imaging frames is employed (Figs. 3 A and 2 B, upper middle). Due to hardware limitations on the maximum number of frames that can be recorded, however, two diffusive components near the domain boundary cannot be clearly resolved as in the case of the low noise simulations (Fig. 2 B, upper right). Given this limitation, we applied Student’s $t$-test to the distances from two-component pixels to the phase boundary to test whether that the number of diffusive components detected is indeed correlated with proximity to the phase boundary (Fig. 3 C, inset). Distributions of estimated diffusivities indicate that the high diffusivity peak from two-component model regions coincides with the high diffusivity peak from the one-component model region in...
FIGURE 3 Membrane heterogeneity in two-component, phase-separated SLBs is resolved by Bayesian ITIR-FCS. (A) TIRF image of RhoPE-labeled two-component supported lipid bilayers (DLPC/DSPC). (Dots) Pixels where multiple diffusing components ($N_D = 2, 3$) are detected. Scale bar: 1 μm. (B) Map of estimated diffusion coefficients from inferred models. Diffusion coefficients corresponding to the slow component are shown for the two-component model. (C) Distribution of diffusion coefficients from inferred models. (Inset) Mean distances of two-component pixels and all pixels from domain boundaries ($p$ value $< 0.01$). (D) Distribution of diffusion coefficients obtained using conventional FCS analysis. To see this figure in color, go online.

the DLPC-enriched phase, and that the low diffusivity peak from the two-component model is clearly in the lower range compared with the slow diffusivity peak from the one-component model in the DSPC-enriched phase. This suggests that the DSPC-enriched phase may have more than one component that is unresolved given the level of measurement noise. This results in an apparent diffusivity that is higher than the slow component from the two-component model regions. The observed heterogeneity in DSPC-enriched domains is consistent with the small number of two-component pixels inside the domains and a previous study that examined cross-correlation of neighboring pixels (ΔCCF) (37). The heterogeneous intensity distribution inside the microdomains also suggests that the observed microdomains might be clusters of smaller domains, as shown by previous AFM studies (40,41). As a control, we also tested the approach using one-component lipid bilayers (DLPC). The two-component model is preferred only for a small fraction of pixels (~5%), but this could have resulted from residual, unfused vesicle that was not washed out in the preparation of the SLBs or from roughness of the coverslip (see Section S5 and Fig. S12 in the Supporting Material).

Organization of domains induced by monomeric hIAPP in the plasma membrane

The above examples illustrate the ability of the Bayesian FCS procedure to resolve heterogeneity in model membranes. Next, we sought to characterize the organization of microdomains induced by monomeric hIAPP forming on live neuroblastoma (SH-SY5Y) cell membranes, which has been used to study the cellular leakage mediated by hIAPP as well as other amyloidogenic peptides (42). A series of ITIR-FCS measurements of the plasma membranes of SH-SY5Y cells stained with fluorescent probe (DiI-C18) was recorded at 5-min intervals, with bright domains becoming clearly visible and increasing in size ~5 min after the addition of monomeric hIAPP. Similar to the microdomains observed in phase-separated SLBs, conventional analysis shows low diffusivity inside the domains. In contrast to conventional FCS analysis, however, Bayesian model selection outcomes for these domains exhibit highly distinct patterns in which two-component diffusion is detected throughout the domains (Fig. 4 A, top row, and see Movie S1 in the Supporting Material) rather than only at domain boundaries (Fig. 3 A). Note that two-component diffusion is observed in some regions on the plasma membrane even before addition of hIAPP, which may result from the complex organization of the plasma membrane itself.

Distributions of diffusivities of the two components reveal a fast component $D_{fast}$ coinciding with diffusion in the domain-free plasma membrane as well as a slower component $D_{slow}$, which presumably reflects the diffusion of aggregate peptide-lipid complexes (Fig. 4 A, upper and lower middle rows, and see Movie S2). While one-component diffusion eventually becomes the preferred model in the central regions of microdomains (post $t = 40$ min), the fact that its diffusivity value lies between the two values of the distinct diffusing components in two-component regions also indicates the presence of two components in this region (arrows in Fig. 4 A, lower middle row). These distinct components are likely not resolved because the amplitude of the fast component is below the level that can be detected by the Bayesian approach given the noise level, which is also consistent with the observed plateau in the fraction of the slow component $\alpha_{slow}$ at $\alpha_{slow} \sim 0.55$ (Fig. 4 B, middle) and simulation results (see Section S6 and Fig. S13 B in the Supporting Material) (26,27). The clear presence of two diffusing components inside microdomains suggests that the observed domains are adsorbed on the surface of the membrane rather than embedded within it, like the microdomains in the phase-separated SLB, resulting in TACFs containing simultaneous contributions from both free membrane diffusion and aggregate peptide-lipid domains. This interpretation is supported by simulations of microdomains carpeting on membranes (see Section S6 and Fig. S13 in the Supporting Material). In contrast, conventional FCS analysis yields a single apparent or effective diffusivity inside the domains, which is approximately one order of magnitude higher than the value of $D_{slow}$ that is observed using model-based Bayesian analysis (Fig. 4 A, bottom row). Experimental controls to test this interpretation would be interesting to pursue in future studies.
FIGURE 4 Bayesian ITIR-FCS supports the carpet model for organization of hIAPP-induced domains forming on the plasma membrane. (A) (First row) TIRF images of DiI-labeled cell membrane as a function of time after addition of peptide. (Dots) Pixels at which multiple diffusing components ($N_D = 2, 3$) are detected. Scale bar: 1 μm. (Second row) Spatial maps of estimated diffusion coefficients from the inferred models. Diffusion coefficients corresponding to the slower component are shown for the two-component model. (Third row) Spatial maps of the slow component fraction. Fractions in one-component regions are set to 1 for pixels located inside domains and 0 for pixels outside domains. (Fourth row) Distributions of diffusion coefficients estimated from inferred models. (Arrows at time-points 40 and 60 min) Peak of the one-component model pixels inside the domain at high fraction of the slow component. (Fifth row) Distribution of diffusion coefficients estimated using conventional FCS analysis that assumes a single component is present throughout the spatial domain. (B) (Left) Temporal evolution of diffusion coefficients estimated using conventional analysis (gray) and Bayesian analysis capturing two-component regions (orange). Medians and quartiles are shown. (Middle) Temporal evolution of the slow component fraction in two-component regions as a function of time. Medians and quartiles are shown. (Right) Temporal evolution of fractions of pixels classified to each model using Bayesian model selection. Four phases can be defined based on the trends of these parameters: I, $t = 1–5$ min; II, $t = 5–40$ min; III, $t = 40–60$ min; and IV, $t = 60–75$ min. (C) Schematic showing the proposed carpet model of hIAPP aggregation on the surface of the plasma membrane. Aggregates increase in size as peptides extract lipids from the membrane. To see this figure in color, go online.
Examination of the temporal evolution of the estimated diffusivity and amplitude parameters (Eq. 2) reveals four distinct phases of hIAPP domain formation (Fig. 4 B):

The first (Phase I: \( t = 1 \) to \( t = 5 \) min) consists of a nucleation phase in which domains begin to form but are not yet clearly visible by eye. Locations of two-component pixels are relatively dynamic in this phase, resulting in variable \( D_{\text{fast}} \) and \( \alpha_{\text{slow}} \).

The second (Phase II: \( t = 5 \) to \( t = 40 \) min) contains two components that are clearly detected within bright hIAPP domains. Changes in \( \alpha_{\text{slow}} \) may result either from changes in domain height or the lipid density in domains, so that the monotonic increase in \( \alpha_{\text{slow}} \) and the areal fraction of the two-component region may be related to the expansion of domains vertically and horizontally, respectively, assuming fixed lipid density in domains (Fig. 4 A, middle row, and see Movie S3).

In the third (Phase III: \( t = 40 \) to \( t = 60 \) min), the areal fraction of the two-component region reaches a plateau even though the size of the domains continues to increase. This is because the contribution from diffusion in the membrane (\( \alpha_{\text{fast}} \)) becomes too small to be detected in the centers of the domains as \( \alpha_{\text{slow}} \) increases, resulting in the one-component regions dominated by the slow component.

Finally, in the fourth phase (Phase IV: \( t > 60 \) min) microdomains cease to expand, coinciding with the plateau in \( \alpha_{\text{slow}} \).

Importantly, conventional ITIR-FCS analysis shows an initial increase in the median of \( D \) from all pixels in the first 10 min after addition of peptide, followed by a constant decrease until the end of Phase III. This is in contrast to the preceding results of the Bayesian ITIR-FCS approach, which reveals, after an initial increase in the first 10 min, \( D_{\text{fast}} \) and \( D_{\text{slow}} \) in the two-component region remain largely constant during the remaining measurement period. These results indicate that monomeric hIAPP association with the plasma membrane increases membrane fluidity (\( D_{\text{fast}} \)), similar to observations in SLBs (30). The observed decrease in the median value of \( D \) from all pixels after 10 min is primarily due to the increasing size of the microdomain area captured in the imaging field of view, as well as the increasing value of \( \alpha_{\text{slow}} \). Given the low diffusivity observed within micro-domains (\( D_{\text{slow}} = 0.1–0.01 \) \( \mu m^2/s \)), previously observed slow fluorescence recovery from fluorescence-recovery-after-photobleaching (FRAP) experiments on SLBs after addition of soluble hIAPP may reflect recovery due to the slow diffusion within peptide-lipid domains that cover the intact membrane rather than due to diffusion of lipids in the membrane (43).

Taken together, these results support a model for microdomain formation in which hIAPP forms a carpet on the plasma membrane. This carpet subsequently extracts lipids from the membrane by forming peptide-lipid complexes as domains expand both horizontally and vertically, consistent with previous observations for the association mode of hIAPP with SLBs (Fig. 4 C) (30). In contrast to that work, however, this analysis reveals dynamical aspects of the spatial-temporal organization of membrane microdomains induced by hIAPP on plasma membranes by resolving multiple diffusing species, which is a unique result of the inference ability of the Bayesian ITIR-FCS approach. A competing model for peptide-membrane interaction in which peptides form porelike structures would involve penetration of the membrane by microdomains and result in a model selection pattern similar to microdomains in phase-separated SLBs, which is not observed under the experimental conditions employed.

CONCLUDING DISCUSSION

Rigorous estimation of the statistical noise present in TACF curves obtained from imaging FCS samples can be automated to perform objective Bayesian model selection and parameter estimation, which are essential to extracting maximum molecular biological information from the FCS data. Application of this approach to ITIR-FCS data enables the investigation of dynamical membrane processes evolving in both space and time, as is typical of complex living systems. Application of the procedure to hIAPP-treated live cells shows hIAPP increases fluidity of the plasma membrane while inducing the formation of microdomains that carpet the membrane, presumably consisting of high-molecular-weight peptide-lipid complexes.

Although the precise nature of these microdomains and their implications on cytotoxicity warrant significant further investigation, these preliminary findings provide in vivo evidence in support of the carpet model for peptide-membrane interactions that lie below the critical concentration for peptide aggregation and peptide-induced membrane disruption. The general nature of the proposed Bayesian FCS inference procedure should find wide applicability to two-dimensional membrane processes as well as three-dimensional molecular processes interrogated using SPIM in diverse living systems that include embryos and tissues (44,45). Our approach is available to the broader scientific community at http://fcs-bayes.org.

SUPPORTING MATERIAL

Thirteen figures, three movies, References (46,47) and supplemental information are available at http://www.biophysj.org/biophys-supplemental/ S0006-3495(13)05717-2.

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REFERENCES


