**Supplementary Information**

**A comprehensive review of fluorescence correlation spectroscopy (FCS)**

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**1 Pulsed Interleaved Excitation Based Dual-color FCCS**

The spectral crosstalk in dual-color FCCS gives rise to an artificial cross-correlation.To suppress the crosstalk, Müller *et al.* proposed pulsed interleaved excitation (PIE)[[19](#_ENREF_19)]. In PIE, two or more excitation pulses (e.g., green and red pulses) are interleaved repeatedly and synchronized by the common master clock. The detection of fluorescence is performed with a time-correlated single-photon counting (TCSPC) board. The TCSPC board[[134](#_ENREF_134)] records for each photon the absolute arrival time (macro-time) and the relative time (micro-time) with respect to the trigger signal from the excitation lasers. The green and red pulses are delayed electronically with each other to ensure temporal separation of the excited fluorescence of two species, as shown in Figure S1a. Then, the crosstalk can be eliminated, and the accuracy of FCS results can be improved validly. Importantly, the delay between the green and red pulses should be varied according to the fluorescence lifetime of the fluorophores to avoid crosstalk in different channels[[135](#_ENREF_135)]. According to the fluorescence lifetime, the excitation rates usually range from 1 MHz to 50 MHz.

As a principle-validation experiment, dual-color FCCS with and without PIE has been performed in glycerol/water solution (1:1.5, v/v) mixed with Atto488 and Atto550 dyes. Figure S1b and c show the autocorrelation and the cross-correlation results without PIE and with PIE, respectively. The results show that the amplitude of cross-correlation without PIE is as large as 16% of the autocorrelation, which is a false positive cross-correlation due to the spectrum crosstalk. However, there is no clear cross-correlation with PIE, and meanwhile, the autocorrelation of Atto550 is enhanced for the improved SNR. The comparison reveals that PIE can remove crosstalk effectively in dual-color FCCS.

Except for the capability of removing crosstalk, the PIE scheme can enhance the time resolution of conventional dual-color FCCS by two folds[[135](#_ENREF_135)]. Hence, PIE based dual-color FCCS[[45](#_ENREF_45)] can be used for the study of fast dynamics processes, such as protein-protein interactions in live cells[[136](#_ENREF_136)]. In this pursuit, Jastrzebska e*t al.*[[137](#_ENREF_137)] used this method to measure the population of receptors that undergo co-diffusion, which is a signature of stable dimerization. They found that human red cone opsin exhibits a high propensity for dimerization. Moreover, PIE has also been applied to polarization-sensitive FCS[[116](#_ENREF_116)], scanning FCS and so on. Recently, Gao *et al.*[[138](#_ENREF_138)] proposed PIE based line-scanning spatial correlation spectroscopy (PIE-lsFCS), which overcomes artifacts due to the crosstalk and membrane movement. They



**Figure S1.** Schematic illustration of PIE based Dual-color FCCS. (a) Two-color excitation and detection, with pulsed interleaved excitation scheme. The correlation curves were obtained for the Atto488 and Atto550 dyes in glycerol/water solution (1:1.5, v/v) without PIE (b) and with PIE (c).

investigated with PIE-lsFCS the lateral diffusion of double-stranded DNA labeled with two fluorophores.

In addition, by virtue of the TCSPC, the fluorescence lifetime of fluorophores can be included in FCS analysis. For example, Lanzanò *et al.*[[97](#_ENREF_97)] combined the separation of photons by lifetime tuning and fluorescence lifetime correlation spectroscopy (SPLIT-FLCS). With this method in STED-FCS, the fluorophore position is determined according to the lifetime of fluorescent photons. They measured the diffusion of EGFP at spatial scales ranging from the diffraction size down to 80 nm in living cells. In a word, the combination of PIE and TCSPC is an important technique to promote the development of two or multi-channel FCS.

**2 Comparison of Different Approaches Targeting Biomolecular Dynamics**

There are several fluorescence-related methods for investigating the molecular dynamics, such as fluorescence correlation spectroscopy (FCS), fluorescence recovery after photobleaching (FRAP)[[139](#_ENREF_139)], fluorescence resonance energy transfer (FRET)[[140](#_ENREF_140)], raster image correlation spectroscopy (RICS)[[57](#_ENREF_57)], and single-molecule tracking (SMT)[[141](#_ENREF_141)], as is summarized in Table S1.

FCS is a powerful tool to measure quantitative information of bio-molecules, including the concentration, diffusion coefficient, and other fluorophore behavior such as blinking and binding by analyzing intensity fluctuation induced by molecules diffusing through an observation volume. FRAP measures the diffusional dynamics of fluorescently tagged molecules by purposely photobleaching a sample region with high laser power, and subsequently recording the recovery of fluorescence as unbleached fluorophores exchange with the bleached ones[[139](#_ENREF_139)]. FRAP has a simple experimental configuration and a simple analysis model, despite the information provided by FRAP is limited to the diffusion coefficient. Recently, a similar technique appears, with which the dynamics of fluorescently-labeled molecules can be observed by suddenly and selectively activating dark fluorescent markers to the bright ones, and tracking their intensity spread in 2D and 3D[[142](#_ENREF_142)]. FRET can quantify the dynamics and interaction of a pair of fluorescently labeled molecules within a distance of 5–10 nm. The major disadvantage of FRET is that a negative result could arise simply from the fluorescent tags being further than 10 nm apart, even if the two tagged proteins are themselves interacting, or due to inappropriate orientation of the molecules relative to each other (*i.e.*, dipole orientation)[[143](#_ENREF_143)]. RICS probes the dynamics by repeatedly scanning a wide area across the sample and performing a correlation analysis of relevant subregions within the acquired images. The limitation of RICS lies in its low spatial resolution, which is typically a few μm2, and the requirement on the uniformity of biomolecular concentration within such subregion. SMT probes the dynamics of biomolecules by analyzing the positions of single molecules in sequential image series[[141](#_ENREF_141), [144](#_ENREF_144)]. The difference between SMT and other approaches lies in that SMT tracks specific molecules by taking image series while the other methods analyze the intensity fluctuation induced by single-molecule behavior by average values. Furthermore, SMT is based on imaging, and therefore, its time resolution is limited to ~ ms, much slower than that of FCS.

**Table S1.** Comparison of Different techniques Targeting the Investigation of Molecular Dynamics

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Techniques** | | **Observables** | **Advantages** | **Disadvantages** |
| FCS | * concentration * hydrodynamic-radius * mobility * fluorophore brightness * kinetics * interaction or kinetics of different molecules | | * time resolution of sub-μs, enabling fast mobilities to be detected (*D* up to 300 m2s-1) * direction and space-related mobilities are attainable by pCF, msFCS, and scanning FCS * dual-color FCCS enables to detect the interaction of molecules with high sensitivity and specification | * measurable concentration range is limited to 1 pM ~ 100 nM * insensitive to single-molecule heterogeneity. * single-point FCS lacks spatial information * results can be handicapped by many factors, such as bleaching, background, crosstalk |
| FRAP | * molecular mobility | | * sensitive to slow dynamics * highly accessible | * lack of spatial information * insensitive to heterogeneity at the single-molecule level |
| FRET | * interaction or kinetics in term of the distance of a pair of molecules | | * specific to a couple of molecules * spatial information attainable | * the distance between the two molecules is limited and no larger than 10 nm |
| SMT | * mobility | | * sensitive to single-molecule heterogeneity * spatial information attainable | * time resolution is limited to ~ms * molecules need to be sparse |
| RICS | * concentration * hydrodynamic radius * mobility * fluorophore brightness | | * spatial-dependent concentration and mobility are attainable | * the spatial resolution is low, *i.e.*, that is typically a few μm2 * the uniformity of biomolecules within the selected subregion is required |

**3 Exemplary Diffusion Coefficient of Commonly-used Fluorophores**

**Table S2.** Diffusion Coefficients of Fluorophores in Solution Commonly used in FCS

|  |  |  |  |
| --- | --- | --- | --- |
| **Fluorophore** | **λEm (nm)** | **Diffusion coefficient (μm2 s-1)** | **References** |
| **Atto655-maleimid** | 686 | 407 ± 10 | [[145](#_ENREF_145)] |
| **Atto655-carboxylic acid** | 685 | 426 ± 8 | [[46](#_ENREF_46), [145](#_ENREF_145)] |
| **Atto655-NHS esther** | 685 | 425 ± 6 | [[146](#_ENREF_146)] |
| **Atto488-carboxylic acid** | 523 | 400 ± 10 | [[147](#_ENREF_147)] |
| **Cy5** | 670 | 360 ± 10 | [[146](#_ENREF_146)] |
| **Alexa 647** | 665 | 330 ± 10 | [[146](#_ENREF_146)] |
| **Alexa 633** | 647 | 340 ± 10 | [[146](#_ENREF_146)] |
| **Alexa 546\*** | 572 | 341 | [[148](#_ENREF_148)] |
| **Alexa 488\*** | 520 | 435 | [[148](#_ENREF_148)] |
| **eGFP\*** | 510 | 95 | [[148](#_ENREF_148)] |
| **Rhodamine 6G** | 550 | 414 ± 5 | [[145](#_ENREF_145)] |
| **Rhodamine 123** | 530 | 460 ± 40 | [[149](#_ENREF_149)] |
| **Rhodamine 110** | 535 | 470 ± 40 | [[149](#_ENREF_149)] |
| **Fluorescein** | 520 | 425 ± 1 | [[150](#_ENREF_150)] |
| **Oregon Green 488** | 550 | 411 ± 6 | [[145](#_ENREF_145)] |
| **TetraSpeck Beads,**  **0.1μm diameter** | 430 | 4.4 ± 7.0 | [[151](#_ENREF_151)] |
| 515 |
| 580 |
| 680 |

Diffusion coefficients measured in water solutions at 25℃, except for those marked with \*, which were obtained at 22.5℃.

**Table S3.** Diffusion Coefficients of Lipids or Proteins Measured with FCS

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Lipids/protein** | **Environment** | | | **Diffusion coefficient (μm2 s-1)** | **References** |
| **lipids labels with DiI-C18a** | GUV (composition) | | | 6.3 ± 0.2 | [[152](#_ENREF_152)] |
| SM | DOPC | Cholesterol |
| 1 | 0 | 0 |
| 0 | 1 | 0 | 2.6 ± 0.2 | [[152](#_ENREF_152)] |
| 0.25 | 0.25 | 0.5 | 1.6 ± 0.2 | [[152](#_ENREF_152)] |
| 0.1 | 0.8 | 0.1 | 4.9 ± 0.4 | [[152](#_ENREF_152)] |
| **annexin A5-mGarnetb** | Membrane of living HeLa cells | | | 17.5 ± 0.6 | [[95](#_ENREF_95)] |
| **cytoplasmic MinDc** | Living Escherichia coli | | | 16.4 ±2.1 | [[153](#_ENREF_153)] |
| **replication protein Ac** | Cytosol of Living HeLa Cell | | | 15.3±2.0 | [[154](#_ENREF_154)] |

a Diffusion coefficients were measured at 25℃, the number of SM/DOPC/Cholesterol represents the molar fraction.

b Diffusion coefficient was measured at 37℃.

c Diffusion coefficient was measured at room temperature.