FRAP vs. FRET vs. FCS

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Fluorescence Recovery After Photobleaching (FRAP), Fluorescence (or Förster) Resonance Energy Transfer (FRET), and Fluorescence Correlation Spectroscopy (FCS) are three fluorescence-based techniques that are commonly used to study molecular diffusion and/or complexing. Below, we describe these techniques and compare the properties obtained from each.

Overview of Techniques

Fluorescence Recovery After Photobleaching (FRAP)

FRAP is performed in a variety of ways. In Spot FRAP, for example, a small volume is rapidly bleached and the recovery of fluorescence intensity in this spot is measured as bleached fluorophores diffuse out of the volume and unbleached fluorophores diffuse in.

Fluorescence (or Förster) Resonance Energy Transfer (FRET)

FRET measures molecular proximity by measuring the dipole induced dipole interaction between donor and acceptor fluorophores. Methods of determining the degree of FRET include: donor quenching, appearance of sensitized acceptor emission, decrease in donor lifetime, depolarization of donor fluorescence, and reduction in donor photobleaching.

Fluorescence Correlation Spectroscopy (FCS)

FCS measures the fluctuations of fluorescence as molecules and/or complexes diffuse in and out of a small confocal volume.

Comparison of Information Obtained

Properties that Describe Molecular Diffusion and Complexing

In **Table I** below we compare the information that can be obtained from these three techniques about molecular diffusion and complexing. We focus on these physical properties:

- **Diffusion Rate:** the ability of the technique to measure diffusion rates, typically expressed as the diffusion coefficient of the molecule or molecular complex
- **Multicomponent Diffusion:** the ability of the technique to detect and distinguish between single and multiple component diffusion

- **Mobile Fraction:** the possibility that only a fraction of the species are free to diffuse, the rest being immobile
- **Concentration:** the ability of the technique to provide an independent calibration-free measure of molecular concentration
- **Complexing:** the ability of the technique to detect molecular complexing
- **Complex Stoichiometry:** the ability of the technique to determine the stoichiometry of molecular complexes, i.e. the fundamental ratio at which molecules combine
- **Binding Kinetics:** the ability of the technique to measure on and off kinetics of complex formation

Table I: A Comparison of Parameters Obtainable with FRAP, FRET, and FCS

	FRAP	FRET	FCS
Diffusion Rates	√		√
Multicomponent Diffusion			√
Mobile Fraction	√		
Concentration			√
Complexing		√	√
Complex Stoichiometry			√
Binding Kinetics	√	√	√

Detailed Descriptions

Fluorescence Recovery After Photobleaching (FRAP)

FRAP is a relatively easy technique to implement. Determination of diffusion coefficients derives from diffusion times and therefore requires careful determination of the beam radius in both the xy plane and along the z axis. It has proven to be very difficult to extract multiple diffusion coefficients from FRAP.

FRAP is unique among the three techniques in its ability to detect mobile fraction. It does not provide an independent measure of concentration. Its ability to detect molecular complexing and to measure stoichiometry of these complexes is limited by its ability to distinguish diffusion coefficients. This is particularly problematic for two-dimensional diffusion, such as in membranes, where the dependence of diffusion coefficient on molecular size is logarithmic. FRAP can be used to measure molecular kinetics.

Because of the high level of bleaching light, it is extremely important to perform controls for photodamage. This applies both on the physical level (is the light damaging structure or inducing complexing?) and on the biological level (is the light damaging cells or biochemical pathways?).

Fluorescence (or Förster) Resonance Energy Transfer (FRET)

FRET may be viewed as a method for proximity sensing. It is not generally implemented as a technique for measuring molecular diffusion. It does not provide an independent measure of donor concentration. With careful calibration it can provide a measure of acceptor concentration.

It is hard to obtain any information about stoichiometry, and this limited information can only be obtained with careful calibration. FRET is extremely powerful as a technique for measuring changes in the degree of complexing and as a result can be used to measure on and off kinetics of complex formation.

Debatably, FRET is more often abused than used properly. FRET refers to Förster Resonance Energy Transfer, the dipole induced dipole interaction between two singlet fluorophores states. This phenomenon requires that the efficiency of transfer between isolated donor acceptor pairs falls off as the reciprocal of the sixth power of the intermolecular distance. This must be demonstrated, or at the very least, one must use fluorophore pairs previously shown to exhibit this dependence. Without this assurance results can be misleading, or at the very least, are purely qualitative.

Fluorescence Correlation Spectroscopy (FCS)

FCS can be used to measure both single and multiple component diffusion. As is the case for FRAP, determination of diffusion coefficients requires knowledge of the beam radii. FCS does not measure mobile fraction. It does provide a direct and independent measure of concentration without calibration. Because of this concentration measure and the ability to distinguish between diffusion coefficients, FCS data provide a measure of complex stoichiometry and binding isotherms. FCS can also be used to measure the on and off rates of complex formation.

FCS measurements depend upon having a significant amount of concentration fluctuations. This limits the concentration range over which FCS measurements can be made. This manifests itself as an upper limit to the concentration range. FCS measurement has historically been difficult to implement and has required a high level of expertise. Sensor Technologies' QuantumXpertTM FCS Spectrometer is designed to change this by providing an easy-to-use intuitive FCS platform.

Selected References

Fluorescence Recovery After Photobleaching (FRAP)

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