X-Cite®
Fluorescence Illumination • In Control

The importance of a stable fluorescence light source in FRET measurements

Challenge

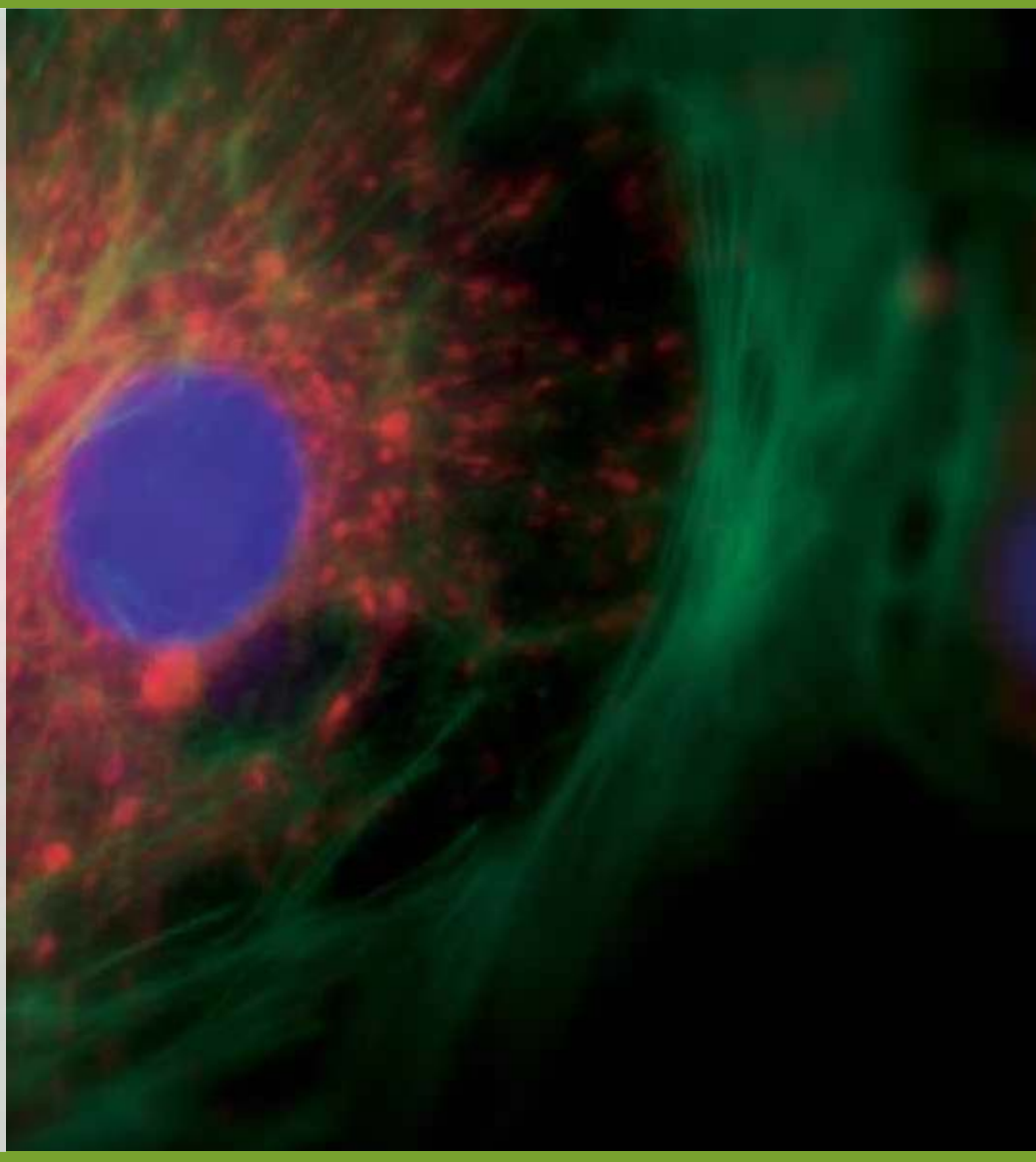
FRET data reliability due to light source-created artifacts

Solution

X-Cite® *exacte* with Closed-Loop Feedback technology used in FRET set-up

Benefit

Reliable and repeatable FRET data



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Introduction

Fluorescence resonance energy transfer (FRET) describes the transfer of energy from an excited fluorescent donor molecule to an acceptor molecule that lies within the Förster distance (< 10nm). When energy transfer takes place, one can measure both a decrease in the light intensity emitted from the excited donor molecule and an increase in intensity emitted from the acceptor molecule. These intensity-based FRET measurements can be taken using either fluorescent dyes or proteins with the appropriate spectral overlap and have become a proven methodology for studying biological signal transduction processes.

FRET imaging provides an effective way to quantify the interactions of biological molecules. Molecular interactions are often quantified using a relative FRET index. Images are taken using the donor excitation wavelength and acceptor emission wavelength (DA), the donor excitation wavelength and donor emission wavelength (DD), and in some cases, the acceptor excitation wavelength and acceptor emission wavelength (AA). The simplest form of the FRET index is calculated using a ratio of the acceptor (DA) to donor emission intensities (DD). However, more complex expressions have been used to correct for spectral "bleed-through."[1,2]

Intensity-based FRET measurements have been successfully used to study both the kinetic and spatial aspects of signal transduction during live cell imaging. Moreover, these measurements do not require equipment and expertise more sophisticated than that normally associated with careful epi-fluorescent imaging. However, like any experimental approach, FRET-based measurements can suffer from experimental errors either inherent to the fluorescent-based probes or originating from imaging equipment. Here we review a few of the most relevant sources of error and point out how having a stable and precisely controllable fluorescent light source, like that offered by the X-Cite® *exacte*, can help to reduce these complications by providing stable fluorescence illumination.

Materials and Methods

8-well Labtek dishes were coated with 10µg/ml human fibronectin at 4°C overnight. HEK cells were transfected with angiotensin receptor II (angRII) and the EKAR FRET probe using Fugene HD. After 16h, transfected cells were seeded into the Labtek dishes using DMEM supplemented with 10% FBS and p/s. After attachment, cells were serum-starved for four hours. Experiments were conducted using a Zeiss Axiovert 200M microscope with either the X-Cite® 120 or the X-Cite® *exacte* light source. Cells were imaged in Leibovitz (L-15) without phenol red and stimulated with 30nM angiotensin. The FRET ratio R was calculated as DA/DD as described above and normalized to the starting ratio at time zero to give Rn.

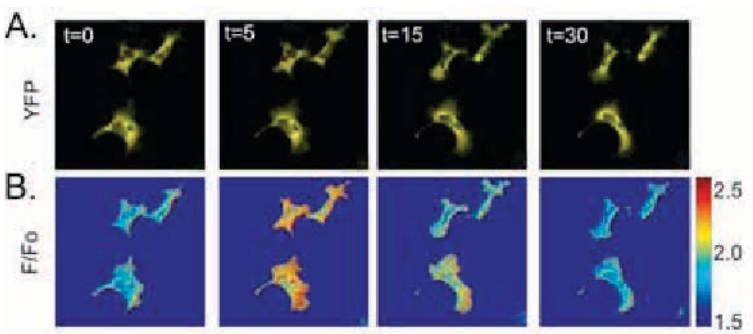

Illustration of FRET measurement. YFP images of HEK cells expressing the EKAR FRET probe (A). Corresponding FRET ratio following stimulation with Angiotensin II (B).

Probe signal to noise ratio

Over the past two decades genetically encoded FRET probes have been developed targeting many signaling pathways including PKC, PLC, ERK, and Akt.[3,4,5] These probes can be either a single protein molecule capable of intramolecular FRET, tethering a donor and acceptor fluorophore together via a regulatory domain, or intermolecular, utilizing separate donor and acceptor molecules.

Moreover, with the addition of localization sequences, these probes can be targeted to specific cellular domains. While these probes have shown great potential in characterizing signaling events, many suffer from a limited dynamic range and a low signal to noise ratio. *Figure 1* shows an experiment using the intramolecular FRET probe EKAR transfected into HEK cells and imaged with a light source that was unstable. Cells were stimulated with Angiotensin II and imaged at 30 seconds intervals for 35 minutes. Data for panels A and B were taken under the same experimental conditions but were sampled at different times during the light source fluctuations. Black lines show the normalized change in the FRET ratio, Rn, for individual cells over time. Variation in the lamp intensity is also formulated using the FRET ratio so that the pattern can be directly compared. The background Rn is given in red and shows up to a 10% fluctuation over time. This background fluctuation superimposes a corresponding pattern on the individual cell measurements. Arrows were inserted into each panel to mark some of the time points where background fluctuation heavily influenced FRET measurements. In contrast, *Figure 2* shows data taken with the X-Cite® *exacte*. Using X-Cite®'s proprietary Closed-Loop Feedback which provides short and long-term intensity stability, background Rn did not fluctuate more than 2%. More importantly, no artificial patterns were superimposed on the cell data.

Photobleaching

An inherent problem with almost all experiments using fluorescent imaging is the irreversible photobleaching of fluorescent molecules.[2] This problem can be even more complex in the case of FRET measurements, as the donor and acceptor fluorophore may bleach at different rates. Although methodologies to correct for photobleaching have been developed, most commonly involving fitting the data to exponential curves, it is very advantageous to minimize photobleaching as much as possible. The X-Cite® *exacte* offers precise digital control over the intensity output of the fluorescent light and allows the researcher to best optimize the intensity to minimize photobleaching. In addition, the stability given by the active feedback control (provided by its proprietary Closed Loop Feedback technology) ensures that the rate of photobleaching does not change due to changes in the lamp intensity and allows easier correction of any photobleaching that does occur.

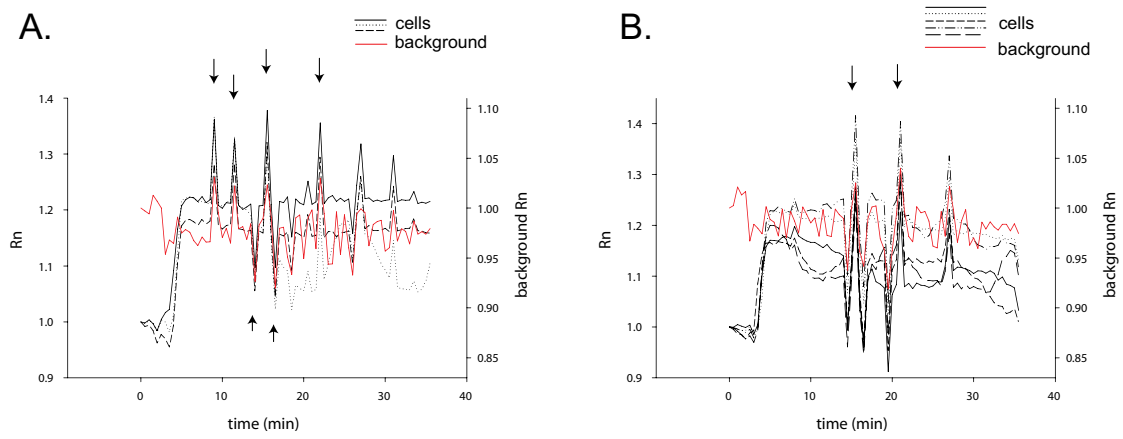

Figure 1: Measuring EKAR activation with an unstable fluorescent light source. Shown in both panels is the normalized FRET ratio (Rn) calculated for individual cells (black, left y-axis) and the image background (red, right y-axis). Data for panels A and B were taken under the same experimental conditions but were sampled at different times during the light source fluctuations. Arrows indicate times when background fluctuations significantly influenced FRET measurements.

Uneven spatial excitation

Often it is desirable to study the spatial pattern of molecular interactions during live cell imaging. Uneven spatial excitation can superimpose artificial gradients in FRET measurements. This is particularly problematic when using bimolecular FRET probes. To remove this effect, it is necessary to perfectly align the fluorescent light source to yield a homogeneous excitation field. The X-Cite® *exacte* was designed such that the lamp is automatically aligned correctly to eliminate the need for cumbersome user alignment and ensure uniform field excitation.

Experimental reproducibility

Day to day experimental variation is a difficult problem to eliminate with most experimental techniques. The X-Cite® *exacte* is designed to prevent the fluorescent light source from being a major contributing factor to this variation. Not only can the intensity output be precisely set, but the output can also be measured and documented using a computer interface. The X-Cite® Optical Power Measurement System (OPMS) is an additional tool that can be used to measure output from a light source at the end of a liquid light guide, or at the objective plane. The Objective Plane Power Meter has a slide format sensor that allows the user to measure power at the sample plane. Knowing the exact power that is being used to excite your sample is useful in order to repeat future experiments requiring the same power. The OPMS can be also be used to calibrate the X-Cite® *exacte* in order to specify the desired current output from the system.

Many fluorescent light sources are stable only over a short time period limiting the number of experimental data points that can be taken in the same day.

The stability offered by the X-Cite® *exacte* allows continuous data collection without interruption caused by light source fluctuations.

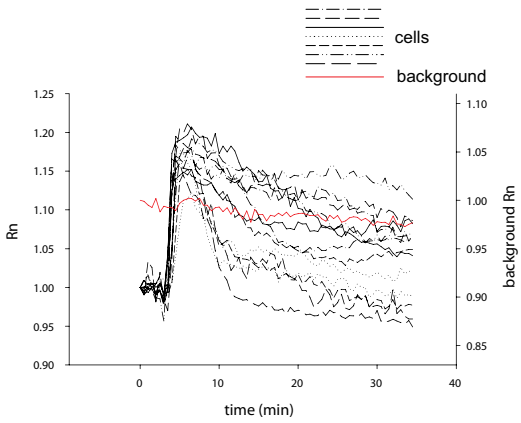

Figure 2: Measuring EKAR activation with the X-Cite® *exacte*. Shown is the normalized FRET ratio (Rn) calculated for individual cells (black, left y-axis) and the image background (red, right y-axis). Data was taken using Closed-Loop Feedback control to assure a steady fluorescent light output.

Summary

FRET-based measurements can suffer from experimental errors due to either the fluorescent-based probes or originating from imaging equipment. In this Application Note, we have reviewed the most relevant error sources by comparing the FRET ratios in HEK cells using two light sources with differing output stability The X-Cite® *exacte* is ideally suited for FRET imaging; it offers stable illumination and precise control to help eliminate unnecessary complications associated with FRET imaging.

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