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Precise calculation of photoactivation kinetics

Measuring rapid kinetics of proteins in living cells requires the capability for fast, accurate measurements. Researchers hoping to obtain precise kinetic data from fluorescence recovery after photobleaching or photoactivation experiments need an easily controllable system for stimulation of a specific region and subsequent imaging, and the Olympus FluoView FV1000 confocal laser scanning microscope (cLSM) with SIM scanner makes this possible. We describe here how to precisely measure off rates using a cytosolic photoactivatable probe that binds endosomal membranes.

The diversity of genetically encoded fluorophores has increased since GFP was first cloned in 1992. There are now a variety of GFP mutants spanning much of the visible spectrum. Some of these variants allow photoactivation, such that they increase their fluorescence intensity after exposure to specific wavelengths of light, and using these variants, one can turn on the fluorescent intensity in a region of interest and study the activated proteins over time (for a review, see ref. 1). The photoactivatable version of GFP (PA-GFP) gains a 100-fold increase in fluorescence (with emission at 517 nm) after exposure to light in the ultraviolet-to-violet range (350–420 nm; ref. 2).

Using standard molecular biology techniques, a protein of interest can be linked to a fluorophore. Depending on the fusion partner, this approach can be used to label subcellular organelles, cells of interest and specific tissue regions. This has opened the possibility for *in vivo* studies of, for example, organelle dynamics and function, protein expression and turnover, protein interaction, and cell motility. Chimeric fluorescent proteins allow studies of dynamic events that range in duration from less than a second up to several days. The measurement and analysis of rapid subsecond kinetics, however, necessitates special requirements for both the microscope hardware and software.

The Olympus FluoView FV1000 cLSM is available with a proprietary SIM scanner, which allows the confocal system to simultaneously stimulate and image. Using this setup one can use an independent laser for light stimulation while recording images with the main scanner. Structures of interest can be selected and stimulated during scanning, facilitating accurate measurements immediately after stimulation. For the highest efficiency in photobleaching or photoactivation, a circular or so-called 'Tornado' scan, is possible with the SIM scanner, maximizing the dose of light in the activation or bleaching area.

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Measuring rapid kinetic events

To measure rapid kinetics after photoactivation, it is essential to know the exact interval between stimulation and measurement. The exposure of the stimulating laser can easily be controlled on a millisecond level, and the images acquired using the main scanner also have millisecond precision. It is therefore straightforward to make an identical, repetitive setup in one line of experiments. However, the location of the stimulated region within the image will usually vary for each measurement, resulting in different delays between stimulation and start of measurement. If the stimulated area appears late in the frame, it will be a longer delay compared to an area early in the frame (**Fig. 1**). It is possible to correct for these varying delays by calculating the exact time from stimulation to imaging of the region of interest, and then adjusting this time for each measurement. With a short interval, the correction will only have minor effects; but if the interval is longer, a correction will be more pronounced.

Measuring in practice

To illustrate this principle we used a coat protein linked to PA-GFP. This coat protein interacts with a specific lipid in the membrane of early

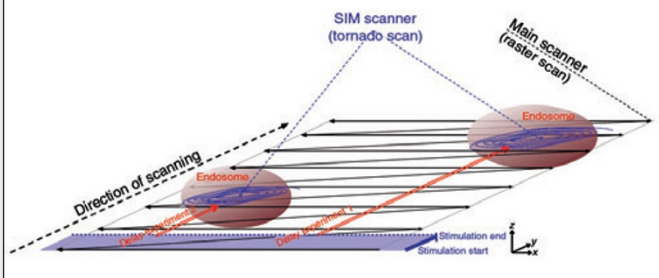

Figure 1 | Varying delays between photoactivation and imaging of activated area. Illustration of how varying locations of a stimulated area can result in different delays between stimulation and scanning of the stimulated area. Blue area indicates start and end of stimulation. The circular enclosed areas indicate two examples where the stimulating light can be exposed.

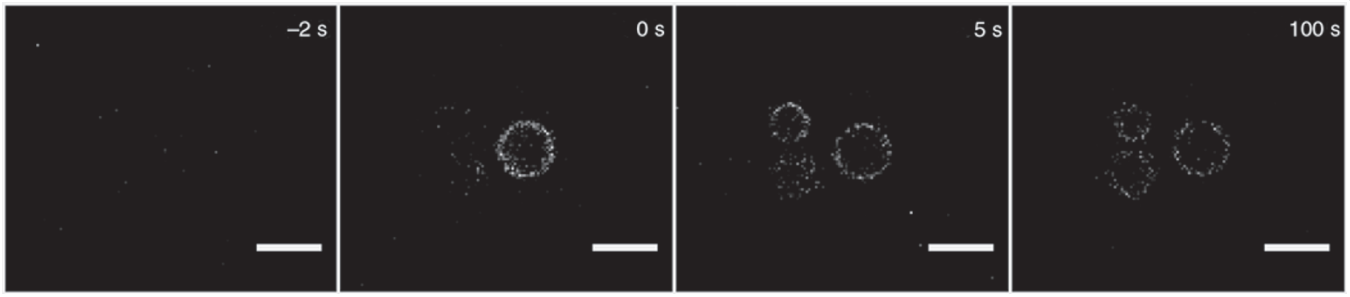

Figure 2 | Activation of PA-GFP linked to a cytosolic coat protein. Maden-Darby canine kidney cells were stably transfected with a cytosolic coat protein linked to PA-GFP. Region of interest was located, and the SIM scanner allows individual activation of this area independently of the image scanning. Cells were monitored before, during and after photoactivation. Scale bars, 5 μm.

endosomes. We marked a region of interest around the area to be activated, in this case an endosome, and the SIM scanner stimulated the area with a short pulse (25 ms) of low-intensity (10%), 405-nm light for photoactivation. The image scanning, however, was continuous prior to, during and after activation, allowing changes in intensity to be monitored over time (**Fig. 2**). The intensities were normalized and plotted against time[3], and the intensity half-life ($t_{1/2}$) and fraction remaining on the membrane were calculated[4]. In the first example, there was a 1.1-s delay between stimulation and imaging of the stimulated area. The $t_{1/2}$ before time correction was 4.90 ± 0.25 s, whereas the corrected $t_{1/2}$ was 3.90 ± 0.16 s. In the next example the $t_{1/2}$ was measured as 3.02 ± 0.19 s before time correction. The delay was 0.33 s, and this resulted in a corrected $t_{1/2}$ of 2.90 ± 0.16 s. The correction is naturally most pronounced for the longer time delays (**Fig. 3**).

Conclusion

In rapid cellular kinetic measurements, it is ideal to monitor a stimulated area immediately after stimulation. But because the location of the region being stimulated will vary in the image scan between experiments, a delay will often appear. If this delay is not taken into consideration and corrected for, the kinetic data are wrong—for example, in the experiment above, the $t_{1/2}$ would appear to be too long. Other techniques may not generate similar delays, and the comparison of different measurements requires accurate data. By using the SIM scanner on the Olympus FluoView FV1000, one can regulate both stimulation and scanning at the millisecond level. As a result, the output data can be subjected to the necessary correction, producing precise calculations of kinetic parameters in photoactivation.

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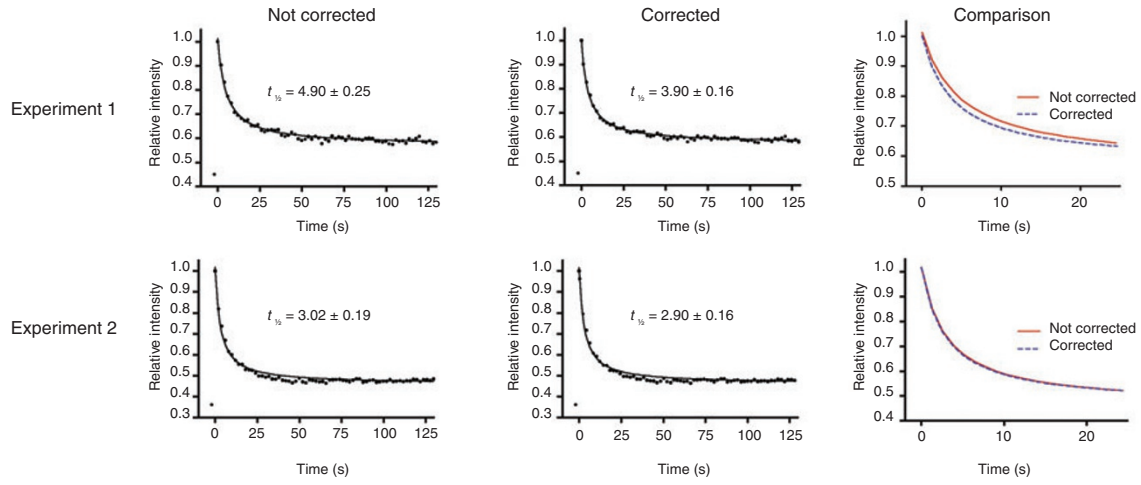

Figure 3 | Intensity curves of photoactivation. Fluorescent intensity was monitored before and after activation, and normalized values were subjected to nonlinear regression and plotted against an uncorrected and a corrected time scale. To visualize the effect of the correction, the curve fit from both the corrected and uncorrected time scale is shown. In experiment 1, the activated region is further away from the image scan than in experiment 2, resulting in a more pronounced correction in the first experiment compared to the second.