

# Precise calculations on rapid kinetics in photoactivation

## Circumventing pitfalls in off-rate measurements of PA-GFP labelled coat protein attached to endosomal membrane using the Olympus FV1000 confocal laser scanning microscope

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Measuring rapid kinetics of proteins in living cells requires microscope set-ups that enable fast and accurate measurements. To obtain precise kinetic data from FRAP (fluorescence recovery after photobleaching) or photoactivation (PA) experiments it is important to have an easily controllable system for stimulation of a specific region and subsequent imaging. A commercial system, the Olympus FluoView FV1000 confocal laser scanning microscope (cLSM) with its original SIM scanner, enables fast bleaching and photostimulation during continuous imaging. We describe here how to precisely measure off-rates using a cytosolic photoactivatable probe that binds to endosomal membranes.

### Advanced Fluorescence

The diversity of genetically encoded fluorophores has increased during the last years. Since the cloning of green fluorescent protein (GFP) in 1994, there are today a variety of GFP mutants spanning over the entire visual spectrum (emission ~450-600 nm). Some of these variants allow photoactivation, such that they increase their fluorescent intensity after exposure to specific light. By 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 [1]). The photoactivatable version of GFP (PA-GFP) gains a 100-fold increase of fluorescence (517 nm emission) after exposure to light in the UV to violet range (350-420 nm) [2].

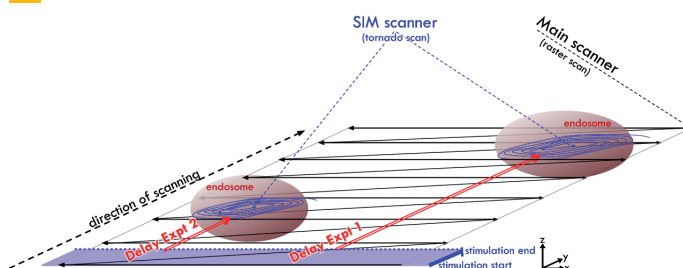
Using standard molecular techniques, a protein of interest can be linked to a fluorophore and, depending on the fusion partner, can label sub-cellular organelles, cells of interest, and specific tissue regions. It has opened the possibility for in vivo studies of organelle dynamics and function, protein expression and turnover, protein interaction, and cell motility. The chimeric fluorescent proteins allow studies of dynamic events that last from sub-second level up to days. To measure and analyze rapid sub-second kinetics, there are special requirements for both the microscope hardware and software.

The Olympus FluoView FV1000 cLSM is available with a proprietary SIM Scanner, which enables 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 following stimulation. For the highest efficiency in photobleaching or photoactivation, a circular or so called “Tornado” scan is possible with the SIM scanner, maximising the dose of light in the bleaching/activation area.

### Measuring rapid kinetics after photoactivation

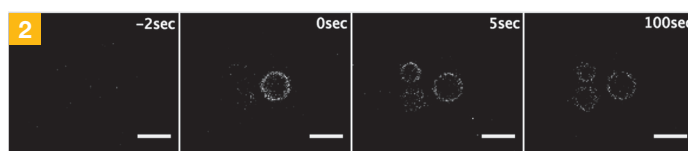
To measure rapid kinetics after photoactivation, it is essential to know the exact interval between stimulation and measurement. The exposure of the stimulating laser can easily be controlled on a millisecond (msec) level and the images acquired using the main scanner also have a msec precision. Therefore it is 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 (Figure 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 adjust this time for each measurement. With a short interval the correction will only have minor effects, however, if the interval is longer a correction will be more pronounced.

1 Varying delays between photo activation and imaging of activated area



### Measuring in practice

To illustrate this principle we used a coat protein linked to PA-GFP. The coat protein interacts with a specific lipid in the membrane of early endosomes. A region of interest was marked around the area to be activated, in this case an endosome, and the SIM Scanner stimulated the area with a short pulse (25 msec) of low intensity (10%) 405 nm light for photoactivation. The image scanning though, was continuous prior to, during and after activation allowing changes in intensity to be monitored over time (figure 2).

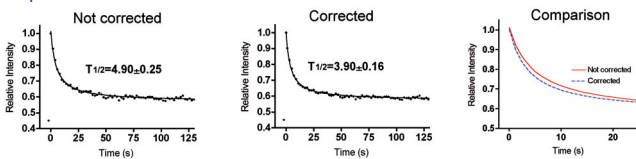


Activation of photoactivatable GFP linked to a cytosolic coat protein

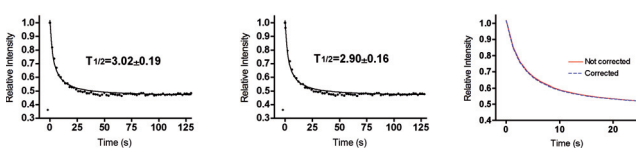
The intensities were normalized and plotted against time [3], and the intensity half life -  $T_{1/2}$  and fraction remaining on the membrane were calculated [4]. In the first example there was a 1.1 sec delay between stimulation and imaging of the stimulated area. The  $T_{1/2}$  before time correction was  $4.90 \pm 0.25$  sec, whereas the corrected  $T_{1/2}$  was  $3.90 \pm 0.16$  sec. In the next example the  $T_{1/2}$  was measured as  $3.02 \pm 0.19$  sec before time correction. The delay was 0.33 sec and this resulted in a corrected  $T_{1/2}$  of  $2.90 \pm 0.16$  sec. The correction is naturally most pronounced for the longer time delays (Figure 3).

### 3 Intensity curves of photoactivation

#### Experiment 1



#### Experiment 2



## Conclusion

In rapid cellular kinetic measurements the ideal situation is to monitor a stimulated area immediately following stimulation. However, since the location of the region to stimulate 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 and for the experiment above the  $T_{1/2}$  appears too long. Other techniques may not introduce similar delays and to compare different measurements the data must be accurate. By using the SIM scanner on the Olympus FluoView FV1000 one can regulate both the light stimulation and scanning on a msec level and therefore the output data gives the opportunity for the necessary correction and precise calculations of kinetic parameters in photo activation.

## ACKNOWLEDGEMENTS

T. Bergeland is a fellow of the Norwegian Cancer Society.

## References

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## Figures

### 1 Varying delays between photo activation 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.

### 2 Activation of photoactivatable GFP linked to a cytosolic coat protein

MDCK cells were stably transfected by a cytosolic coat protein linked to photoactivatable GFP. Region of interest is located, and the SIM scanner allows an individual activation of this area independently of the image scanning. Cells were monitored prior to, during and after photo activation. (Scale bar  $5\mu\text{m}$ )

### 3 Intensity curves of photoactivation

Fluorescent intensity was monitored prior to an activation, and normalized values were subject to non-linear 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 compared. 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.

## Information

Further information on the Olympus FluoView FV1000 system is available at:

[www.olympus-europa.com/microscopy](http://www.olympus-europa.com/microscopy)