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3D localized photoactivation of pa-GFP in living cells using two-photon interactions.

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Abstract— **We report about two-photon activation of a photoactivatable derivative of the Aequorea Victoria green fluorescent protein (paGFP). This special form of the molecule increases its fluorescence intensity when excited by 488 nm after irradiation with high intensity light at 413 nm. The aim in this work was to evaluate the use of two-photon interactions for confining the molecular switching of pa-GFP in the bright state. Therefore experiments were performed using fixed and living cells which were expressing the paGFP fluorophore and microspheres whose surface was modified by specific adsorption of the chromophores. The molecular switches were activated in a range of wavelength from 720 nm to 840 nm. The optimal wavelength for activation was then chosen for cell imaging. A comparison between the conventional activation and two-photon mode demonstrates clearly the better three-dimensional (3D) confinement and the possibility of selection of cell volumes of interest. This enables molecular trafficking studies at high signal to noise ratio.**

I. Introduction

The use of green fluorescent protein (GFP) from Aqueorea Victoria grew dramatically over years thanks to the possibility to explore the protein distribution in living cells and especially the potential to investigate molecular kinetics such as diffusion and/or dissociation rates [1]. Since, particle tracking inside the cell requires the ability to spatially and temporally mark specific structures to follow their signal in time over a "dark" background the advent of photo-activatable green fluorescent proteins (pa-GFP) opened new perspectives for molecular imaging in living cells [2, 3]. There are now two promising photo-activatable molecules known which can serve as such a marker. Normally, wild-type GFP exists as a mixture of neutral phenols and anionic phenolates leading to a major absorption peak around 397 nm and a minor peak around 480 nm. Site specific mutagenesis substituting threonine 203 with histidine successfully lead to an excellent photo-convertible molecule producing a 100 fold increase in 488nm excited-fluorescence after irradiation with high energy flux at 405 nm. Selective photoactivation by means of Confocal Laser Scanning microscope (CLSM) immediately demsotrated that pa-GFP can be considered an optimal tool to study spatial and temporal dynamics of proteins in vivo, as demonstrated by tracking of lysosome and mitochondria using targeted fusion proteins [4, 5]. Now, in terms of spatial confinement of the photo activation process, the use of two-photon or even multiphoton excitation [6] provides several favorable aspects compared to single photon confocal microscopy in photomarking biological structures to be tracked [7]. The small excitation volumes, of the order of magnitude of subfemtoliter, due to the non-linear requirements [8] provide a unique control of the excitation and consequently photoactivation in the 3D space. Even though single photon CLSM can efficiently modulate excitation power in planar sub-micron region, it fails to elicit the same control along the optical axis, being the excitation volume extended to the entire depth of focus of the objective. The ability to mark specific cells in living embryos by photoactivating biomolecular markers can provide a unique tool in developmental biology studies to understand cell fate and mechanisms of differentiation [9].

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II. Materials and Methods

A tunable Ti:sapphire laser source (Chameleon XR, Coherent, USA) was used. Pulse-widths are ≤ 140 fs full-width at half maximum (FWHM) at a repetition frequency of 90 MHz. The laser beam was coupled directly into the confocal spectral scanning head of a Leica TCS SP2 AOBS using the infrared port. The measurements were performed using an average power of $\langle P \rangle_{min} \sim 2.5$ mW up to $\langle P \rangle_{max} = 12.5$ mW at the focal plane. Imaging of the activated protein has been obtained by using the 488 nm line of a 20 mW Argon ion laser. Single photon photoactivation has been performed using a Leica TCS SP2 AOBS equipped with a 405 nm 10mW laser diode.

Phoenix and HeLa cells were grown on cover slips in standard culture conditions at 37 °C, 5% CO2 in DMEM medium supplemented with 10% North American Fetal Bovine Serum (Gibco Europe, Paisley, UK).

PA-GFP was a generous gift from Dr George Patterson. HeLa transient transfection was performed using FuGene (Boehringer-Ingelheim Italia S.p.A., Milan, Italy) reagent according to manufacturer instructions. Cells were harvested and fixed after 48 hours. Before mounting, cover slips were stained with the DNA dye TOPRO 3 (Molecular Probes Europe, Leiden, Netherlands) to facilitate cell identification.

Phoenix cells were transfected with PA-GFP encoding DNA using Calcium Phosphate. After 24 hours, cells were lysed in cold 1X PLC buffer (50mM HEPES, 1.25% glycerol, 150mM NaCl, 166mM MgCl2, 1% TRITONX-100, 0.001% EGTA,10mM Napyruvate, 0.1 mM NaOrtovanadate, 0.01 mM PMSF, Aprotinin, pepstatin and leupeptin) on ice for 1 hour. Cell lysates were incubated with anti GFP polyclonal antibody and subsequently with ProteinA sepharose beads. For imaging, beads were resuspended in a 90% glycerol solution containing diazabicyclo-(2.2.2)octane antifade (Sigma-Aldrich S.r.l., Milan, Italy).

Images were collected with a 100x oil NA = 1.4 objective (HCX PL APO, Leica Microsystems S.p.A., Milan, Italy). Green fluorescence was initially excited with λ = 488 nm to acquire the pre-activation intensity. The scanning process on this area took 1.28 s and the dwell time per pixel was ~5 µm. The activation process was then primed by focusing a pulsed infrared laser beam on a 22 µm² region (512 x 512 pixel). For the activation process the dwell time per pixel was varied between 4.88 µs and several milliseconds (ms). Subsequently, the unzoomed area was imaged with the acquisition parameters used before the activation process. The image before and after activation were analyzed for determination of mean intensity values. The ration of mean fluorescence intensities of the activated areas were taken as a measure for the efficiency of the photoconversion process. The modified spheres were sandwiched between ethanol/acetone cleaned cover slip and glass slide. To avoid drying, the sample was sealed with ordinary nail polish.

III. RESULTS

Two-photon activation, similarly to two-photon excitation, is expected to be a three-dimensional confined process. This can be proved by imaging selected volume of interest within artficial samples containing the pa-GFP or living cells able to express the protein. We have checked the photoactivation properties as function of activation wavelength and power.
Due to the locally restricted non-linear excitation probability, activated volumes of finite thickness were expected in dependence of the provided laser light intensity. Whereas single photon induced activation should extend along the whole beam path of the light in the sample. A comparison between one-photon (λ = 405 nm) and two-photon activation process is shown in Figure 1. The thickness of the two-photon activated volume is limited to a narrow region inside the cell, while one photon activation results in a fully activated cell in axial direction (Figure 1b).

In the two-photon stimulation, the activation volume can be tuned precisely by varying the energy of the incoming laser beam. Several areas (performing a scan in the focal plane) were activated using different laser light energies.

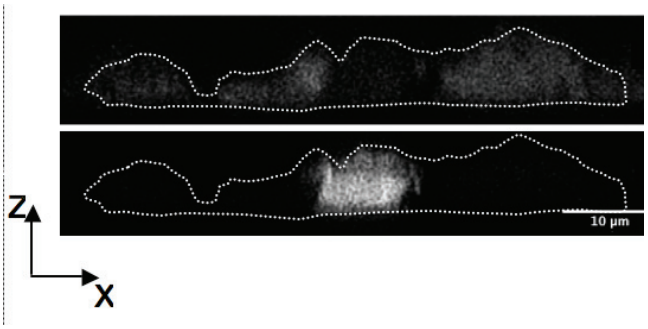

Figure 1: Photoactivation extension along the z-axis using two-photon (a-above) and conventional (b-below) photoactivation of pa-GFP within a living cell (shape is contoured by white lines).

The intensity profiles of these areas were used to determine the extension of the process along the optical axis. Irradiation with a laser intensity of 5 mW and 10 mW caused an increase in the thickness of the activated area.

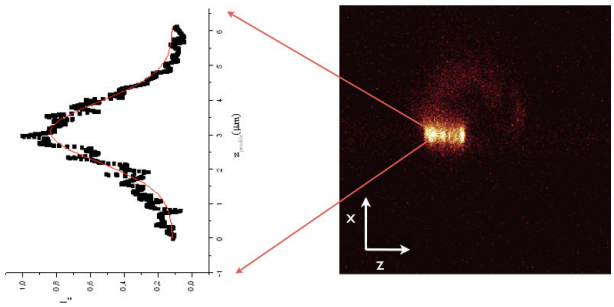

Figure 2: Intensity profile along the z-axis to evaluate the full-width-half-maximum value of the photoactivation shape.

The change of the activated volume is in accordance with the expectation based on the change in the intensity distribution I(z) that follow an inverse fourth power law as function of the distance, z, from the geometrical focus of the lens. Using parameters like the excitation wavelength, the numerical aperture, the refractive index of the sample, it is possible to predict an arbitrary intensity value in dependence on the distance from the focal plane. For λex = 750 nm, NA = 1.4, n = 1.518 and an excitation power of 10 mW an intensity value of ~ 0.36 at a distance of 3.5 µm can be estimated. This intensity value represents the edge between activated and not-activated volume and can therefore be considered as a threshold for the activation. The intensity value will be reached at different distances from the focal plane for different laser powers. With this value the approximate extension of the activation volume in z-direction can be calculated. Using 5 mW as activation power the intensity threshold value is obtained at a distance z = z5

= 2.4 μm from the focal plane. This value is in good accordance with the one extracted from the measurements, see figure 2. Several areas were excited and the resulting thickness of the activated areas were around ~ 2.35 μm. The thickness of the activated volume increased as function of the laser light energy and was affected by the number of activation cycles ranging from ~ 3.5 μm to ~ 3.0 μm. Activating two areas with a laser energy 1.5 times higher but with a reduced repetition rate results in slightly different activated volumes and intensities. The values for different volume of interest can be summerized acoording to the following triplets reporting scan time per pixel [μs], activation power [mW] and FWHMI(z) [μm], calculated as shown in figure 2: (19.6, 5, 2.35); (4.9, 17, 3); (9.8, 10, 3.5). To quantify the error that can be made due to the location on the sphere the activation of different areas, covering the whole sphere, with the same settings were performed. The difference in the fluorescence intensity after irradiation with 488 nm was found to be 10%. This insecurity of the measured intensity values is intrinsic and will not be displayed additionally to the statistical errors. To identify the best working efficiency a full activation spectrum would be deserving. With our set-up, a range of wavelength between 720 nm and 920 nm is accessible which covers very well the known absorption cross sections of wild-type GFP and at least the absorption peak of EGFP. For the acquisition of the spectrum care was taken for the different probability to absorb two photons in dependence on the wavelength (power correction for higher wavelengths). Further the power density and time per pixel was kept constant for all investigated wavelengths to keep the activation comparable. To measure the success or efficiency of the activation process the ratio of the fluorescence intensities (irradiation with $\lambda = 488$ nm) after the two-photon activation and before were considered. It has been found that the conversion efficiency drops dramatically for wavelengths above 830 nm (for high powers and long times it is nevertheless possible) [7]. Further, the efficiency shows a high level for wavelength between 720 nm and 750 nm. Up to 800 nm another efficiency level. Unfortunately, the following values show strong fluctuations so nor a clear assignment to the former level, nor an assignment to the low level efficiency shall be made at this point without further investigation.

IV. Conclusion

In the present work, the photophysical properties of PA-GFP under multiphoton excitation have been reported to demonstrate the possibility to employ two-photon microscopy in combination with photoactivatable markers for protein dynamic studies. PA-GFP exhibited same photoconversion properties when excited in far red wavelength range using two-photon excitation in comparison with photoactivation at 405 nm: the absorption of photons can induce an increase of absorption cross section at 488 nm. This effect can be efficiently employed to mark submicron regions in the whole cell selecting, case by case, the appropriate volume of interest. Examination of the activated spatial volume as a function of the excitation energy showed that intensity modulation can be efficiently used to induce spatially controlled protein photoconversion along the optical axis providing a unique possibility to dynamically identify single 3D structures. Two-photon activation can be efficiently used to track the fate of proteins and other biological macromolecules in living cells following cell cycle steps, and applications in terms of 3D memories could be pursued [10].

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