HYBRIDIZATION OF LASER-INDUCED SPECTROFLUORESCENCE ANALYSIS (LIFS), MATRIX-ASSISTED LASER DESORPTION / IONIZATION MASS SPECTROMETRY (MALDI), FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP) AND FLUORESCENCE LOSS IN PHOTOBLEACHING (FLIP) MICROTECHNICS

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Abstract. Novel MALDI MS + FLIP approaches for verifying continuity of membranous structures and measurements of nucleus-cytoplasm exchange rates are proposed. Novel approaches for the measurements of lateral diffusion / molecular mobility and bindings using MALDI + FRAP hybridization are proposed. FRAP (Fluorescence Recovery After Photobleaching) is a method for the diffusion kinetics measurements in living cells using fluorescence microscopy which allows to estimate quantitatively the two dimensional lateral diffusion in molecularly thin film containing fluorescent-labeled probes, or for single cell examination (i.e. the study of lateral mobility of cellular molecules). Fluorescence Loss in Photobleaching (FLIP) is a microscopic technique predominantly performed using laser scanning microscopy (e.g. for tagged protein local photobleaching by short, intensive laser excitation on CLSM platform) used for the studies on molecular mobility inside the cells and membranes. MALDI (Matrix-Assisted Laser Desorption / Ionization) is a soft ionization technique used in mass spectrometry, allowing for the analysis of biomolecules (biopolymers such as DNA, proteins, peptides and sugars) and large organic molecules (such as polymers, dendrimers and other macromolecules), which tend to be fragile and fragmented when ionized by more conventional ionization methods (according to encyclopedic definition). The above laser-based technique is readily compatible with MALDI using the same laser beam for MALDI and FLIP.

Keywords: Matrix-Assisted Laser Desorption / Ionization Mass Spectrometry (MALDI), Laser-Induced Spectrofluorescence Analysis (LIFS), Fluorescence Loss in Photobleaching (FLIP), Fluorescence Recovery After Photobleaching (FRAP), Confocal Laser Scanning Microscopy (CLSM)
Introduction

FRAP (Fluorescence Recovery After Photobleaching) is a method for the diffusion kinetics (and also, correctly, reaction-diffusion kinetics) measurements in living cells using fluorescence microscopy (Zlatanov, 1987; Lopez, 1988; Swaisgood, 1989; Anders, 1990; Nagy, 1990; Bryers, 1998; Salomé, 1998; Tinland, 1998; Higgs, 2000; Reits, 2001; Carrero, 2003; Cha 2004; Joubert, 2004; Fukano, 2004; James, 2004; Sprague, 2005; Lele, 2006; Abbaci, 2007; Febo-Ayala, 2007; Travascio, 2007; Dushek, 2008; Kang, 2008; McNally, 2008; Tolentino, 2008; Lambert, 2009; Travascio, 2009; Hagman, 2010; Mueller, 2010; Mai, 2011; Wachsmuth, 2014; Yapp, 2016) which allows to estimate quantitatively the two dimensional lateral diffusion in molecularly thin film containing fluorescent-labeled probes, or for single cell examination (i.e. the study of lateral mobility of cellular molecules). Similar, though less common techniques (also referred to as FRAP) have been developed for the three dimensional diffusion and molecular binding investigation inside the cell. Modern confocal scanning laser microscopes (CSLMs) (Hardingham, 2000; Gribbon, 2001; Loerke,2005; Seiffert, 2005; Braeckmans, 2007; Campbell, 2007; Maung, 2007; Mazza, 2008; Tsibidis, 2008; Kang, 2010; Roberti, 2011; Kang, 2012; De Los Santos, 2015; Lemcke, 2016) are equipped with the system for local photobleaching in selected regions (Figure 1). But currently there are no completely satisfactory methods for molecular-chemical characterization of diffusion / binding kinetics for FRAP using MALDI (Matrix-Assisted Laser Desorption / Ionization) imaging techniques (for example, see Figure 4).

Figure 1. Principle of FRAP. A) The bilayer is uniformly labeled with a fluorescent tag; B) This label is selectively photobleached by a small (~30 μ) fast light pulse; C) The intensity within this bleached area is monitored as the bleached dye diffuses out and new dye diffuses in; D) Eventually uniform intensity is restored. From Wikipedia, the free encyclopedia [URL: https://en.wikipedia.org/wiki/File:Frap_diagram.svg]
FLIP (Fluorescence Loss in Photobleaching; Figure 2) is a microscopic technique predominantly performed using laser scanning microscopy (e.g. for tagged protein local photobleaching by short, intensive laser excitation on CLSM platform) used for the studies on molecular mobility inside the cells and membranes. The primary application of FLIP is the determination of the continuity of membranous organelles based on the fluorescence intensity in the region of interest and the second less common application of FLIP is the observation of the protein shuttling from the cytoplasm to the nucleus and the determination of the shuttling rate. The above laser-based technique is readily compatible with MALDI (Figure 3) using the same laser beam for MALDI and FLIP and laser spot assisted mapping (Figure 4).

**Figure 2.** FLIP – decreased fluorescence in a defined region (the red box) adjacent to a bleached region (the circle). From Wikipedia, the free encyclopedia [URL: https://en.wikipedia.org/wiki/File:Fluorescence_Loss_in_Photobleaching_Schematic.jpg]

**Figure 3.** MALDI (Matrix-Assisted Laser Desorption / Ionization). Scheme from MSU tutorial presentation.
**Figure 4.** Hierarchical clustering of a mouse kidney data set achieved by MALDI-MSI:

A – full dendrogram of all spectra in a mouse kidney data set. B – optical image of the mouse kidney analyzed by MALDI-MSI. C and D – reconstruction of selected dendrogram branches and corresponding images. The three main branches reflect the renal cortex (blue), medulla (green), and pelvis (red). C, the medulla branch separates into two distinct areas, whereas the cortex branch further differentiates into fat and connective tissue of the renal capsule and hilus and the actual cortex (D). (Walch et al.; from the Archive of Internet)

**Methods**

In this report we propose to design a new scheme for FRAP measurements based on a coordinate-positioning two-axis stage for MALDI imaging (from typical MALDI heads) and focusable laser sources with programmable microbeam / millibeam positioning. The method is appropriate for standard MALDI heads with nitrogen lasers (337 nm), excimer ArF (193 nm), KrF (248 nm), XeCl (308 nm) and frequency-tripled and quadrupled Nd:YAG lasers (355 nm and 266 nm, respectively). The method can also operate at non-conventional wavelengths, such as 532, 307, 317, 327, 347, and 357 nm (Ahn, 2012), according to the photobleachable carrier spectrum.

Also we propose to design a novel scheme for FLIP measurements using the coordinate-positioning 2D-stage for MALDI imaging (from MALDI head, Feodorov (Notchenko, 2013; Notchenko, 2014; Oganessian, 2014; Gradov, 2015; Gradov, 2015a; Notchenko, 2015) or CLSM stages) and focusable laser sources with microbeam / millibeam positioning. The method is appropriate for standard MALDI heads with nitrogen lasers (337 nm), excimer ArF (193 nm), KrF (248 nm), XeCl (308 nm) and frequency-tripled and quadrupled Nd:YAG lasers (355 nm and 266 nm, respectively). The method can also operate at non-conventional wavelengths if they are compatible with the photobleachable agent spectrum (532; 307, 317, 327, 347, and 357 nm (Ahn, 2012)).

**Preliminary data**

Many *in situ* MS-experiments were designed to obtain detailed information on the molecular-chemical basis of fluorescence recovery. Following these studies, computational experiments were carried out which can be used for one-to-one mapping of cytological /
histological FRAP patterns and MALDI imaging results with temporal resolution (as well as photobiophysical interactions with soft matter in photobleaching can be correlated with the local MALDI ionization in the same cellular regions). Multi-wavelength hybrid MALDI imaging + MS-FRAP setups can be used for molecular biochemical co-localization studies in hyperspectral (or spectrozonal) imaging regimes. Interactions in far ultraviolet, vacuum ultraviolet and extreme ultraviolet bands may be partially simulated using Geant4 (CERN) in the frames of Geant4-DNA approaches or in terms of particle physics methods (for example: World initStep = laser photon source; World = cellular media; Tank = photo-bleachable vacuole; OutOfWorld = MALDI background target (or wafer); Target = local microscopic FRAP zone) (McCormick, 2003; Rannou, 2004; Dollan, 2005; Lo Meo, 2009; Meo, 2009; Boschi, 2011; Grevillot 2011Blake, 2013; Dooraghi, 2013; Glaser, 2013; Joshirao, 2013; Cuplov, 2014; Nozka, 2014; Tendeiro, 2014; Nilsson, 2015; Pibelet, 2015; Ogawara, 2016).

The above simulations based on Monte-Carlo methods can be also performed using FLUKA particle transport codes under FORTRAN (in contrast to Geant4 based on C++), but with the known physical / technical and algorithmic limitations. We cannot give a complete list of our performed and planned works due to the limited size of this abstract, but the complex model computational and technical considerations prove our preliminary conclusions about the prospects of hybridization between FRAP and MALDI imaging approaches in the framework of standard serial manufactured MALDI-MS setups.

Conclusion

Since the shuttling rate of a cytoplasm component is directly proportional to its fluorescence decay rate, and if the transport from the nucleus to the cytoplasm occurs, photobleaching will occur in the nucleus as well, the exchange rate between the nucleus and the cytoplasm can be determined from this data. We have developed a novel mathematical apparatus (together with O. Gradov ex-group (Gradov, 2014; Grädow, 2014; Alexandrov, 2015; Gradov, 2015; Jablokow, 2015; Jablokow, 2015a)) required for the investigation of the FLIP molecular mechanisms using kinetic MS studies (including oscillatory and autowave reaction-diffusion regimes), based on temporal resolution and adaptive spatiotemporal resolution of MALDI and MALDI imaging, respectively. Also we have proposed to use the conveyor system for serial slice analyzing (after the automatic microtome / vibratome sectioning module) for MALDI-FLIP-MS-based screening of different tissues on discretely shifting MALDI sample carriers (or a “sample lent” in this case) unwinding from the reel / spool. Thus, several new methods of analysis may be proposed from hybridization of MALDI and FLIP approaches.

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