The method of intracellular fluorescent species *in situ* production within living cells by femtosecond laser pulses

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**Abstract.** Here we report on a novel non-invasive technique of *in situ* fluorescence species creation with the use of tightly-focused femtosecond laser beam with 800 nm wavelength. Fluorescent species can be created in a strictly defined area inside a living cell, without damaging the plasma membrane and surrounding structures. Species are characterized by bright fluorescence with a maximum at 520 nm, high stability and low toxicity. No significant effect of fluorescent species on cell viability and development is observed. The technique of *in situ* fluorescence species creation is an attractive tool for *in vivo* cell visualization and intracellular tracking because of its high accuracy and low damage effect.

1. Introduction

Studying of intracellular organization, organelle interaction, and dynamics of intracellular processes are fundamental problems in cell biology. The topical direction in cell imaging is developing non-invasive high-specific labels for the tracking of cellular components. Nowadays multiple methods of *in vivo* and *in vitro* imaging are available, and most of them work on the principle of selective chemical binding with specific molecules. Moreover, delivery of fluorescent species into the cell is required in the most methods. Another principle of fluorescent labeling is production of fluorescent species within biological material, and the most promising instruments for this purpose are tightly focused ultrashort lasers [1]. They allow us to create fluorescent particles within tissue from its material in a strictly defined area [2, 3] by the non-invasive manner, in a very localized volume with minimal chemical, mechanical and thermal stress [4, 5].

In this research we describe a novel technique for creating fluorescent species using femtosecond lasers inside a living cell. Fluorescent species retained for more than 24 hours and did not affect viability and development of mouse GV oocytes. These features make the method of *in situ* fluorescence species creation an attractive tool for intracellular visualization and tracking.

2. Methods

2.1. Experimental setup

In the experiments, we used the inverted optical Olympus IX71 microscope with the objective 60× and NA = 0.7. Fluorescent species creation was performed using a femtosecond laser Tsunami (Spectra Physics) generating the radiation at the wavelength 800 nm. The diameter of the beam waist was 1.39 μm. The laser pulse duration was 30 fs, pulse energy 100 nJ, and the pulse repetition rate was
The estimated power density for the pulse in the beam waist was 2.2×10^{14} \text{ W cm}^{-2}. The visual control was implemented using the CMOS 1.3Mpix Thorlabs camera.

2.2. Fluorescence registration

We used two methods to record fluorescence signal from the sample. In the first method fluorescence was excited by single-photon absorption of laser diode radiation with \( \lambda = 462 \) nm (Nichia NDB7675), which was coupled to the Olympus microscope and focused by the same objective lens as femtosecond radiation. The laser diode beam was focused on the objective lens to give an about 500 \( \mu \)m wide and uniform field of illumination. The fluorescent image was also recorded by the same cameras as described above with exposure time up to 1 second.

In the second method, fluorescence was excited by two-photon absorption of femtosecond laser pulses focused by the objective lens. In order to avoid damage to the biological material or chemical reactions induced by laser radiation its average power was reduced below 10 mW. Fluorescence signal was collected by the same objective lens and directed via a beamsplitter cube and then was coupled to an Acton SP300i monochromator and then to a PI-MAX 2 CCD camera (Princeton Instruments) used to record the fluorescence spectra.

2.3. Sample preparation

Mouse germinal vesicle (GV) oocytes were obtained from 4-week-old C57Bl/CBA female mice. Mice were superovulated with 10 IU of pregnant mare’s serum gonadotropin intraperitoneally 48 h before preparation. The ovaries were removed from the mice, transferred to M2 medium («Sigma»), and were prepared with thin tweezers. Cumulus-oocyte complexes were collected with a glass capillary and placed into 0.1% hyaluronidase solution to remove cumulus. For the experiments, GV oocytes were placed in a 50 µl drop of M2 medium onto Petri dish with a cover glass and central hole («SPL Lifesciences»). Drop was covered with 2 ml of paraffin oil («Origio»).

2.4. In vitro maturation

Oocytes were cultivated in DMEM medium («PanEco») supplemented with 15% (v/v) fetal bovine serum (Gibco), 1 IU/ml gentamicin («Sigma») and 1 IU/ml of PMSG («Intervet») at 37°C, 90% humidity, 5% CO\(_2\) for 24 h. After IVM, oocytes were examined for the presence of polar bodies and metaphase plates.

3. Results

Fluorescent species were created in the cytoplasm, nucleus and nucleolus of GV oocytes using tightly focused femtosecond laser beam (Fig. 1, A-C). Area of fluorescent species creation was set by focusing to a place of interest. Minimal spot size was estimated as 500 nm. Fluorescent particles did not diffuse through the cell, but retained their original shape for the long time and even after oocyte maturing.

The luminescence of fluorescent species were able to be excited both one-photon (at the wavelength 460 nm) and two-photon (at the wavelength 800 nm). The maximum of the luminescence corresponds to 520 nm and seems to be the same for cytoplasm, nucleus and nucleolus (Fig. 2).

Fluorescent particles persisted in a living cell for 24 hours and even more (Fig. 1, D-F). Presence of fluorescent species in the cell did not affect the ability of oocytes to develop up to the metaphase II (MII) stage during in vitro maturation. Oocytes of both experimental and control groups developed to the MII stage with 80-90% rate. This indicates that the method of in situ fluorescent species production has low toxicity for living cells.
Fig. 1. Fluorescent species in the cytoplasm (A), nucleus (B) and nucleolus (C) of GV oocyte. Arrows indicate the fluorescent spots.

Fig. 2. Luminescence spectra of fluorescent species, produced in the cytoplasm, nucleus and nucleolus.

Intracellular tracking was performed on GV oocytes. Fluorescent spot created within a nucleus seemed to be anchored to the chromatin filament. Spot movement revealed by the time-lapse imaging shows chromatin restructuring (Fig. 3) during the experiment (~6 minutes). Bleaching of fluorescent species was not observed during this time.
Fig. 3. Fluorescent spot migration within the nucleus of GV oocyte. Arrows indicate fluorescent particles, the dotted line indicates nucleus region. A – 0 s, B – 56 s, C – 231 s, D – 350 s.

4. Conclusion.

We have developed a novel method of non-invasive intracellular labeling, which allows to perform tracking of structures of the interest and does not require delivery to the cell. Tightly focused femtosecond laser beam creates fluorescent spot right in the focus area and does not disturb surrounding intracellular contents. It seems that fluorescent species are formed due to the carbonization of intracellular organics. Presumably, the composition of fluorescent particles is similar for species, produced from cytoplasm, nucleus and nucleolus, because its luminescence spectra are the same.

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References

[1] Damalakiene L., Karabanovas V., Bagdonas S., Valius M., Rotomskis R. 2013. International journal of nanomedicine, 8, 555-568.
[2] Ding P, Wang H., Song B., Ji X., Su Yu, He Y. 2017. Anal Chem. 89 (15), 7861-7868
[3] Sun Q., Qin Z., Wu W., Lin Y., Chen C., He S., Li X., Wu Z., Luo Y., Qu J.Y. 2018. Biomed Opt Exp. 9 (2), 581-590
[4] Gattass R.R., Mazur E. 2008. Nature Photonics. 2 (4), 219-225
[5] Hovhannisyan V., Lo W., Hu C., Chen S.-J., Dong C. Y. 2008. Opt Exp. 16 (11), 7958-7968