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High-resolution multiphoton cryomicroscopy

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ABSTRACT

An ultracompact high-resolution multiphoton cryomicroscope with a femtosecond near infrared fiber laser has been utilized to study the cellular autofluorescence during freezing and thawing of cells. Cooling resulted in an increase of the intracellular fluorescence intensity followed by morphological modifications at temperatures below −10 °C, depending on the application of the cryoprotectant DMSO and the cooling rate.

Furthermore, fluorescence lifetime imaging revealed an increase of the mean lifetime with a decrease in temperature. Non-destructive, label-free optical biopsies of biomaterial in ice can be obtained with sub-20 mW mean powers.

1. Introduction

Biobanks have become a key resource for research in genomics, personalized medicine (e.g. tissue transplantation, stem cell treatment), as well as the conservation of endangered species. Typically, biobanks make use of cryogenic storage. More than 20 million new samples are conserved per year. Currently, hundreds of millions specimens are stored in biobanks [1]. Cryopreservation refers to the technique of storing living cells and tissues at low temperatures for long periods of time. Cryopreservation techniques have been shown to be useful for a variety of mammalian cells such as lymphocytes, granulocytes, gametes, hepatocytes, bone marrow stem cells as well as human tissues such as cornea, skin, pancreatic tissue, liver slices, and heart valves [2,3]. In addition, plant cells and plant tissues have also been cryopreserved [4–6].

To minimize cell damage due to ice crystal formation, Polge et al. introduced glycerol as a cryoprotective agent in 1949 [7]. Some years later, dimethyl sulphoxide (DMSO) was used to prevent freezing damage to living cells [8]. The first *in vitro* fertilization with cryopreserved human spermatozoa was performed in 1978 [9]. The first baby from a cryopreserved embryo was born in 1984 [10].

When the cell in a medium is cooled below freezing temperature, the initial ice formation takes place within the extracellular microenvironment. The cell membrane acts as a barrier and prevents intracellular ice formation. Super-cooled intracellular water remains at a higher chemical capability than the partially frozen extracellular solution. This leads to a thermodynamic non-equilibrium and provides the driving force for two biophysical processes that occur during freezing, cellular dehydration and intracellular ice formation [2,11–20]. When the cooling rate is slow, the intracellular liquid has enough time to flow out of the cell through the semi-permeable membrane and join the extracellular matrix. This results in changes, such as pH and ion concentrations within the extracellular region and potentially causes the tertiary structure of proteins to fold. Hence, some of the original properties of the cell are either diminished or eliminated [2].

Severe damage may take place through mechanical interactions between the extracellular ice crystals and cells [2,8]. Intracellular ice formation is generally considered to be harmful as it causes injury to the intracellular structures as well as to the cell membrane [10]. The balance between the membrane's water permeability and probability of intracellular ice formation results in different optimal cooling rates for different cell types [11,15,16]. Typically, a slow freezing process with cooling rates between 0.3 K/min and 10 K/min with cryoprotective agents comprising 5–10% of the medium that is chosen in cryopreservation [16]. An alternative approach is the immediate placing into liquid nitrogen (vitrification, cooling rate >40 K/min) [16]. This fast-freezing process requires a high cryoprotective agent concentration of 40–70%. Unfortunately, not all cells survive this procedure. Cryomicroscopy has been performed since 1860, when the pioneer of plant physiology Sachs studied freezing plant cells in his cold lab with opened windows on a winter day [21]. His PhD student Müller placed the microscope in a box filled with ice. He invented the cold-resistant grape variety "Müller-Thurgau" [4].

Cryo electron microscopy (EM) was introduced in 1984 when intratissue water was preserved by high pressure freezing [22].

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For example, recent high-resolution cryo EM studies revealed the structure of the ribosome of *Trypanosoma brucei*, the parasite that is transmitted by the tsetse fly [23]. Correlations between light cryomicroscopy and cryo electron microscopy have been studied to a degree [24].

A current novel light cryomicroscope employs additional electrostatic fields to influence the ice crystal formation. In particular, this allows the control of the nucleation temperature [25]. Other light cryomicroscopes are described in references [26–29]. However, high-resolution 3D label-free imaging of cells and tissues during freezing/thawing and within ice blocks is not possible with these "one-photon" microscopes. Although the absorption coefficients of water and ice is low in the spectral range between 200 nm and 400 nm [45], there is the limitation due to enhanced scattering in the short-wavelength range and the hazard of bleaching and photodamage also in out-of-focus regions when using one-photon excitation. This limitation can be overcome when using two-photon/multiphoton imaging techniques.

Two-photon 3D high-resolution laser microscopy was invented by Denk et al. revolutionized the imaging of living cells and tissues at room temperatures [30]. A highly focused near infrared (NIR) femtosecond laser beam provides a tiny sub-femtoliter excitation volume at a high transient GW/cm^2 intensity that is scanned through the specimen. The use of NIR light enables deep material or tissue imaging. Nowadays, multiphoton microscopy and multiphoton tomography is routinely performed. Multiphoton effects include two-photon and three-photon excited fluorescence, second harmonic generation (SHG), third harmonic generation (THG), and coherent anti-Stokes Raman spectroscopy (CARS). Multiphoton imaging has become also a clinical tool to provide non-invasive, label-free, high-resolution optical biopsies [31,32].

Besides the fluorescence intensity, the spatially resolved fluorescence lifetime τ can be imaged. Fluorescence lifetime imaging (FLIM) in the life sciences was introduced by the author and coworkers in Jena in 1980's [33]. One decade later, a two-photon FLIM was employed [34,35]. In 2007, König's group developed a non-linear cryomicroscope based on a tunable femtosecond Ti:sapphire laser [6,36–39].

In this paper, we report on a novel ultracompact, upright 3D scanning multiphoton cryomicroscope with additional fluorescence lifetime imaging based on a frequency-doubled NIR femtosecond fiber laser. Fiber lasers in general provide the well-known advantages of being compact, robust and resistant against thermal and mechanical disturbances at a comparably low cost [40]. Resonator and amplifying medium within these lasers are integrated into the optical fiber. For ultrashort pulse generation, the fibers are typically doped with rare earth ions from ytterbium, erbium or neodymium [41]. The rare-earth ions are excited into long-lived metastable levels by pump light that allows for light amplification by stimulated emission. A strong optical confinement can be reached inside the waveguide structure of the fiber in a particularly with high-gain efficiency. However, maximum output energies are typically limited to about 1 nJ by strong optical non-linearities of the fibers [41]. Often such a low laser output is (with further power losses in the optical setup) insufficient in practice. Furthermore, particularly the optical nonlinearities present a hurdle for the generation of ultrashort pulses [41]. By contrast, a typical Ti:sapphire laser with a pulse duration of 100 fs at an 80 MHz repetition rate and with an output power of 1 W delivers pulse energies of about 12.5 nJ with a peak power of 125 kW. Nevertheless, improved fs-fiber laser technology in recent years has made fiber lasers capable of competing with the conventional solid-state lasers. Improvements in particular concern the dispersion management and techniques used to achieve mode-locking in fiber lasers which have made it possible to reach pulse energies around 25 nJ and pulse durations of 150 fs [42]. The rare-earth doped fiber lasers emit between 1 and 2 μm regions, however, the high output power enables efficient nonlinear frequency generation to generate a laser output with sub-100 fs pulses around 750–810 nm in commercial systems. With these extended wavelengths and energy options, naturally the range of possible applications of fiber lasers widens [43]. In the future, fiber lasers will become even easier to use and will find their way into many applications within biophotonics.

The novel ultracompact two-photon cryomicroscope based on a NIR erbium-doped-fiber femtosecond laser system was employed to study the freezing effects on label-free human and animal living cells with submicron spatial resolution and picosecond temporal resolution.

2. Materials and methods

2.1. Multiphoton cryomicroscope

The multiphoton laser scanning cryomicroscope is based on a compact and robust erbium-doped-fiber oscillator that provides sub-100 fs pulses at 1560 nm central wavelength and a repetition rate of 250 MHz. An amplifier and a frequency doubling SHG crystal generated output laser pulses at a center wavelength of 780 nm with a pulse duration of about 100 fs and a pulse energy of 0.6 nJ. This corresponds to an average power of about 150 mW. The oscillator, amplifier, and SHG crystal is packed into one common compact turnkey system (M-Fiber 780 nm, Menlo Systems GmbH, Martinsried, Germany). In order to compile an ultracompact 4D laser scanning multiphoton cryomicroscope (x,y,z,τ), a standard upright fluorescence microscope is attached with a scan module (JenLab GmbH, Jena, Germany) with x/y-galvoscanners. A temperature-controlled motorized stage (MDS 600, Linkam, Waterfield, UK) is added that allows cooling and heating between −196 °C and +600 °C (77 K/873 K). The controller keeps the sample at a stable temperature or enables temperature modifications at an adjustable rate from 0.1 K/min to 150 K/min by dynamically counterbalancing electrical heating and liquid nitrogen flow. The temperature is measured within the metallic cooling block. A photomultiplier (PMT Hamamatsu 7732) with a fast picosecond rise time in combination with a short-pass filter SP750 in front after passing a 700 nm-dichroic beam splitter is employed as a non-descanned photon detector. FLIM was accomplished through time-correlated single photon counting (TCSPC) using a SPC830 module (Becker&Hickl GmbH, Berlin, Germany).

Fig. 1 shows a scheme and a photograph of the non-linear laser scanning cryomicroscope. The NIR femtosecond laser pulses were employed to excite the fluorescence of the reduced coenzyme NAD(P)H (emission maxima at 440 nm/460 nm) as well as flavins/flavoproteins (530 nm) through two-photon absorption. The maximum mean laser power and the pulse width at the sample were 18 mW and 200 fs, respectively. A 40× oil immersion objective with a numerical aperture of 1.3 (minimum z-steps: 40 nm) was used for high-resolution multiphoton cryosectioning of cells. The system provided a lateral resolution of more than 0.5 μm and 1–2 mm in axial direction when probed with fluorescent nanobeads and point-spread function measurements for non-freezing conditions. The temporal resolution was about 200 ps as determined by the measurement of the instrumental response function (IRF) using the SHG active substance urea. The whole optical system, including monitor and keyboard fits onto an optical breadboard of 0.9×0.6 m^2 and has a total weight of 80 kg (40 kg microscope, 30 kg laser, 5 kg scanner control box, 5 kg cryostage with controller). For comparisons and for FLIM measurements based on a longer temporal pulse-to-pulse distance of 12 ns, the microscope was also combined with a femtosecond laser at lower