Project title: Imaging of biological structures by combined usage of second- and third harmonic generation multiphoton microscopy

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Current state of the art

The use of structure-identifying microscopy techniques that work without dyes (label-free) is preferable, especially when analysing vital samples, i.e. fresh biological tissue or organoids. Multiphoton microscopy is therefore a highly relevant technology in biophysics, which is also expected to be used in pathological diagnosis in the future [1-13].

The technology uses non-linear optical effects to create contrast in the sample. Signals generated by frequency doubling (second-harmonic generation: SHG) and frequency tripling (third-harmonic generation: THG) are suitable for identifying and differentiating specific biological structures in vital samples without the use of dyes [1-13] (Fig. 1). Unlike dye-based fluorescence, nonlinear optical signals like SHG are generated through elastic scattering without photon absorption and re-emission, resulting in a direct link between excitation and emission properties (wavelength, polarization, and propagation direction). This process typically reduces photodamage and photobleaching compared to fluorescence approaches [14].

SHG only occurs in structures that are strictly hierarchically organised and whose molecular structure is non-centrosymmetric. Particularly prevalent biological examples here are collagen of the extracellular matrix and myosin of the skeletal muscles [1-4] (Fig. 1A). However, THG can be triggered at interfaces between molecules with different refractive indices and thus a larger variety of biological structures. In principle, THG makes it possible to visualise the edges of different cell structures in biological tissue [5-9].

Furthermore, an important factor relevant to biological investigations is that THG as well as SHG causes hardly any light damage. Only the higher-energy THG wave (in the ultraviolet frequency range) can cause damage [10, 11]. To generate a THG signal that is as gentle on cells as possible, a longer excitation wavelength is better. With an excitation wavelength of, for example, 1300 nm, the THG signal at 433 nm is in the visible range and is therefore less damaging to cells than light in the ultraviolet range. A longer wavelength also means that deeper tissue layers can be reached. The detection depth for THG is therefore greater than for SHG. The excitation of higher harmonics offers a further advantage. The THG and SHG signal source cannot bleach because no fluorophores are excited [12, 13]. In concrete research applications, this means that changes in the tissue can be specifically detected over time.

While SHG is already a well-established technique, the use of THG as a method for analysing biological samples is still in its early stages. With the increasing use of THG microscopy, tissue structures such as lipids, hydroxyapatite, and oxyhemoglobin have been characterized using this method, complementing SHG-based detection of collagen and myosin, whose non-centrosymmetric molecular structures produce strong SHG signals. [15, 16]. In future, the challenge will be to develop a setup that combines the three imaging techniques: two-photon autofluorescence (TPAF), SHG and THG, to examine complex tissue organisations in a completely label-free manner.

Research goals and working program

The aim of this project is

- to establish three modality (TPAF, SHG and THG) multi-photon microscopy on the FVMPE-RS System (EVIDENT) with an Insight X3 (Spectra Physics) dual-line laser. [Month 1-6]
- to adapt the setup to investigate murine tissue samples (e.g. skin, kidney and liver) and separate the different structures (e.g. collagen, myosin, lipids, membranes, organelles). [Month 7-12]
- to adapt the setup to investigate with *Caenorhabditis elegans* a complex organoid structure. *Caenorhabditis elegans* is a tiny, transparent nematode worm that serves as an important model organism in biomedical research. It is the ideal target structure for this approach because it has a simple anatomy (Fig. 1B). The low cell count and transparent body, which allows cellular processes to be observed, are ideal for highlighting the potential of the methodological approach [17]. As it is already used for studies on ageing, diseases and genetics, since many of its genes and signalling pathways also occur in humans, the expected results are highly relevant. [Month 13-36]

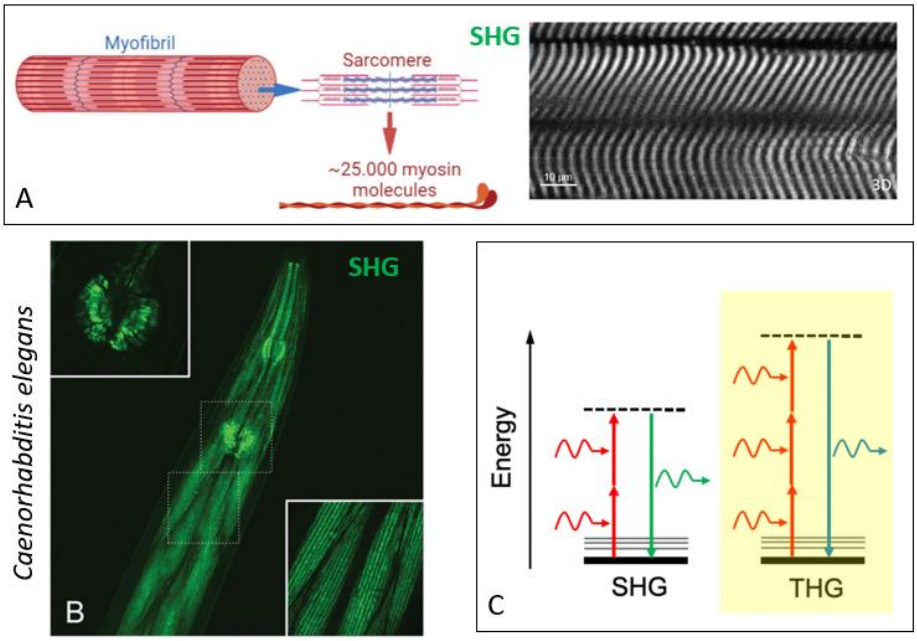

Fig. 1 A: Second-harmonic generation (SHG, frequency doubling) in myosin (Schematic view created in BioRender.com) B: SHG signal in Caenorhabditis elegans (Figure 11.19.2 in [17]) C: Comparison between SHG and Third-harmonic generation (THG, frequency tripling).

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