

# Microstructured imaging windows for live histology

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## ABSTRACT

Ex-vivo histology is time consuming, subjective, and invasive. We discuss how, by coupling non-linear excitation, structured illumination and implantable micro-optics, we can obtain in-vivo tissue histology.

**Keywords:** Non-linear excitation, bioimaging, histology, in vivo imaging

## 1. INTRODUCTION

Ex-vivo histology is time consuming, subjective, and invasive. It involves the sacrifice of lab animals at specific time points, the extraction of tissues, fixing and embedding them in paraffine. Then thin (4-6 micrometers) slices are cut and stained with, for example, eosin (cytoplasm) and hematoxylin (nuclei) or Masson trichrome (for collagen). They can be scanned by optical scanner and read by expert pathologists. A more efficient type of reading can be done via immunohistochemistry by labeling with fluorescently labeled antibodies. Several tens of proteins can be tagged in this way with the newly developed strip technology [1]. The possibility to store digitally at high resolution the optical microscopy image of the tissue sections has revolutionized the pathology methods. However the whole workflow suffers from three main disadvantages: the animals must be sacrificed, they are, despite automatization, time consuming and the efficiency is low (only about 10% of the cells can be segmented and quantified on the image).

## 2. RESULTS

If we could transpose digital pathology to an in-vivo and label free approach we would overcome at least two of the above drawbacks. Our approach to this goal is based on the use of high order non-linear excitation microscopy. In this way we can perform optical sectioning of the tissue and visualize different compartments of the cells. Beside using second harmonic generation and two-photon excitation microscopy, we exploited here third harmonic generation. Two-photon excitation fluorescence (TPEF) and second harmonic generation (SHG) can be performed with excitation at  $\lambda = 800 \text{ nm}$  and 150-200 fs pulsed lasers at high repetition frequencies (typically 80 MHz). They offer the possibility to visualize collagen and cytoplasm, as obtained from histo-chemical analysis with Eosin and Masson trichrome. The laser peak intensity can reach values as high as a few  $\frac{\text{GW}}{\text{cm}^2}$ . Third harmonic generation (THG) requires the use of high pulse energies (in the order of 1-10 nJ) and can be performed with low repetition rates short pulses (30-60 fs). It offers the possibility to visualize the nuclei, as obtained from histo-chemical analysis with Hematoxylin. The laser peak intensity can reach values as high as a few  $\frac{\text{TW}}{\text{cm}^2}$ . It is clear that at these high values of the intensity thermal damage of the tissue can occur and should be carefully monitored exploiting methods like the photo-thermal scanning reported by Bouzin et al. [2]. Preliminary data (data not shown) indicate that in the conditions adopted for imaging in this report the maximum temperature increase is below  $1^\circ$ .

The second line of approach that we adopt to reach our goal is the use of implantable microlenses to reduce the spherical aberration induced by inhomogeneity of the tissue. Finally we will implement AI methods to cluster the data according to

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features that allow us to separate different types of cells. In particular we will focus on fibroblasts, monocytes and granulocytes (or polymorphonuclear cells) that together with collagen that we can monitor by means of SHG, will allow us to fulfill most of the requirements of the ISO10993-6 norm (“Biological evaluation of medical devices. Part 6: Tests for local effects after implantation”) [3].

The microlenses were fabricated by two-photon laser polymerization as described in detail in ref.[4]. They were built on one side of a microscope thin glass slide (thickness = 0.17 mm) on whose opposite side a regular micro-lattice was fabricated. The lattice has a overall size of  $0.5 \times 0.5 \times 0.1$  mm, with a porosity of  $50 \times 50 \times 20 \mu\text{m}^3$ . The shape of the lenses was designed to maximize its numerical aperture and to allow imaging all the microlattice. Examples of the shape of the microlenses are reported in Figure 1A, together with the sag function that describes the shape of the lenses. The shape is aspherical but described by a single parameter,  $f_n$ . This parameter determines the focal length, also depending on the refractive index in which the microlenses works. In this proceeding we will consider only the face  $f_n = 250 \mu\text{m}$ . Given the average index of refraction of the tissue ( $n_{\text{tissue}} \approx 1.42$ ), we obtain in water a focal length of about  $320 \mu\text{m}$ .

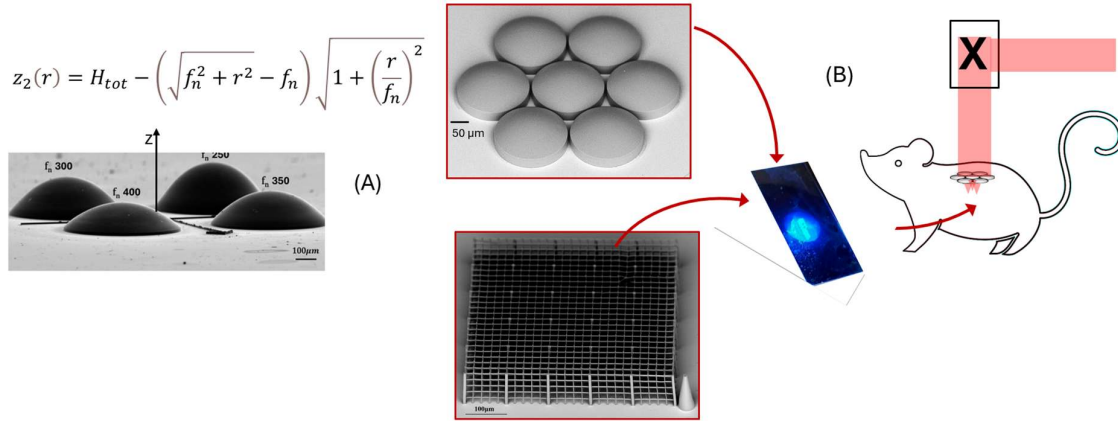


Figure 1. Panel a: electron microscopy (SEM) image of a set of 4 microlenses fabricated with  $f_n = 250, 300, 350$  and  $400 \mu\text{m}$ . The sag function of the lenses is reported above the image. Panel B: SEM images of an array of microlenses and the microlattice that are fabricated on the opposite side of a microscopy glass slide (5mm in diameter) and implanted in mice. The sketch on the right of panel b illustrates the principle of use of the implanted chip on which an almost collimated laser beam is scanned.

By exploiting non-linear excitation we are able to obtain label free images of the tissue. An example is given in Figure 2A, where the THG images of a device implanted in chicken embryo is shown. The circular shapes segmented on the image are cell nuclei whose average size is  $4 \mu\text{m}$ . Smaller structures are taken as lipid infiltrates. Once the nuclei are segmented we can investigate the correlation between several couples of parameters and perform a clusterization analysis. As reported in Figure 2B, by searching correlation between the ratio of the cell area and the nuclear area and the nuclear eccentricity, we can clearly single out two population of cells, that can be ascribed to monocytes and lymphocytes.

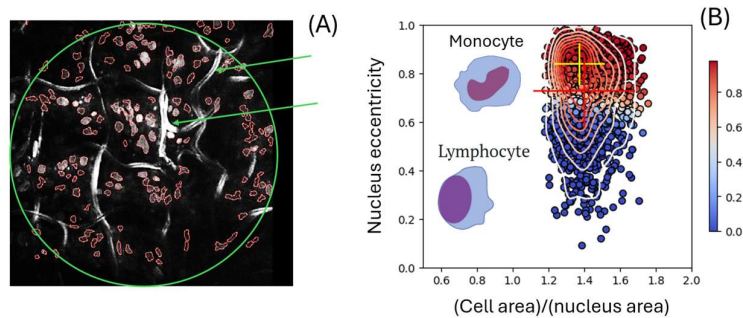


Figure 2: example of THG imaging of a device implanted in a chicken embryo at 7 days after fertilization taken through one microlens. Panel A: the microlens size is outlined by the large green circle. A few beams of the microlattice originally implanted and distorted by the chicken embryo development are clearly visible (arrows). The small circular shapes are cell nuclei segmented on the image. Panel B: example of the clusterization of about 1000 cells detected on a set of images as in panel A. The clusterization of the data in two populations has been done by means of the Gaussian mixture decomposition (from `sklearn.mixture import GaussianMixture`). The colorbar reports the probability to belong to class A (Monocytes), characterized by larger values of the nuclear eccentricity at high values of the ratio of the cell to nucleus area.

An example of THG imaging of a full device (micro-lattice + microlenses) implanted in a chicken embryo at 7 days after fertilization taken through one microlens is shown in Figure 2A. A few beams of the micro-lattice originally implanted appear distorted by the chicken embryo development and are clearly visible in Figure 2A (arrows). The small globular entities marked in red are cell nuclei segmented on the image with the aid of Qupath [5].

The cells segmented on the image are characterized by the cellular and nuclear area and eccentricity. An example of the clusterization of about 1000 cells detected on a set of images as in Figure 2A are reported in Figure 2B. The clusterization of the data in two populations has been done by means of the Gaussian mixture decomposition (from `sklearn.mixture import GaussianMixture`). According to the known average morphology of the cells and nuclei of immune cells (in terms of the ratio of the areas of the cell to the area of the nucleus and of the eccentricity of the nucleus), we ascribe these populations to monocytes and lymphocytes.

### 3. CONCLUSIONS

In this paper we discuss how the use of high numerical aperture implantable microlenses allows to get good quality images of tissue. The numerical aperture of the microlenses fits the requirement to image an implantable microlattice with  $xy$  size = 0.5 mm and thickness 0.1 mm. The wide radius of the lens, 0.3 mm, and the effective focal length (about 0.32 mm) implies a numerical aperture  $\approx 1$  or even larger in water. It is this feature together with the good quality of the lens surface that allows us to get high resolution, high contrast images with non-linear excitation, up to third harmonic generation. Similar implantable microlenses, combined with cell and nuclei segmentation and data clustering could be used to obtain perform digital pathology in-vivo

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