Application Note

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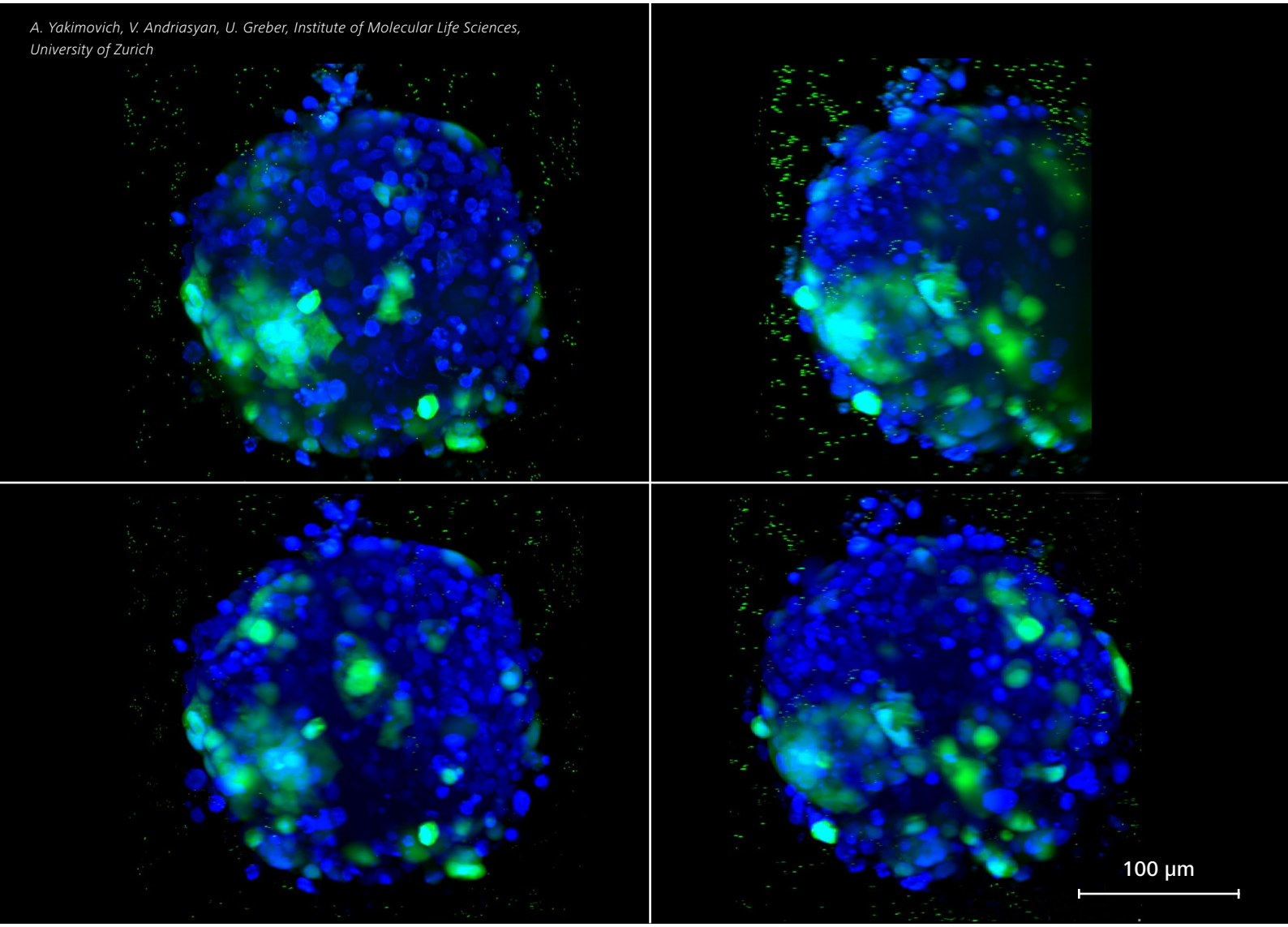

Fast Imaging of Cellular Spheroids with Light Sheet Fluorescence Microscopy

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Fast Imaging of Cellular Spheroids with Light Sheet Fluorescence Microscopy

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Introduction

A light sheet based microscope obtains an optical section of a fluorescent specimen by illuminating only a section of the sample with a thin sheet of light. The emitted fluorescence is recorded with a camera through a wide-field microscope that is oriented perpendicularly to the light sheet (Huisken et al., 2004; Siedentopf and Zsigmondy, 1902; Voie et al., 1993). This microscopy technique provides much faster image acquisition while exposing the specimen to relatively low amounts of light, thereby reducing photobleaching and photoxicity. The improvement is especially significant with large specimens that require many sections in order to generate a three-dimensional image, such as entire organisms, organoids mimicking tissues or three dimensional cell cultures. Finally, mounting of specimens in transparent aqueous gels allows them to be imaged from multiple directions (Multi-view imaging), generating high contrast images of highly scattering specimens.

Cultured cells behave differently if grown on a flat substrate compared to those grown in three-dimensional gel matrices. Such three-dimensional cell cultures are believed to be more physiological than cells grown on flat surfaces, and provide deep insight into the physiology of real tissues (Griffith and Swartz, 2006).

Light sheet fluorescence microscopy (LSFM) in combination with Multiview imaging was quickly identified as a useful tool for imaging of three dimensional cell cultures, which are known to be highly scattering (Verveer et al., 2007). In this article we present an application of the commercially available high-resolution light sheet based microscope, Lightsheet Z.1 from ZEISS, to the investigation of large cultured organotypic tissues, so called cellular spheroids.

Experimental procedures and results

Sample preparation

Human bronchial epithelial cells (HBE cells) were cultured as scaffold-free organotypic spheroids using a hanging drop method and infected with a transgenic human adenovirus type C2 with E3B region substituted by an eGFP cassette expressed under the major CMV promoter (HAdV-E3BGFP) (Yakimovich et al., 2012). Spheroids were fixed using para-formaldehyde and stained for DNA with Hoechst 33342. Fixed spheroids were mixed with a suspension of 0.5 µm fluorescent beads (Merck Millipore, Catalogue No.: 80380016, www.merckmillipore.com) in low-melt agarose solution (Carl Roth GmbH, Product No.: 6351.5, www.carlroth.com). The mixture was then sucked into glass capillaries with inner diameter of 1 mm. The agarose was allowed to gel at room temperature for five minutes before imaging.

Imaging

Spheroid samples were imaged using Lightsheet Z.1 with 20x/1.0 detection optics and two-sided 10x/0.2 illumination optics. To counteract the degradation of the light-sheet by the highly scattering spheroid, the specimen was sequentially

illuminated through each of the two opposing illumination objectives, generating pairs of single-side illumination images, which were instantaneously combined into optical sections with considerably improved penetration depth.

To increase the acquisition speed, the specimen was illuminated simultaneously with two wavelengths. The fluorescence emission was split by a beam splitter and recorded simultaneously by two PCO.edge cameras.

Lightsheet Z.1 features rotation of the specimen around the axis perpendicular to the illumination and detection objective axes, which enables imaging of the specimen from different directions. Four three-dimensional images or "views" of the spheroid were recorded along directions that were offset in steps of 90^0, and later registered and fused into a single three-dimensional image.

Processing of the Multiview image sets was done using ZEN imaging software. Four views were first registered by the ZEN landmark-based algorithm, using coordinates of the fluorescent beads embedded in the agarose to bring the four views into the same orientation. The four views were finally fused using a mean-intensity algorithm. The resulting single image showed a good and homogeneous contrast across the entire periphery of the spheroid.

Results

Especially in case of highly-scattering large specimens, such as the spheroids used in our study, any single view image depicts only a fraction of the entire volume of the specimen in high quality (Fig. 1a). By Multiview imaging, views from different angles are combined and thereby provide high quality information of enhanced specimen volumes (Fig. 1b-c).
In our study, image improvement by Multiview imaging shed light onto significantly deeper regions of the light-scattering organoid. This, in turn, allowed identification of infected cells within the organoid (Fig. 1c, Fig. 2).

Multiview imaging not only provided good quality data over a larger part of the spheroid, but it also increased axial resolution of the resulting images. This could be easily shown on the images of the fluorescent beads, which in a single-view image appeared elongated along the direction of the microscope's detection axis (Fig. 2, top row). In an image fused from four views (Fig. 2, bottom row), the images of the beads appeared shorter and more isotropic, and thus better reflected the real shape of the beads.

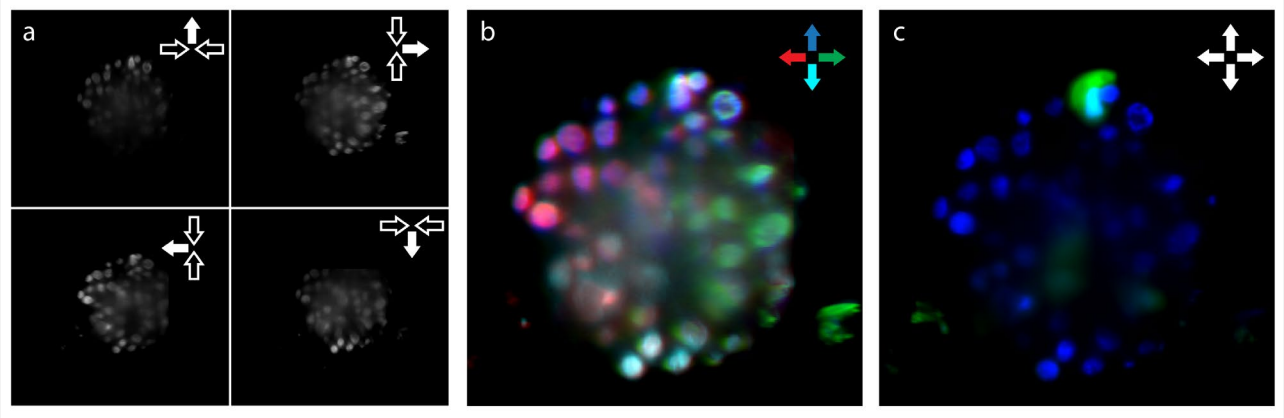

Figure 1 *Demonstration of Multiview image acquisition and image fusion of a cell spheroid*

a) A section perpendicular to the rotation axis through four three-dimensional images (Hoechst staining of DNA) of a Multiview dataset shows image degradation along illumination (empty arrows) and detection axis (solid arrows). Each image demonstrates best quality only in the part of the specimen that faces the detection optics.

b) Overlay of four Multiview images after image registration. Each view demonstrates good quality in only a part of the specimen (colored arrows indicate direction of each view's detection optics), while all images together cover the entire volume of the spheroid.

c) High quality image fused from the input images in Fig 1a-b (blue) overlaid with an image of cytoplasmic eGFP, expressed by cells that were infected with transgenic human adenovirus C2 (in green).

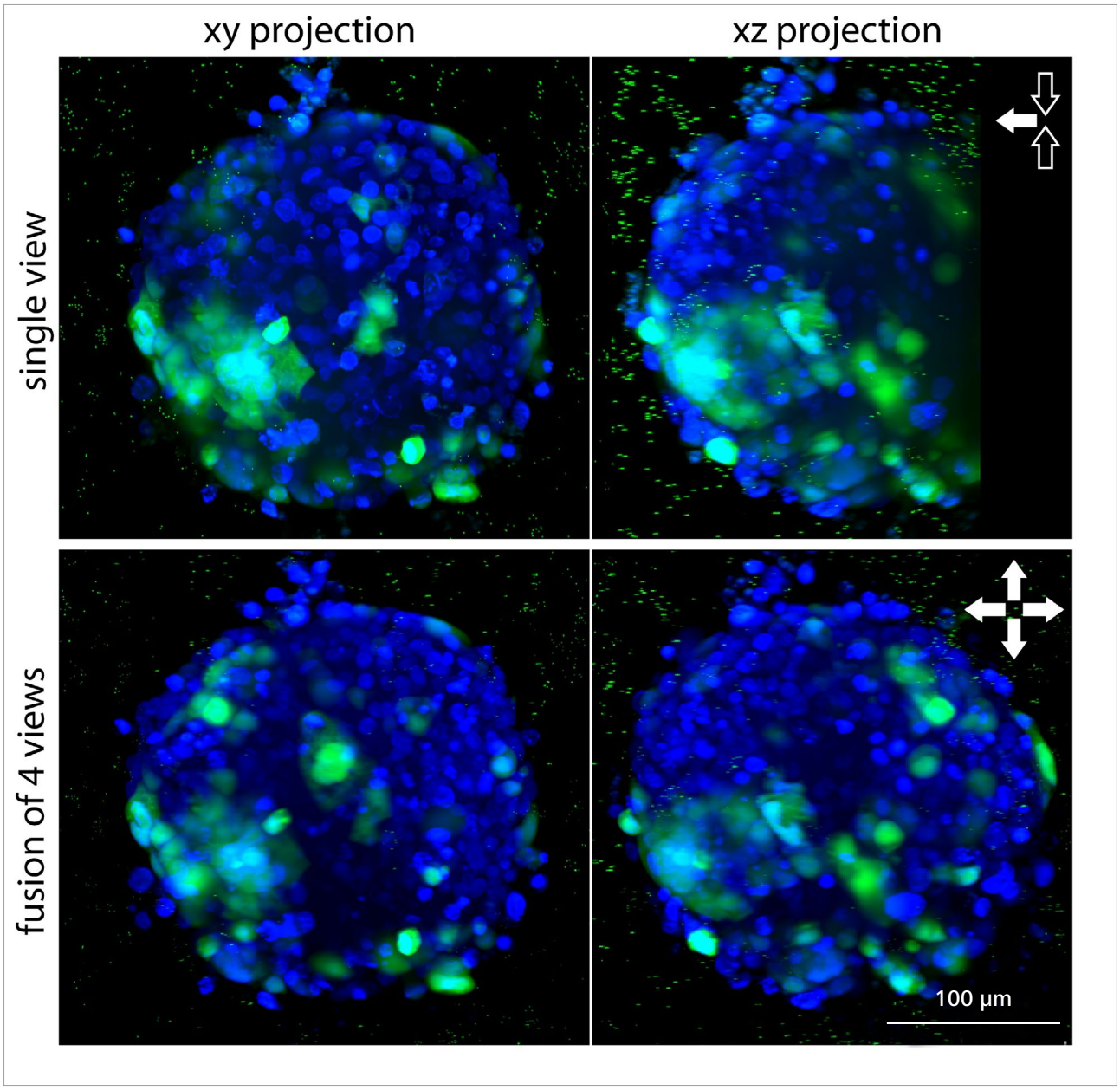

Figure 2 *Hoechst stained Human Bronchial Epithelial cell (HBE) spheroids infected with transgenic human adenovirus C2 expressing eGFP. The upper row shows maximum intensity projections (MIP) of a single stack along z-axis (xy projection) and y-axis (xz projection). The spheroid is surrounded by small green fluorescent puncta representing the fiducial beads. The lower row illustrates MIP of image fused from four registered stacks acquired at different angles. The xz projection demonstrates the improved resolution in z and increased penetration depth of the Multiview fused image compared to the single view stack.*

Conclusion

Light sheet fluorescence microscopy enables scientists to image large spheroidal cell cultures with high frame rate and low light exposure. Additionally, the light scattering characteristic of large spheroid cell cultures can be efficiently circumvented by dual side illumination and Multiview imaging. Lightsheet Z.1 combines the advantages of a light sheet microscope with easy specimen mounting, full experimental control and robust image processing tools provided by ZEN imaging software. This makes Lightsheet Z.1 a perfect tool for imaging of large and highly scattering specimens. The high acquisition rate and simple specimen preparation enable imaging of many samples in a short amount of time. Furthermore, we show that Lightsheet Z.1 can be a tool for the development of novel assays in infectious disease biology.

References:

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