Application note

Large volumetric imaging of intestinal organoids via ExM and *RE*scan

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Organoids offer physiologically relevant platforms for studying disease, but their dense architecture impedes deep, high-resolution imaging. Expansion microscopy (ExM) physically enlarges samples, enhancing resolution, epitope accessibility, and optical clarity. We combine ExM with AION, a next-generation confocal system featuring switchable pinhole, fast large field-of-view imaging, and minimal phototoxicity. This approach enabled rapid volumetric imaging of expanded mouse small intestinal organoids more than 600 µm deep in super resolution. The system's flexibility allowed contrast optimization without sacrificing resolution. Coupled with automated sample preparation and deformation correction, this integrated workflow has potential as a robust, scalable platform for deep tissue imaging in organoid-based research and translational applications.

Keywords: Expansion microscopy, Organoids, Super resolution, Confocal microscopy

INTRODUCTION

Organoid cultures are increasingly being used as physiologically relevant models for studying disease. High resolution volumetric imaging is necessary to fully understand the molecular mechanisms of disease and the spatial relationships in these models. However, the complex architecture of organoids poses significant challenges, including light scattering and limited antibody penetration.

Expansion microscopy (ExM) (Chen et al., 2015) presents a unique solution to these issues. In ExM, organoids can be embedded into a swellable hydrogel, physically enlarging the sample and replacing much of the structure with water.

This approach, based purely on sample preparation, not only boosts the resolution of the imaging system, but also reduces sample density to provide optical clearing, and increases epitope accessibility. Therefore, ExM is a powerful technique to facilitate high resolution deep tissue imaging.

One of the main obstacles for high resolution deep tissue imaging, for example expanded organoids more than 200 µm in depth, is using a microscopy approach capable of imaging deep with minimal susceptibility to the adverse effect of the scattered light and minimal photobleaching while, at the same time, maximizing signal-to-noise

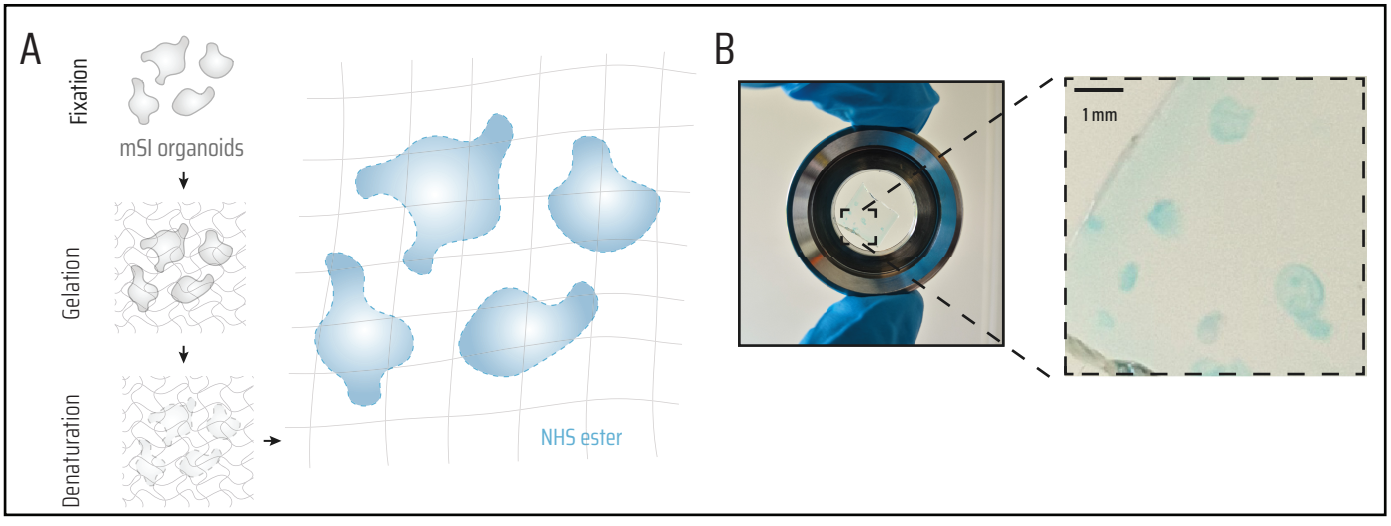

Figure 1. A) Schematic of the U-ExM method used for expansion of mouse small intestinal organoids. Organoids were first fixed, embedded in a swellable hydrogel, homogenised using denaturation and expanded. NHS-ester was added post expansion. B) Image of the expanded hydrogel mounted in an imaging chamber with single organoids visible.

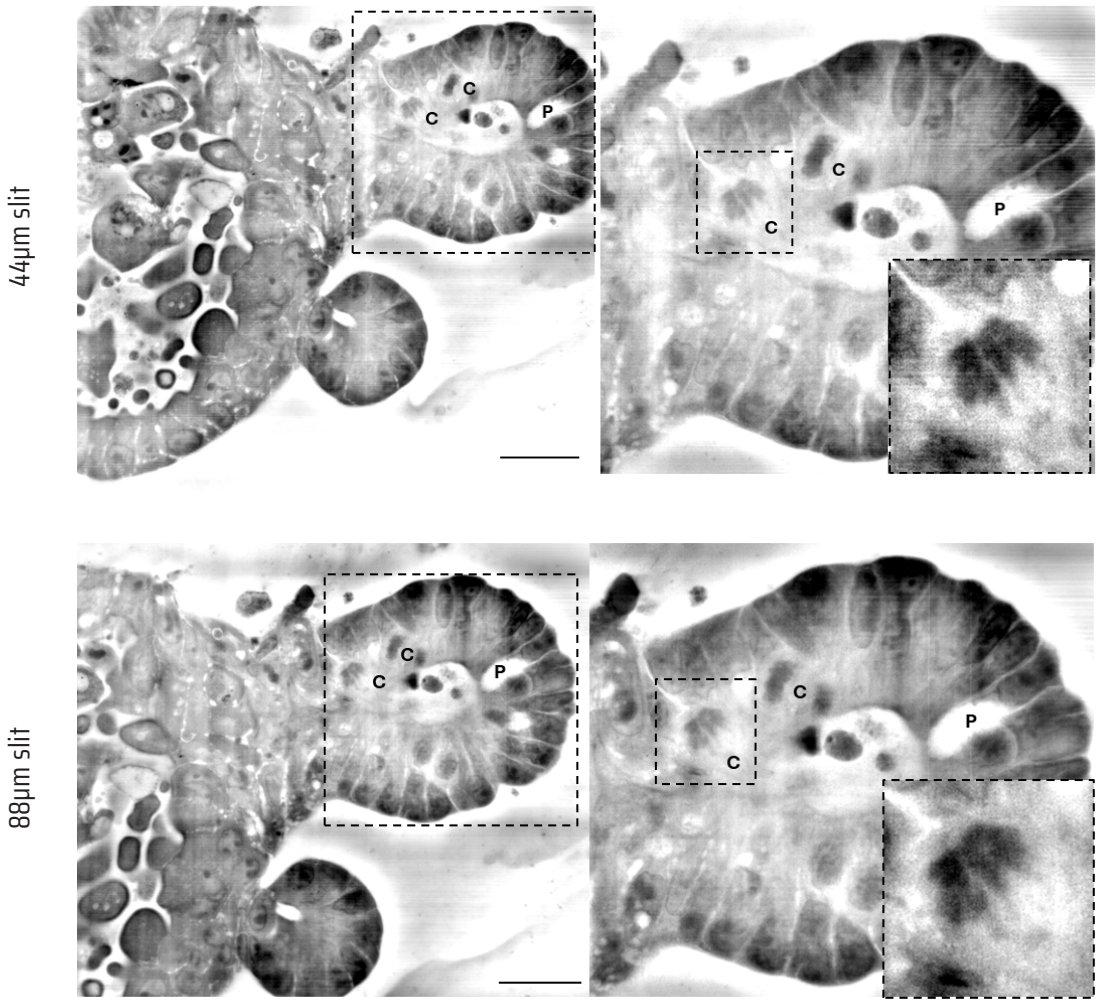

Figure 2. Representative images of mouse small intestine organoids, after 4X expansion. The images represent a single z-stack acquired with Line *RE*scan AION system and 30x Sil /1.05NA Objective with a WD of 800µm. These images compare different pinhole sizes (top panel 44 µm and bottom panel 88 µm). Inserts on the right demonstrate how a smaller pinhole size allows for better visualization of the chromosomes in the highlighted mitotic cells. The letters identify the different cell types visible in the crypts (C - chromosomes in mitotic cells; P - Paneth cells). Scale bar 100 µm.

ratio. Here, we demonstrate the powerful combination of ExM in organoid cultures with Line *RE*scan AION.

SAMPLE PREPARATION

Mouse small intestinal organoids (mSI) were received as a gift from Saskia Suijkerbuijk (Utrecht University), and cultured as previously described (Garcia et. al., 2021). Organoids were processed for expansion microscopy using a variation on the U-ExM protocol (Gambarotto et al., 2018). Organoids were labelled with the total protein marker NHS-ester, allowing volumetric visualisation of cellular ultrastructure in these samples (Fig. 1A). In particular, ATTO 647N NHS-ester (ATTO-TEC GmbH) was added to the sample post-gelation. NHS-esters bound to primary amines on proteins leading to the total body labelling with ATTO 647N fluorescent dye.

Following expansion and labelling, the gel was affixed to a 1.5H glass coverslip using poly-L-lysine and mounted in an Attofluor cell chamber (Invitrogen) submerged in water for imaging (Fig. 1B). Macroscopic measurement of the gel indicated a 4X expansion factor.

ExM WITH LINE *RE*SCAN CONFOCAL

The decrowding effect of ExM enabled a visualisation of detailed structures combined with broader context. The structures within the expanded organoid were investigated using Line *RE*scan AION. AION is a cutting-edge confocal microscope that excels in live cell and organoid imaging, combining gentle 3D imaging with unprecedented speed and field of view.

Importantly, AION is the most gentle confocal microscope with a switchable pinhole, in existence, reducing photobleaching and preserving fluorescence in fixed samples. We have used this feature of AION and imaged mSI using 44 µm and 88 µm wide slit that provided us with, when using 30x/1.05 NA objective, confocal imaging with 1.8AU and 3.6AU, respectively (Fig. 2).

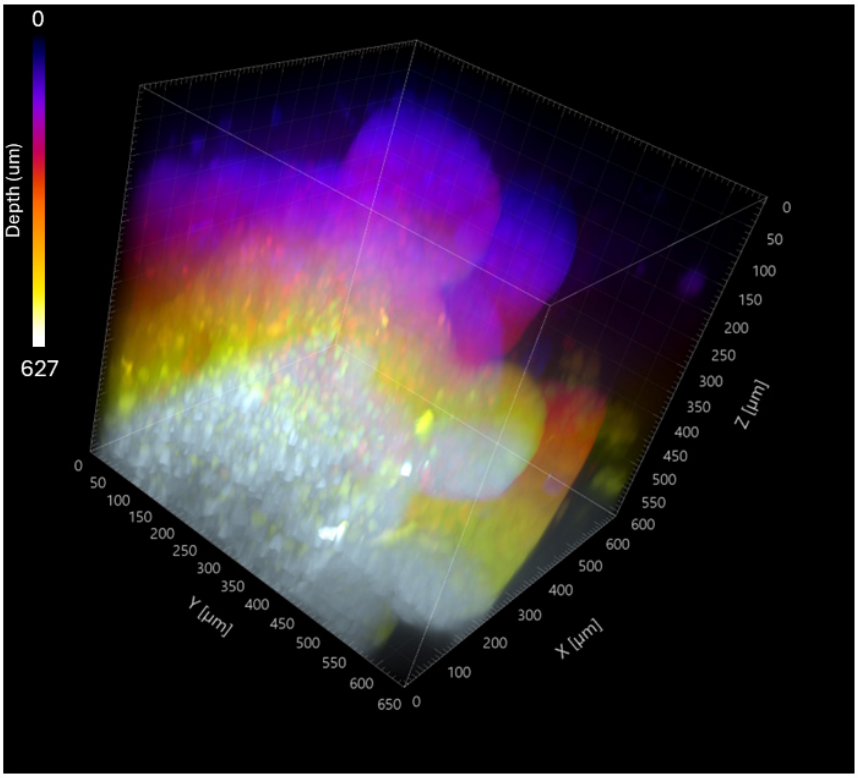

Figure 3. Ultra-deep imaging in mouse small intestine organoids. The deep color coding of the whole z-stack acquired (from dark blue close to the cover slip to white 627 µm deep into the sample) allowed for the 3D reconstruction of the majority of the expanded organoid. Expanded mouse small intestine organoids were imaged with 30x Sil Objective/1.05NA with a WD of 800µm and a slit size of 44µm.

Observing the broader context of the expanded mSI organoid has not uncovered visible differences with different slits. As *RE*scan belongs to image scanning microscopy (ISM) techniques, opening the pinhole beyond 1AU does not affect the resolution, but it does affect confocality. Therefore, upon closer inspection of details observable only in high resolution, we identified small differences in the chromosomal structures. This emphasizes the importance of flexibility provided by the switchable pinhole.

High volumetric imaging was achieved while acquiring Z stack more than 600 µm in depth. As AION provides a field of view (FOV) of 26.5 mm (FN26.5) without vignetting, using Kinetix camera with 3200x3200 pixels and pixel size 6.5 µm, we imaged a cube of approximately 0.301 mm^3(690 µm x 690 µm x 627 µm) in less than 3 minutes (Fig 3.). This demonstrates how a large FOV in combination with an objective with a large working distance enables the acquisition of largest possible volumes without stitching.

DISCUSSION

The combination of ExM and Line *RE*scan confocal AION has big potential in future disease investigations. Minimal photobleaching, largest FOV in the field and high speeds of acquisition make AION the optimal tool for analysing the large volumes of ExM samples. Importantly, manipulating the chemistry of ExM allows for the preparation of samples where different features are emphasized. Therein the flexibility of AION comes into play. In other words, one might choose between a closer pinhole, relevant for increasing the contrast, and a more open pinhole that is important for longer or imaging with less laser power.

While we have shown that this combination of techniques is powerful, it is important to note limitations. One key challenge with ExM is uncertainty around isotropy and deformation, unknown without a precise internal scale bar for measurements. As is standard, we provide an approximate calibration of the expansion factor based on macroscopic gel size.

Due to the fact that the organoids are increasingly used in applications such as drug screening and pathology, this underscores the need for (i) more accurate, validated measurement strategies, and (ii) greater standardisation and automation to ensure reproducibility between samples.

CONCLUSION

In the future, we hope to demonstrate how the powerful combination of ExM with Confocal NL's AION microscope (see box below) can be made more accessible through integration with Visualise.bio's GelCast system. The GelCast sample processing robot provides standardisation to the expansion microscopy field by automating and parallelising sample handling, reducing variability. Furthermore, the intrinsic scale bar and deformation mapping enabled by GelMap (Damstra et al., 2023) from Visualise.bio will offer further validation, crucial for more applied studies in organoids with ExM. Together, the complementary technologies of Confocal NL and Visualise.bio open new opportunities for scalable, high resolution volumetric imaging workflows with organoid models.

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AION LINE *RE*SCAN CONFOCAL SYSTEM OVERVIEW (BOX1)

AION Line *RE*scan is a confocal add-on unit designed for fast and deep imaging with minimal phototoxicity. AION can be combined with a number of different laser combiners, most sCMOS cameras and virtually any widefield microscope to create the confocal imaging system with a switchable pinhole that is optimized for live cell imaging.

- Acquisition speed of over 140fps at full FOV of more than FN26.5
- Compatible with Upright and Inverted Microscope
- Optical confocality with a wide range of objectives (4x-100x)

GELCAST SAMPLE PROCESSING ROBOT OVERVIEW (BOX2)

The GelCast expansion microscopy sample processing robot from Visualise.bio automates all steps of the ExM workflow, from sample in to gel out. By processing samples in a well-plate format, it ensures increased throughput and reliability – with no need for manual intervention.

- Automated sample gelation and buffer exchange
- Flexible routines, with temperature range 4 - 95°C, accommodating multiple ExM recipes up to 10X expansion
- Compatibility with multiple samples: cells, organoids, and tissues