Setting up an in vitro assay for drug testing based on 3D tumor spheroid imaging by Light Sheet microscopy

Implementation of a protocol for spheroid observation and analysis

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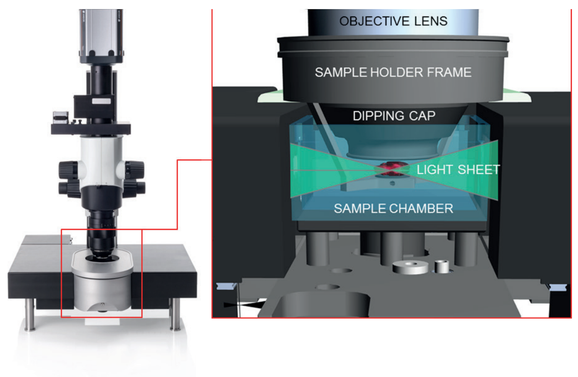
Abstract

Spheroids, 3D structures formed by cells, is a model that is more representative of *in vivo* tumors than 2D culture while avoiding complexity, heterogeneity and low transparency of whole tissues[1].

Our Transversal Project at Sup'Biotech aims at elaborating an antineoplastic drug assay using spheroids observed by Light Sheet Fluorescence Microscopy (LSFM). This first study optimizes experimental parameters and protocols from HeLa-YFP spheroid generation to post-acquisition processing of the images. The use of clarification (Ethyl Cinnamate) was deemed necessary. As drug assays using spheroid usually track their volume changes, an early comparison between estimation techniques suggest that 3D modellisation-based estimations are more precise than diameter-derived estimations, potentially due to the relative non-sphericity of the observed cell aggregates. The next steps of this project will be the introduction of drug treatment.

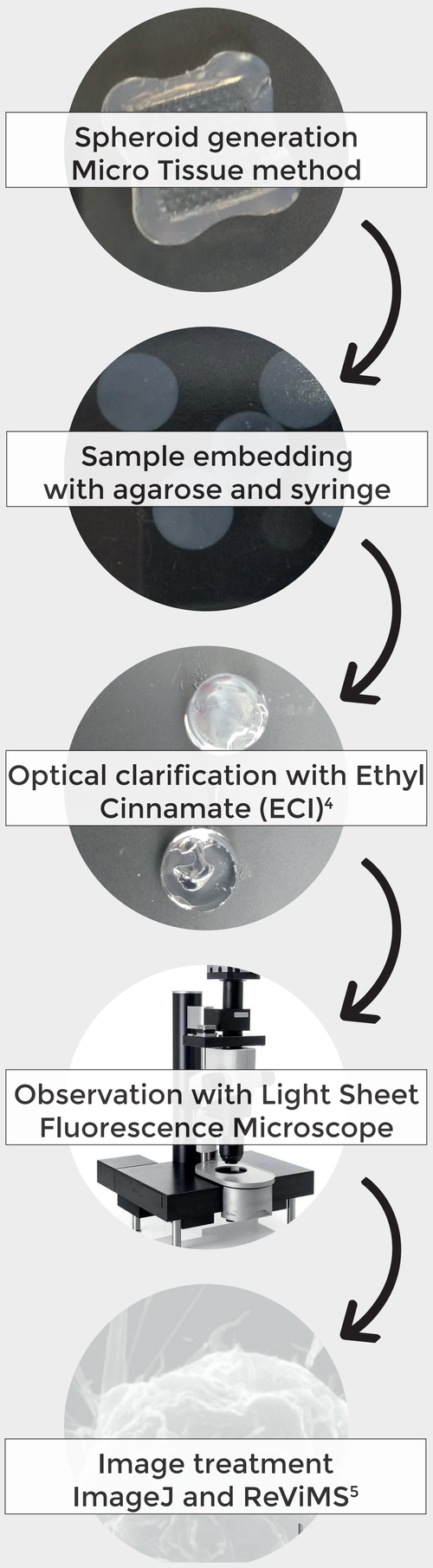


Why using LSFM ?

- **3D imaging**
- **Reduced photobleaching**[1]
- **Larger work distance** than confocal
- Better light penetration than confocal microscopy

Material and methods

Steps



Optimized parameters

Several initial cell seeding number of HeLa-YFP tested.

200, 300 and 400 µm expected diameter (according to manufacturer)[3]

Embeddeding in agarose using a syringe
(Best container for homogenous repartition of spheroids in gel)

Imaging before and after optical clarification.

ECI :
preservation of endogenous fluorescence (YFP)

Parameters optimized:
Laser intensity, exposure time, numerical aperture, width of light sheet, origin of laser (left and/or right), stack step size.

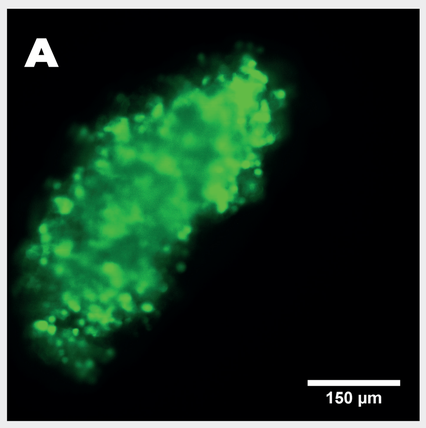
«400 µm» spheroids are more convenient to handle

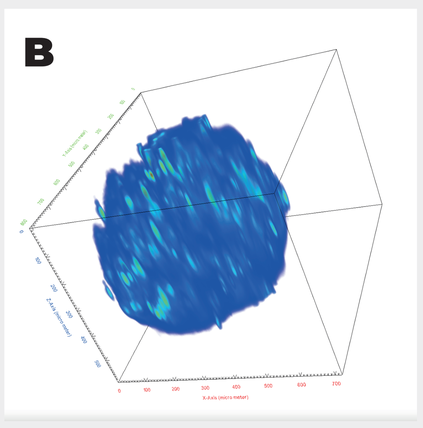
Parameters optimized:
Best computer profile, optimal format for ReViMS and segmentation parameters

Results

Protocol optimization: LSFM imaging before and after clarification

Observations at 488 nm (YFP signal) with a 12.6x magnification, at a Z-step of 2 µm, with LSFM.

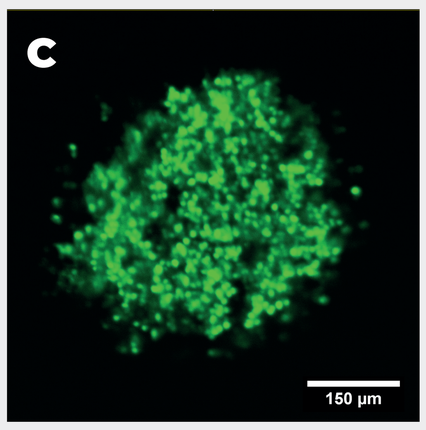


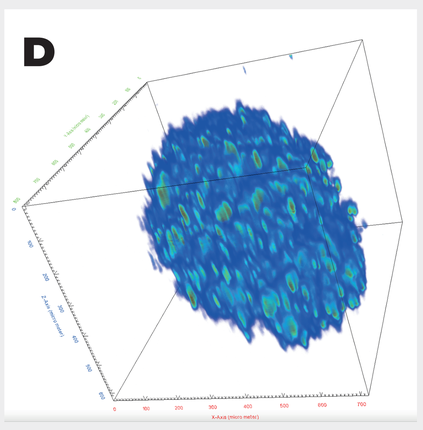


Before clarification step
LSFM numerical aperture was set at 0.035 and light sheet width at 20 µm.

(**A**) «300 µm» spheroid Z-projection (max intensity) before optical clarification obtained with ImageJ.

(**B**) «300 µm» spheroid before optical clarification, reconstructed in 3D using ICY.





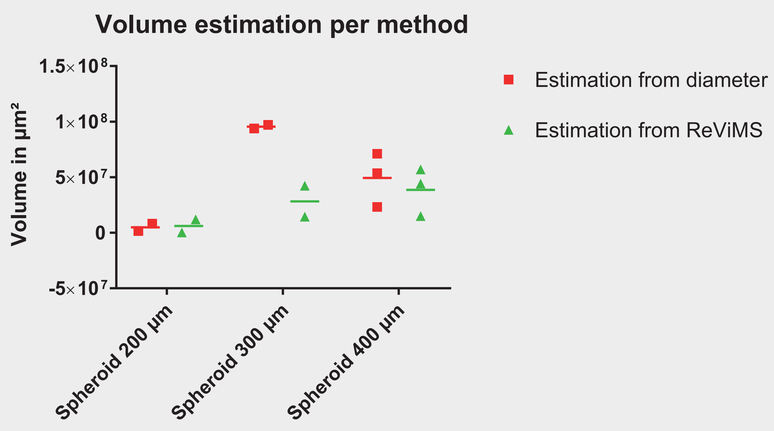
After clarification step
LSFM numerical aperture was set at 0.156 and light sheet width at 20 µm.

(**C**) «300 µm» spheroid Z-projection (max intensity) after optical clarification obtained with ImageJ

(**D**) «300 µm» spheroid after optical clarification, reconstructed in 3D using ICY.

Images show that blurring occur on non-clarified «300 µm» spheroids, depending on the size of the sample. Similar blurring was observed on other spheroids.
Images of spheroids after optical clarification were systematically clearer and more detailed.

Comparison of volume estimation techniques



Clarified spheroids were imaged by LSFM and volume was estimated using either the diameter of the spheroid or by quantification technique of the volumes using 3-D multicellular aggregates Reconstruction and Visualization from Multiple Sections (ReViMS)[5].

Comparison between diameter-derived volume and ReViMS[5] estimations might suggest that volume calculation using diameter are biased, potentially due to the relative non-sphericity of the observed cell aggregates.

Discussion

Several steps of the protocol were optimized, including the optimum size for spheroids (diameter of 400 µm), optical clarification step as well as acquisition on the LSFM and post-acquisition processing of image using ImageJ followed by ReViMS[5]. Using the samples we imaged, we compared commonly used classical volume formula using radius and volume estimation through stack-by-stack 3D modelling (ReViMS). There were important differences between the two estimations, which could be explained by the un-even shape of observed spheroids that could bias diameter-based volume calculations.

Perspectives

During the following Transversal Projects semester, we shall advance towards an oncology-model drug assay. We will observe the effect on spheroids volumes (using the optimized protocol developped) of a well-studied cancer therapy, Paclitaxel (assay positive control) ; and observe impact on viability using Propidium Iodide.

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References:
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[4]Klingberg, A., Hasenberg, A., Ludwig-Portugall, I., Medyukhina, A., Männ, L., Brenzel, A., … Gunzer, M. (2017). Fully Automated Evaluation of Total Glomerular Number and Capillary Tuft Size in Nephritic Kidneys Using Lightsheet Microscopy. Journal of the American Society of Nephrology. https://doi.org/10.1681/ASN.2016020232
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