Optimisation of fluorescent staining protocols for the examination of myocardial restructuring in rabbit model of MI

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Light-sheet microscopy of cleared tissues

- Light scattering limits axial depth limits in tissue imaging.
- optical clearing produces transparent tissues via matching of refractive indices (RIs).
- light-sheet microscopy (LSM) permits isotropic resolution, large field of views, and high data throughput [1].
- protocols for the preparation, clearing, staining, and mounting of cardiac tissue slices difficult to repeat due to their delicate nature
- results that prove reliable and accurate require highly optimized protocols to mitigate tissue changes in vitro.

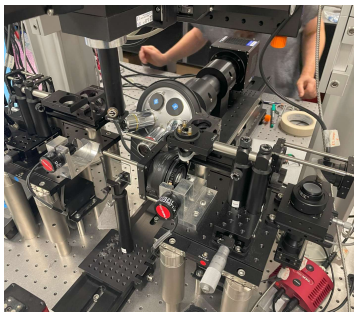
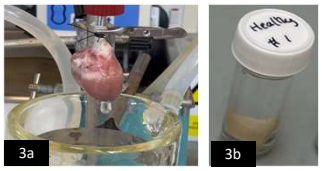


Figure 1: Benchtop mesoSPIM LSM in the Advanced Research Centre at the University of Glasgow

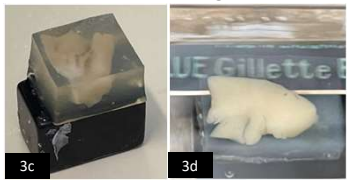
Cardiac tissue fixation and slicing

Tissue Extraction and Fixation



- Langendorff perfused rabbit heart (Fig 3a)
- tissue fixation done with Paraformaldehyde (PFA)
- stored in Phosphate Buffered Saline (PBS) with Sodium Azide to preserve (Fig 3b) [2]

Tissue Slicing



- fixated tissue embedded in agar block
- tissue block glued onto Vibratome platform (Fig 3c)
- slicing at 400µm thickness using ceramic blade (Fig 3d)
- agar removed; slices returned to PBS

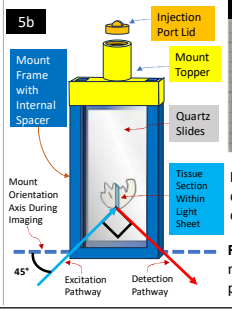
Staining, mounting of cleared samples

Staining

- four staining combinations :
  - WGA – AF488, SYTO Deep Red
  - WGA – Fluorescein, SYTO Deep Red
  - WGA – AF647, SYTOX Green
  - SYTO Deep Red (2x Concentration)
- washed in 1x PBS/Triton-X100, PBS/3% PFA solutions (fig. 5a) [2]
- concealed from light after staining to prevent photobleaching

Mounting

- between two quartz slides using custom 3D printed mount and epoxy
- tissue held in place with mount's 400um wide internal spacer
- immersed in Refractive Index matching solution (RI = 1.45) for 24 hours
- mounted at 45° from excitation/detection paths (Fig 5b)



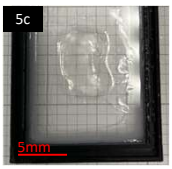


Fig 5c (above): Cleared cardiac tissue sample in custom mount

Fig 5b (left): 3D model of mount and optical pathways oriented at 45°

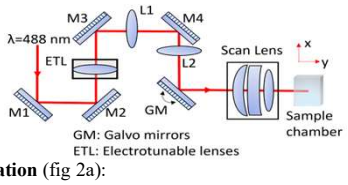
Future work

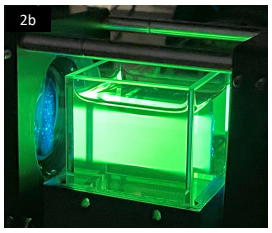
- Implement optimized staining on samples from diseased hearts to perform quantitative structural analysis, assess structural changes relative to healthy hearts
- Examine staining optimization results from alternative stain concentrations and clearing protocol CUBIC-L/RA, assessing image quality relative to CLARITY

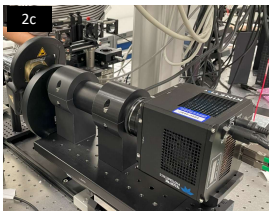
References

[1] Dean, Kevin M et al. *Biophysical journal* **108.12** (2015): 2807-15.
[2] Olianti, Camilla, et al. *Progress in Biophysics and Molecular Biology* **168** (2022): 10-17.
[3] Tomer *et al*, *Nature protocols* **9.7**, (2014): 1682-1697.

Optical pathways of mesoSPIM







Excitation (fig 2a):

- focus of the Gaussian beam shifted across sample using ETLs (Axially Swept Light-sheet Microscopy mode) [1].
- Galvo Mirrors shift beam vertically to form light sheet across imaging plane (fig 2b)

Detection (fig 2c):

- ETL sweep synchronised with Teledyne Photometrics Kinetix sCMOS camera's (diagonal = 29.4 mm) rolling shutter.
- detection pathway from the light sheet through Mitutoyo objectives (2x, 5x, 10x) in a turret, filter wheel, and tube lens to the sCMOS camera

Tissue clearing protocol

CLARITY Protocol [2,3]
(Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging Tissue hYdrogel)

1. wash in PBS to remove remaining non-tissue molecules
2. wash in Hydrogel solution (PFA, hydrogel monomers), ensuring homogeneity.
3. degas in N_2, polymerizes monomers into hydrogel mesh.
4. wash in clearing solution (detergent, weak acid) to expel lipids.
5. lipid saturated clearing solution changed out every 2-3 days (for up to 6-7 months)

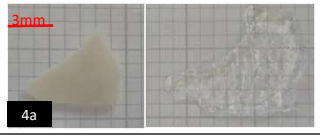


Fig 4a: Cardiac Tissue Sample Before and After CLARITY Tissue Clearing Protocol (≈20 weeks)

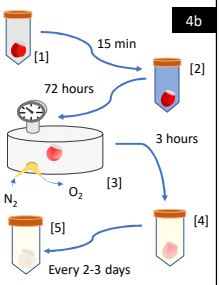


Fig 4b: CLARITY Protocol Steps and Timeline. [1,3]

Comparison of cleared, stained tissue slice images

- image stacks of dual stained tissue samples across 1200µm
- mean 16-bit pixel value across z-stack raw signal used to quantify signal intensity
- data determined WGA conjugated stains have higher intensity
- SYTO/SYTOX stains lower, but unaffected by other stains

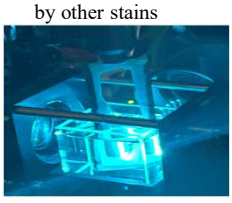
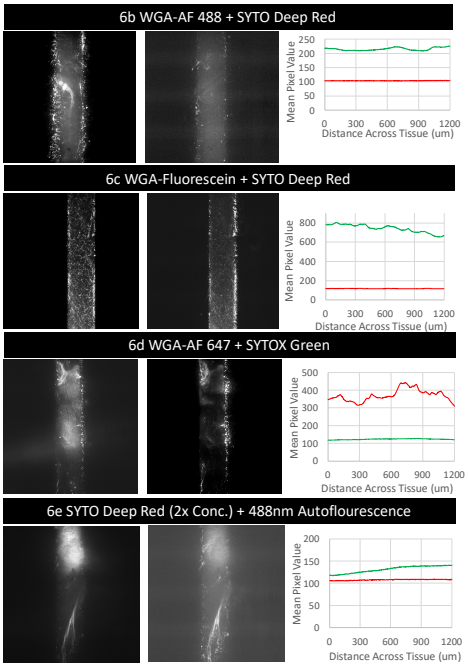


Fig 6a (above): Mounted sample imaged using the LSM at 45°

Fig 6b-e (right): Contrast Adjusted Z-plane images at 5x using 488nm (left) and 647nm (right) laser. Green Line = 488nm; Red Line = 647nm



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