Three-dimensional (3D) Imaging of Biomarkers in Human Core Needle Biopsies of Cancerous Breast Tissue

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Abstract

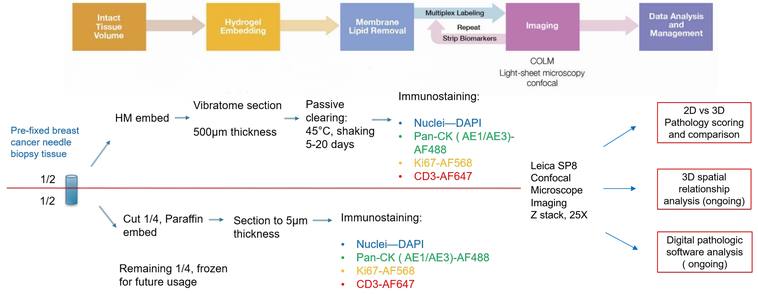
Background: The tumor microenvironment is spatially and compositionally very heterogenous, which introduces great challenges to characterize the underlying factors using standard 2D diagnostic methodologies. Capturing high resolution 3D quantitative biomarker data, while simultaneously preserving morphology of the tumor microenvironment, could lead to a better understanding of key spatial relationships and may lead to better prognostic and predictive clinical outcomes. In this study, we utilized a novel technique, CLARITY, to transform core needle biopsies from patients with breast cancer, into optically transparent tissues, followed by multiplex immunostaining and 3D imaging of molecular markers. This data was compared to the conventional methods of immunofluorescence staining on FFPE thin sections.

Methods: Formalin-fixed (less than 24 hours) human breast cancer core needle biopsy tissue pairs (tumor and adjacent normal) were obtained from patients undergoing excisional surgery. Tissues were subsequently embedded in 4% paraformaldehyde containing a 4%/0.05% ratio of acrylamide/BIS for 48 hours, and polymerized to form an intact hydrogel/tissue matrix. The samples were sectioned to a thickness of 500 µm and lipid-cleared in a solution of 0.2M borate buffer containing 8% SDS, pH 8.5 at 45˚ C. The tissues were then immunostained for various cellular markers (Pan-cytokeratin, Ki67 and CD3) and counterstained with DAPI. Samples were refractive index matched prior to 3D imaging on a Leica SP8 laser scanning confocal microscope or a Lavision BioTec Ultramicroscope II, light sheet microscope.

Results: During the process, the samples remained intact and the cellular morphology was well preserved. The average passive lipid-clearing time for breast cancer core needle biopsy tissue was 5-20 days depending on the size of the tumor. The majority of the samples reached visual optical transparency, with the exception of some regions that contained heavy fibrotic tissue. Preliminary results demonstrated that specific staining of various cellular and nuclear markers was successful as evidenced by 3D imaging. As compared to the images obtained from 2D thin sections, the CLARITY procedure followed by 3D imaging yielded significant imaging depth, with the potential to greatly enhance the understanding of the heterogeneity of the tumor microenvironment.

Conclusion: This is the first study demonstrating that other than fresh or frozen tissues, pre-fixed clinical tissue from patients with breast cancer, can be successfully processed by the CLARITY method and 3D imaged, indicating that the potential power of the technique for core needle biopsy tissue processing and in the identification of biomarkers based on tumor cell heterogeneity.

Method



AF: Alexa Fluor

1. Tissue Lipid-Clearing by CLARITY Technique

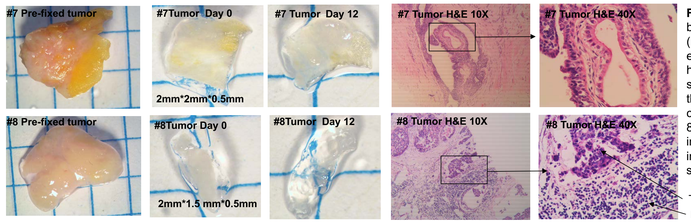


Fig 1 Breast cancer core biopsy tissues #7 and #8 (left two images) were embedded in the hydrogel matrix and sectioned to 500 µm thickness. Tissues were cleared by 8% SDS, pH 8.5 at 45˚ C (middle four images). The four right images are H&E stained samples (10X, 40X).

2. 3D and Optical Slices of Immunostained Tissue

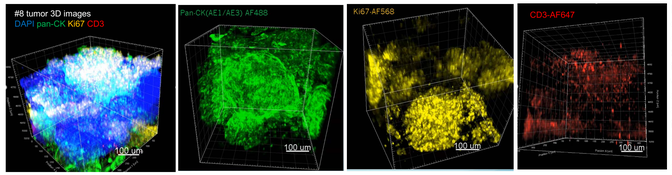
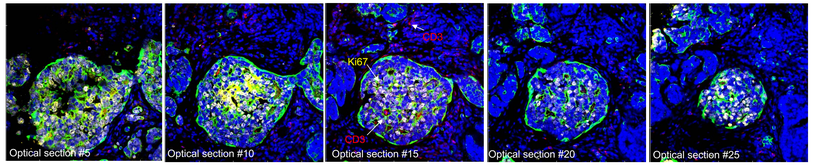


Fig 2 3D images (top) and optical section images (bottom) of #8 breast tumor tissue (2mm*1.5mm*0.5mm). Breast tissue cleared as shown in Fig 1 were immunostained with antibodies against pan-CK, Ki67 and CD3 (optimized doses) followed by a DAPI counterstain and imaged with a Leica SP8 confocal microscope (25X, Z stack). Images were visualized by Imaris Version 9.0.



3. Ki67 Antibody Titration in Tonsil Tissue

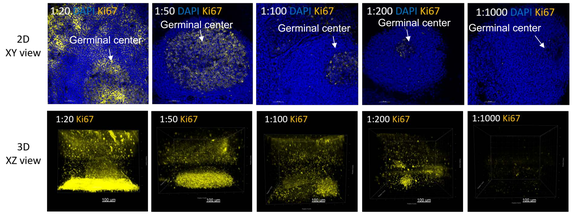
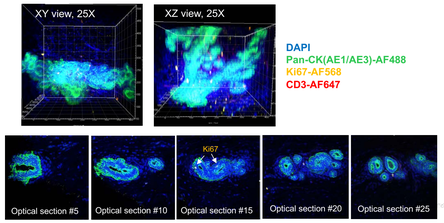
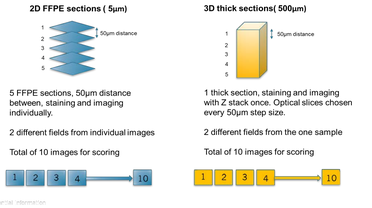


Fig 3 Tonsil tissues (3mm*3mm*0.5mm) were immunostained with varying dilutions of a primary antibody against Ki-67, followed by an Alexa Fluor 568 labeled secondary antibody. Samples were imaged using a Leica SP8 confocal microscope (Z stack, step size 3µm). **Top**: 2D XY view images of nuclear Ki67 expression around germinal center. **Bottom**: 3D XZ view of Ki67 staining depth. The staining pattern of dilutions <1:200 becomes weak and sparse. And the dilution >1:50 has significant increased noise. These results indicate that dilutions of 1:50-1:200 are optimal antibody concentration to use.

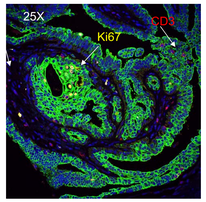
4. Comparison of Ki67 Expression in 3D and 2D images by Conventional Pathology Scoring

#7 Tumor HM embedded 3D sections





#7 Tumor FFPE 2D sections



#7 and #8 Tumor Ki67 positive epithelium

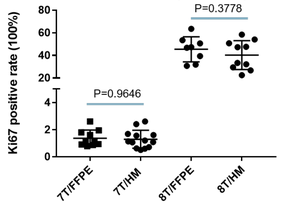


Fig 4 Comparison of 2D images from FFPE sections (5µm) (left, bottom) and 3D images from HM embedded thick sections (500µm) left, top) . Ten images from both thin and thick sections were taken for Ki67 scoring as an initial comparison (top, right). Blind scoring was done by independent pathology professionals and results were analyzed by Graphpad Prism 7.0 (right, bottom). Based on this initial data, equivalent tissue sections from the thicker CLARITY processed, immunostained tumor tissue is concordant with a mirrored FFPE 2D tissue.

Conclusions and Future Directions

1. Pre-fixed clinical tissue from patients with breast cancer can be successfully processed by the CLARITY method and 3D imaged.
2. Pathology scoring of 3D images from HM embedded cleared tissues showed concordant results compared to 2D images from FFPE samples surveyed throughout the same sized block indicating CLARITY is a powerful tissue processing technique that is compatible with clinical sample immunostaining.
3. Further image analysis will be aimed at quantifying target expression levels as well as key spatial relationships (infiltrating T cells) using 3D digital quantitative analysis tools.

Acknowledgement and References

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