#5915 Three-Dimensional (3D) Multiplex Imaging of Biomarkers In Tumor Tissues

Sharla L. White, Sarah McCurdy, Laurie J. Goodman
ClearLight Diagnostics, LLC, 428 Oakmead Parkway, Sunnyvale, CA 94085

ABSTRACT

Background: Obtaining high-resolution information from solid tumors, while maintaining the spatial perspective needed to understand the heterogeneous tumor microenvironment, represents a key challenge for preclinical and clinical cancer applications. **Methods:** We utilized a novel method CLARITY that has been shown to transform intact rodent tissues into an optically transparent and permeable hybridized-hydrogel form that can undergo immunostaining followed by 3-D imaging of nucleic acids and proteins markers, without the need to separately analyze hundreds of conventional FFPE thin sections. In this study, human breast and lung cancer excision biopsy samples were obtained commercially. Murine orthotopic MCF-7 xenograft and patient-derived-xenograft (PDX) breast tumors were explanted and prepared as fresh samples. The intact tissues were incubated in a mixture of 4% paraformaldehyde/4% acrylamide/0.05% bis-acrylamide for 48 hours, and polymerized in the presence of a thermal initiator, VA-044, for 3 hours at 37°C to form an intact hydrogel/tissue matrix. The tissues were then lipid-cleared in a solution of 0.2M borate buffer containing 8% SDS, pH 8.5 at 45-55°C from 5-35 days, depending on sample size. The cleared tissues were then stained with two multiplex antibody panels that were selected to highlight the microenvironment of the tissue including, immune cells (CD-3 or CD-8), vasculature (Lectin-Texas Red or CD-31), and tumor (cytokeratin [8+18] or PD-L1, directly conjugated to Alexa Fluor® 647). For non-directly conjugated antibodies, the tissues were then subjected to a secondary goat anti-mouse or goat anti-rabbit antibody conjugated to either Alexa Fluor® 568 or 700 followed by a nuclear counterstain, Sytox Blue. Samples were then placed in an appropriate refractive index solution for imaging on a laser scanning confocal microscope. **Results:** The tissues remained intact throughout the procedure and the cellular morphology of the tissue was well preserved. The individual components of the microenvironment could be identified, demonstrating that the tissues could be successfully interrogated with a multiplex of indirect and directly conjugated commercially available antibodies. These preliminary studies, provide evidence for the use of this novel technology for detecting the heterogeneity of biomarker expression within the tumor microenvironment. Most notably, the analysis of a lymph node from a patient with metastatic breast cancer revealed variation across the tissue which may have implications for the detection of micro-metastasis in patients with early stage disease when using conventional thin section histological methods. **Conclusion:** These results implicate CLARITY as a powerful next generation tissue processing technology for profiling the intact tumor microenvironment, eliminating the need to recapitulate this spatial and quantitative information with standard thin section techniques.

1. CLARITY Process and Applications

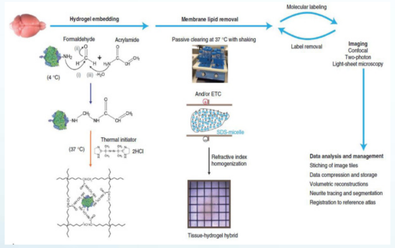

Figure 1a: The CLARITY process involves the fixation/embedding of tissues as indicated in diagram above, which creates a hydrogel tissue matrix. The hybrid tissue matrix can then undergo lipid removal through either passive or active (electrophoretic) clearing, rendering a visually transparent tissue. The tissue can then be immunostained and 3D imaged using confocal, 2-photon, or light-sheet microscopy.(Tomer, Ye, Hsueh, & Deisseroth, 2014)

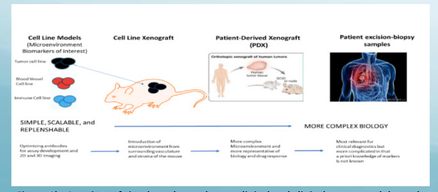

Figure 1b: A variety of simple and complex preclinical and clinical tumor models can be utilized for 3D multiplex imaging. Simple 3D cell line pellets can be generated to verify specific biomarkers of interest and establish antibody feasibility. The working antibodies can then be employed to evaluate murine and patient-derived xenograft tumors, as well as human cancer tissues.

2. Orthotopic MCF7 Cell Line Xenograft

Figure 2: An orthotopic murine MCF7 xenograft tumor that has undergone hydrogel embedding prior to passive clearing (a) is predominantly opaque; however, following passive clearing (b) the removal of lipids, the opacity is significantly reduced allowing the tumor to be immunostained deeply.

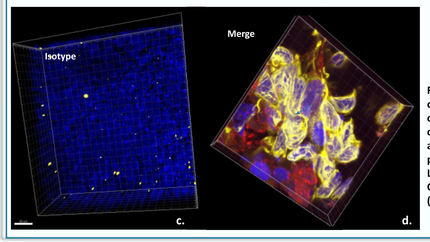

Figure 2: Specific staining can be seen when compared to the isotype control. Isotype panel (c) and merged multiplex panel [Sytox Blue (blue), Lectin (red), Cytokeratin[8+18]] (yellow) (d).

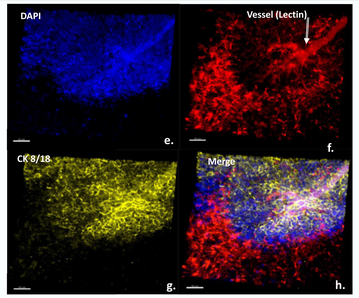

Figure 2: Confocal imaging of MCF7 xenograft tumor (20x objective). A vessel within the tumor is clearly stained with lectin (see arrow); however, while lectin is robust for staining vasculature, it also binds non-cellular sugar-based structures. DAPI (blue, e). Lectin (red, f). Cytokeratin[8+18] (yellow, g). Merged image (h).

3. Patient Derived Xenograft (PDX) Mouse Breast Cancer Model

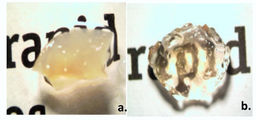

Figure 3: Jackson Laboratories PDX mouse breast cancer model (invasive ductal carcinoma, poorly differentiated grade 3, ER$^-$/PR$^-$/HER2$^+$. HM-embedded tumor, ~10x5x2 mm precleared (a). HM-embedded tumor after passive clearing (b).

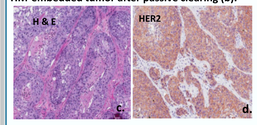

Figure 3: Jackson Laboratories PDX mouse breast cancer model (invasive ductal carcinoma, poorly differentiated grade 3, ER$^-$/PR$^-$/HER2. FFPE staining of mouse P1 tumor provided by Jackson Laboratories: H&E (c) and HER2 (d) staining.

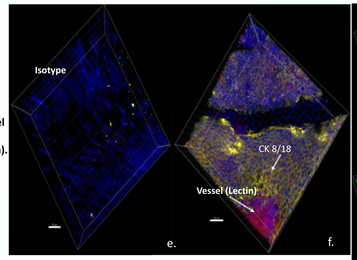

Figure 3: Confocal imaging illustrating general tumor architecture of PDX xenograft tumor (20x objective). Isotype multiplex panel (e) and multiplex panel (f). DAPI (blue), Lectin (red), Cytokeratin[8+18] (yellow).

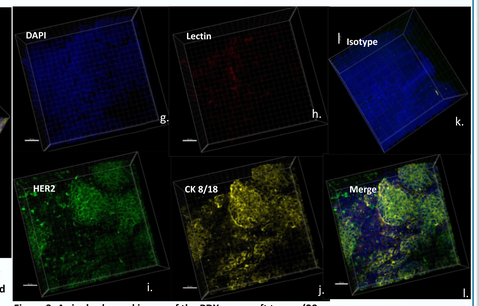

Figure 3: A single channel image of the PDX xenograft tumor (20x objective). DAPI (blue, g), Lectin (red, h), HER2 (green, i), Cytokeratin[8+18] (yellow, j) Merge images in k and l.

Figure 3: PDX xenograft tumor isotype (k) and merged multiplex panel (l).

4. Frozen Human Breast Cancer Tumor

Figure 4: Fixation/embedding of a frozen excision biopsy sample from a patient with a comedocarcinoma, non-infiltrating, T2N0M0, ER/PR negative /HER2 positive (~11x10x7 mm) (a); Hydrogel-embedded tumor (b); and passively cleared tumor (c).

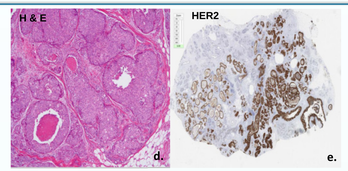

Figure 4: 2D thin section of an FFPE mirrored sample from the same patient. H&E staining 20x (d) and HER2 IHC (e).

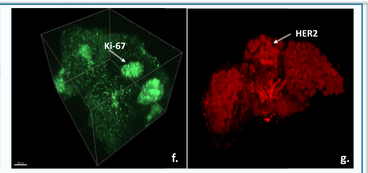

Figure 4: 3D imaging using Lightsheet microscopy: Ki67 staining (f) and HER2 staining (g). 4x magnification.

5. Frozen Human Metastatic Breast Cancer Associated Lymph Node

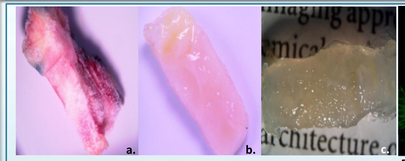

Figure 5: Frozen human metastatic breast cancer associated lymph node (a) HM-embedded lymph node (b) and passively cleared (c).

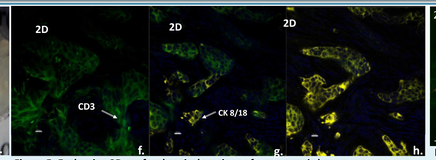

Figure 5: Evaluating 2D confocal optical sections of a metastatic breast cancer associated lymph node, separated by 5 μm, demonstrates the transition from a predominant CD3 (f) to mixed CD3/Cytokeratin[8+18] expression (g) to predominantly Cytokeratin[8+18] expression (h).

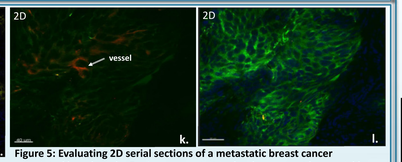

Figure 5: Evaluating 2D serial sections of a metastatic breast cancer associated lymph node, 5 microns apart, it is clearly apparent how quickly a transition from a CD31 (k) to a predominant HER2 (l) microenvironment can occur.

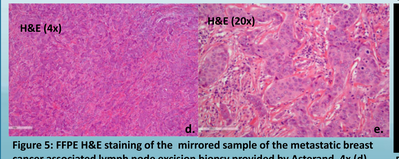

Figure 5: FFPE H&E staining of the mirrored sample of the metastatic breast cancer associated lymph node excision biopsy provided by Asterand. 4x (d) and 20x magnification (e).

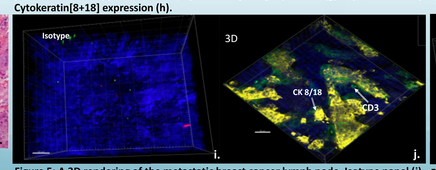

Figure 5: A 3D rendering of the metastatic breast cancer lymph node. Isotype panel (i) and merged multiplex panel [CD3 (green), CD31 (red), Cytokeratin[8+18] (yellow)] (j).

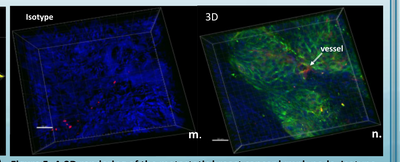

Figure 5: A 3D rendering of the metastatic breast cancer lymph node. Isotype panel (m) and multiplex panel [HER2 (green), CD31 (red), Cytokeratin[8+18] (yellow)] (n).

6. Frozen Human NSCLC Tumor

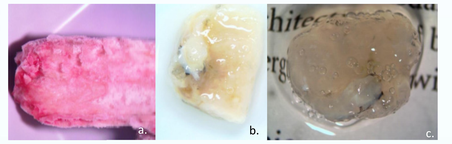

Figure 6. Frozen human lung adenocarcinoma, Stage 1B EGFR$^-$/KRAS$^-$ excision biopsy tumor (a), HM-embedded (b), and passively cleared (c).

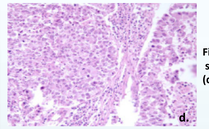

Figure 6. 2D thin section of an FFPE mirrored sample from the same patient. H&E staining 20x (d)

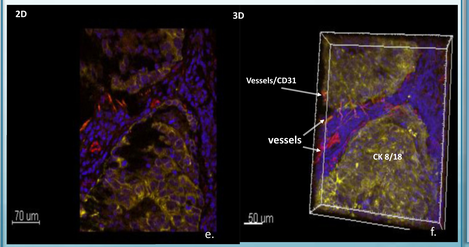

Figure 6: A 2D (e) versus 3D (f) multiplex panel of a lung adenocarcinoma sample(20x objective) with reduced cytokeratin expression when only looking at specific cytokeratin types. Sytox Blue (blue), CD31 (red), Cytokeratin[8+18] (yellow).

SUMMARY

- We demonstrated feasibility for using the CLARITY and 3D imaging methods in a variety of preclinical mouse models as well as frozen human tumor tissue to identify major components of the tumor microenvironment.
- The embedded tissues remained intact throughout the procedure with good preservation of cellular morphology allowing the identification of individual components of the microenvironment in multiplex using a variety of indirectly or directly conjugated commercially available antibodies.
- Early proof of concept studies provide evidence for the use of this novel technology to capture the heterogeneity of biomarker expression within the tumor microenvironment in an intact tissue.
- These results implicate CLARITY as a powerful next generation tissue processing technology for profiling the intact tumor microenvironment, eliminating the need to recapitulate the spatial and quantitative information using conventional 2D thin section techniques.
- Future studies are aimed at cross-validating and understanding the sensitivity/specificity/dynamic range and reproducibility of this method, as well as automating the front-end tissue preparation process and 3D imaging and image analysis.
- Through preservation of the 3D structure and quantitatively measuring key components of the tumor microenvironment, it is proposed that this technique will be a better method for determining prognosis, prediction of response and resistance to therapeutics.

CLEARLIGHT DIAGNOSTICS