**3D molecular pathology with open-top light-sheet microscopy**


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**Background:** Glass slide-based pathology has been the gold standard for prostate cancer diagnosis for many decades. However, this process is resource-intensive, decreases nucleic acid yield for molecular assays, and diagnostically useful 3D information is lost. We have designed a protocol which produces a 3D digital microscopic image of entire prostate biopsy cores while preserving nucleic acids for downstream testing. We expect that this protocol will improve prostate cancer diagnostic precision, enhance molecular diagnostics, and lead to new biologic insights with 3D structural information.

**Methods:** Ex-vivo core needle biopsies taken from radical prostatectomies were dehydrated, chemically clarified to render the tissue transparent, and stained with nuclear (TO-PRO3) and cytoplasmic (eosin) fluorescent dyes. 3D immunofluorescence (IF) staining for CK5 and CK8 was performed using a week-long incubation protocol. Biopsies were imaged using a custom-built open-top light-sheet microscope (OTLS). The images were reconstructed and false-colored to simulate H&E-staining. Custom Python scripts and BigStitcher were used for image processing, and Imaris software for 3D visualization.

**Results:** Biopsies were entirely imaged in 3D after clarification (~6-12 hours). Pseudo-H&E staining showed the benefits of 3D microscopy, including avoidance of overgrading ambiguous regions (well-formed glands vs. poorly-formed glands) and the identification of novel 3D vascular patterns, i.e. a spiral arrangement of smooth muscle cells in arterioles. 3D IF for CK5 and CK8 confirmed the presence of benign and carcinoma regions identified using pseudo-H&E. We optimized our clearing protocol to preserve RNA by using DMSO, NaCl, pH=7, and ethyl cinnamate. The RNA integrity number, used to assess the quality of the RNA, was similar to that of samples immersed in RNAlater (7.9-8.1 with our clearing protocol vs 8.3 with RNAlater).

**Conclusion:** Examination of prostate cancer 3D histoarchitecture yields more precise grading than conventional grading of 2D sections. Most importantly, tangentially sectioned Gleason pattern 3 glands can be distinguished from the poorly formed gland variant of Gleason pattern 4. This finding has major implications for patient care, particularly the decision to remain in active surveillance versus being offered intent-to-cure therapy. In addition, our protocol preserves RNA for potential downstream molecular assays to further improve risk stratification. Further work to characterize novel 3D cancer morphology, including machine learning with 3D IF and integration of RNA and DNA sequencing is in progress.

**Conflicts of interest:** The authors (NPR, AKG, JTCL, and LDT) have a patent application for the OTLS and have a start-up company (LightSpeed Microscopy, Inc).

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