Multiplexed analysis of fixed tissue RNA using Ligation *in situ* Hybridization

Joel J. Credle[1], Christopher Y. Itoh[1], Tiezheng Yuan[1], Erick R. Scott[2], Rachael E. Workman[3], Yunfan Fan[3], Franck Housseau[4], Nicolas Llosa[4], W. Robert Bell[5], Heather Miller[5], Sean X. Zhang[5,6], Winston Timp[3] & H. Benjamin Larman[11]

[1]Division of Immunology, Department of Pathology, Johns Hopkins School of Medicine, Baltimore, MD 21205, USA.
[2]Department of Genetics & Genomic Sciences, Icahn Institute for Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, New York, NY 10078, USA.
[3]Department of Biomedical Engineering, Johns Hopkins University, Baltimore, MD 21218, USA.
[4]Sidney Kimmel Comprehensive Cancer Center, Department of Oncology, and the Bloomberg-Kimmel Institute for Cancer Immunotherapy, Johns Hopkins University School of Medicine, Baltimore, MD, 21205, USA.
[5]Division of Medical Microbiology, Department of Pathology, Johns Hopkins School of Medicine;
[6]Microbiology Laboratory, Johns Hopkins Hospital, Johns Hopkins Medical Institutes, Baltimore, MD 21287,

Background: Clinical tissues are prepared for histological analysis and long-term storage via formalin fixation and paraffin embedding (FFPE). The FFPE process results in fragmentation and chemical modification of RNA, rendering it less suitable for analysis by techniques that rely on reverse transcription (RT) such as RT-qPCR and RNA-Seq.

Results: Here we describe a broadly applicable technique called "Ligation *in situ* Hybridization" ("LISH"), which is an alternative methodology for the analysis of FFPE RNA. LISH utilizes the T4 RNA Ligase 2 to efficiently join adjacent chimeric RNA-DNA probe pairs hybridized *in situ* on fixed RNA target sequences. Subsequent treatment with RNase H releases RNA-templated ligation products into solution for downstream analysis. We demonstrate several unique advantages of LISH-based assays using patient-derived FFPE tissue. These include >100-plex capability, compatibility with common histochemical stains, and suitability for analysis of decade-old materials and exceedingly small microdissected tissue fragments. High throughput DNA sequencing modalities, including single molecule sequencing, can be used to analyze ligation products from complex panels of LISH probes ("LISH-seq"), which can be amplified efficiently and with negligible bias.

Conclusions: LISH analysis of FFPE RNA is a novel methodology with broad applications that range from multiplexed gene expression analysis to the sensitive detection of infectious organisms.

Conflict of Interest: HBL and JJC are listed as co-inventors on a patent application currently pending review at the USPTO.

Funding Acknowledgements: Acknowledgments: We would like to thank Chad Locklear at Integrated DNA Technologies, Inc. for helpful discussions and synthesis of all oligonucleotides used for this study. We are also grateful to Marc Halushka for his assistance with this project. Funding: This work was made possible by support from the Johns Hopkins Medicine Discovery Fund, the Jerome L. Greene Foundation, an NCI IMAT Program R21 award (CA202875), an NIAID U24 award (AI118633), and a Prostate Cancer Foundation Young Investigator award. HBL is a recipient of the Catherine and Constantinos J. Limas Research Award.