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TITLE: Next-Generation Molecular Histology Using Highly Multiplexed Ion Beam Imaging (MIBI) of Breast Cancer Tissue Specimens for Enhanced Clinical Guidance

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have completed the goals of the project timeline, in large part, in this second period of the grant funding. Specifically, we have developed the reagents, cell lines and tissues needed for the assessment of the dynamic range of protein quantitation . We have analyzed the lines with roughly 2/3rds of the planned assays, with the remaining l/3rd in progress . We have optimized multiplexed IF for comparison to MIBI secondary ion mass spectrometry. And we have evaluated mRNA ISH probes for use in both IF and MIBI detection . The Stanford group in related but not funded by this proposal work has developed a second generation SIMS instrument which will enable higher throughput, and facilitate the further aims of the proposal in the coming grant periods.

15. SUBJECT TERMS

Nothing listed

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INTRODUCTION:

Current breast cancer diagnosis includes predictive assays to guide therapy decisions, involving a minimum of 3assays: ER, PR, and HER2. Many labs also include a marker of proliferation (Ki67), and sometimes myoepithelial (SMA), epithelial (CK8/18), and lobular markers (ECAD). Recently, a host of new multi-marker panels developed. The "Mammostrat" assay (Clarient) uses a panel of five IHC markers (P53, SLC7A5,NRDG1, HTF9C, CEACAM5). Gene-expression assays using qRT-PCR, array hybridization, and RNA sequence assays have also been developed. The OncotypeDX, for example, uses a panel of 21 genes (16 analytical, 5 controls: Ki67, STK15, Survivin, CCNB1, MYBL2, MMP11, CTSL2, HER2, GRB7, GSTM1,CD68, BAG1, ER, PGR, BCL2, SCUBE2, ACTB, GAPDH, RPLPO, GUS, TFRC) to stratify risk of recurrence, and relative benefit of adjuvant chemotherapy. This explosion in biomarkers poses both cost and logical selection challenges. In addition, these assays generally lose all spatial context information (including heterogeneity). MIBI technology provides the potential to simultaneously assay all of the relevant analytes in an intact tissue architecture, with

submicron resolution and a greatly expanded dynamic range of quantitation. We propose to develop assays and analysis tools to evaluate breast cancer tissues using formal fixed and paraffin embedded tumor tissues from the clinic, and we will compare the utility of the MIBI platform assays to the current assays. Our *objective* is to validate MIBI as an alternative to current standard multi-gene assays. We also *hypothesize* that MIBI breast cancer data will improve the ability to stratify risk and predict therapy responses by taking into account the distribution and heterogeneity of molecularly defined cell populations in breast cancer.

KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*
Breast Cancer Diagnosis
Pathology
Immunophenotype
Multiplex
Morphology
RNA In Situ Hybridization
Immunohistochemistry/immunofluorescence
Predictive Biomarkers
Quantitative Image Analysis

Body/ Key Research Accomplishments/ Reportable Outcomes:

A. What was accomplished under these goals?

Notes: *For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

SEE ATTACHED.

B. What opportunities for training and professional development has the project provided? *Nothing to Report.*

C. How were the results disseminated to communities of interest? *Data was reported at an NCI sponsored meeting for Innovative Molecular Analysis Technologies in December 2016.*

D. What do you plan to do during the next reporting period to accomplish the goals?

Statement of Work Progress Update:
Elements of each of the specific aims require work performed at both UC Davis and Stanford. Briefly, the division of labor falls into the following breakdown: All tissue procurement, tissue and cell culture handling, tissue sectioning and mounting, probe labeling, tissue probe incubations and standard curve measurements (western and qRT-PCR) will be performed in the Borowsky lab at UC Davis. All nanoSIMS imaging, initial image analysis, image segmentation and data output will be performed in the Nolan lab at Stanford. Subsequent analysis and risk stratification algorithms will be done in collaboration of all groups with the informatics team lead by Dr. Levenson at UC Davis. The following is a breakdown of specific aims into individual tasks over the three years of the grant period.
Specific Aims: In order to achieve the objectives we will develop two new multi-gene panels of MIBI multiplexed *in situ* detection reagents, and compare the quantitative data to the conventional clinically derived "one at a time" and/or "grind-it-up" assays. Meanwhile, our data analysis will provide complex cell population distributions, which will be compared to clinical outcomes. We anticipate that new discoveries of specific cell populations associated with specific outcomes or tumor biologies will require larger retrospective, and eventually prospective trials, but this proposal will enable such studies to proceed rapidly and efficiently.

Aim I: One slide complete IHC analysis: Develop the multiplex panel of the following 13 mass tagged primary antibodies for simultaneous diagnosis, categorical predictive assessment and calculation of current algorithms for risk prediction: ER, PgR, HER2, Ki67, BAG1, SMA, CK8/18, ECAD, P53, SLC7A5, NRDG1,HTF9C, CEACAM5.

Ia. Complete the currently developed 10 antibody panel (see preliminary data and pending publication *revisions submitted*, Nature Medicine) with additional antibodies to complete the 13 antibody panel.

Tasks: Ia.1 (Davis) Choose, order and test by conventional DAB/secondary antibody detection the new antibodies to complete the panel. For each, control tissue sections and breast cancers (deidentified) using conventional formalin fixed and paraffin embedded tissue blocks will be used.

Completed.

Ia.2 (Davis) Optimize titers using conventional immunohistochemistry.

Completed for 10 of 13 proposed antibodies. 3 additional in progress.

Ia.3 (Davis) Prepare mass tagged primary antibodies.

Year 2 planned.

Ia.4 (Davis) Prepare tissue samples with mass tagged antibodies: single label, double label and complete panel (13 label).

Year 2-3.

Ia.5 (Stanford) nanoSIMS MIBI imaging of single, double and panel labeled samples.

Pilot work completed, test samples Year 2.

Ia.6 (Stanford) Initial image analysis of MIBI images for display of categorical and quantitative signals.

Pilot work completed, test samples begin Year 2.

Ia.7 (Stanford) Image cell segmentation and cell distributions by 13x immunophenotype and cell morphology.

Follows Aims Ia.6 and 7.

Ib. Measure standard curves for each analyte against western blots using cell lines and tumor samples. Compare quantitation dynamic ranges to conventional IHC.

Ib.1 (Davis) Prepare cell culture samples and define standard clinical samples with matched frozen tissue as controls for each antibody.

Completed.

Ib.2 (Davis) Conduct quantitative western blot analysis for cell/ tissue quantitative protein determination for each antibody.

Completed.

Ib.3 (Davis) Prepare matched samples used in western blots for conventional and MIBI IHC.

Completed.

Ib.4 (Davis) Conduct conventional IHC.

Completed.

Ib.5 (Davis) Use Aperio image analysis tools to quantify signal intensity and distribution of conventional IHC.

Pilot testing completed, reoptimized using IMARIS image analysis. Additional tool sets under evaluation.

Ib.6 (Stanford) MIBI imaging of matched samples.

Year 2.

Ib.7 (Stanford) Use MIBI image analysis tools to quantify signal intensity and distribution of the MIBI IHC.

Pilot analyses completed. Test samples begin Year 2.

Ib.8 (Davis) Prepare standard curves of western quantified analyte concentration v. conventional IHC quantitiation.

Completed for IHC markers, in progress for new IHC markers to replace ISH.

Ib.9 (Davis and Stanford) Prepare standard curves of western quantified analyte concentration v. MIBI IHC.

Curve data available to optimized MIBI IHC when ready.

Ib.10 Reiterate (1-9) with additional samples at high and low concentrations as needed to define the dynamic range limits as needed (find the curve plateaus to determine the full linear detection ranges).
Samples identified, analyses Year 2-3.
Ib. 11 (Davis and Stanford) Report technical applications findings—manuscript.
Year 3. One MS in submission now, describing methods for signal/noise enhancement in multiplex IHC.

Ic. Automate IHC4 + BAG1 score, and "Mammostrat" score using one slide 13 marker quantitative image. Continue development of the analysis software.
Ic.1 (Stanford) Utilize cytokeratin and/or ECAD channels to segment epithelium from stroma.
Completed.
Ic. 2 (Stanford) CellProfiler segmentation using hematoxylin channel (aluminum peak) or addition of dsDNA antibody if needed (Davis prep, Stanford analysis).
Completed.
Ic. 3 (Stanford) Import segmented multiparemeter data into SPADE software package for population analysis.
In progress.
Ic. 4 (Davis) Develop cell position matrices for aim 3 evaluation.
Year 3.
Ic. 5 (Davis and Stanford) Test display utility, and modify for user/pathology interface.
Year 3.
Ic. 6 (Davis) Use standard curve quantified (ER, HER2) and categorical percentages (PR, KI67) to provide input for IHC4 score algorithm.
Measurements have been developed, algorithm testing and comparison to standard methods underway year 3.
Ic. 7 (Davis) Use standard curves and categorical percentages to provide input for Mammostrat score algorithm.
Year 3.
Ic. 8 (Davis) Compare output scores to clinically derived conventional scores.
Year 3.
Ic. 9 (Davis and Stanford) Report utility findings—manuscript.
Year 3.

Aim II: MIBI Oncotype mRNA in situ: Develop the multiplex panel of the following 21 gene mRNA in situ hybridization for quantitative analysis and recalculation of the current algorithms for recurrence risk: (16 analytical, 5 controls: Ki67, STK15, Survivin, CCNB1, MYBL2, MMP11, CTSL2, HER2, GRB7, GSTM1, CD68, BAG1, ER, PGR, BCL2, SCUBE2, ACTB, GAPDH, RPLPO, GUS, TFRC)

IIa. Compare hybridization results for mass tagged probe designs from both collaborating companies (ACD and Biosearch). Develop hybridization conditions for mixing probe types.
IIa. 1 (Davis) Choose and prepare FFPE tissue sections and control FFPE cell line pellet sections for hybridizations.
Completed.
IIa. 2 (Davis) Test pre-optimized conditions (from collaborating company data) using conventional fluorescent label detection.
19 of 21 targets optimized.
IIa. 3 (Davis) Prepare mass tagged ISH probes.
Year 3.
IIa. 4 (Davis) Prepare tissue samples with mass tagged ISH probes: single label, double label and half panel and full panel.

Year 3.
Ia.5 (Stanford) nanoSIMS MIBI imaging of single, double and panel labeled samples.
Year 3.
Ia.6 (Stanford) Initial image analysis of MIBI images for display of quantitative ISH signals.
Year 3.
Ia.7 (Stanford) Image cell segmentation and cell distributions by ISH phenotype and cell morphology.
Year 3.

IIb. Measure quantitative ISH imaging against real-time PCR to develop standard curves across different tissue preparations for each probe. Assess pairwise interference.
IIb.1 (Davis) Prepare cell culture samples and define standard clinical samples with matched frozen tissue as controls for each ISH probe.
Completed.
IIb.2 (Davis) Conduct quantitative RT-PCR analysis for cell/ tissue quantitative mRNA determination for each transcript.
In Progress. 19 of 21 targets quantified.
IIb.3 (Davis) Prepare matched samples used in RT-PCR for MIBI ISH.
Year 3.
IIb.4 (Stanford) MIBI imaging of matched samples.
Year 3.
IIb.5 (Stanford) Use MIBI image analysis tools to quantify signal intensity and distribution of the MIBI ISH.
Year 3.
IIb.6 (Davis and Stanford) Prepare standard curves of RT-PCR analyte concentration v. MIBI ISH quantitiation.
Year 3.
IIb.7 Reiterate (1-6) with additional samples at high and low mRNA concentrations as needed to define the dynamic range limits as needed (find linear detection ranges).
Year 3.
IIb. 11 (Davis and Stanford) Report technical applications findings—manuscript.
Year 3.

IIc. Normalize quantitative ISH imaging using control genes for algorithm development. Compare clinical samples using MIBI Oncotype mRNA *in situ* to Oncotype DX recurrence score.
IIc.1 (Stanford) Compute average intensity/dot count for each analyte over the area of tumor.
Year 2-3.
IIc. 2 (Stanford) Compare computed average to qRT-PCR values.
Year 2-3.
IIc. 3 (Stanford) Normalize values with control probes for input into algorithm.
Year 2-3.
IIc. 4 (Davis) Reverse engineer an *in situ* Oncotype DX recurrence score by comparing to clinically derived (deidentified) score values across multiple tumors.
Year 2-3.
IIc. 5 (Davis) Validate *in situ* recurrence score against an additional test set and define the variance parameters.
Year 2-3.
IIc. 6 (Davis) Determine features associated with standard v. *in situ* score discrepancies.
Year 2-3.
IIc. 7 (Davis and Stanford) Report utility findings—manuscript.
Year 3.

Aim III: Heterogeneity as an additional tumor virulence measure: The data generated in aims I and II above provide a complex matrix with each analyte quantity per cell as well as cell morphology and size information, as well as microanatomic location information. Aim III will examine computational approaches to assess heterogeneity.

IIIa: Finding minority populations of virulent cancer cells. Do small numbers of cells with high risk calculations embedded in otherwise low risk tumors imply a greater risk?
IIIa. 1 (Stanford) Use CellProfiler with both MIBI IHC and MIBI ISH data to identify subsets of cells with "high scores" or "low scores".
Year 3.
IIIa. 2 (Davis) Construct score histograms of tumor cell populations to compare distributions across multiple breast cancer phenotypes.
Year 3.

IIIb. Multiparameter topology assessment: Using more advanced statistical methods like principal component analysis taking into account not just individual cell paramenters, but molecularly defined populations proximity and relationship to the tumor shape and intersection with the surrounding tissue structures.
Year 3.

E. CHANGES/PROBLEMS: The most significant aspect of the project that is being modified is to move away from ISH approaches in favor of IHC. The most relevant considerations here are a. One industry partner (Agilent) has closed the development and sourcing of their ISH probe reagetns. We have instead been evaluating exclusively ACD probe sets. These have become the industry standard, but are not ultimately suitable for higher dimension multiplexing as they rely on a limited set of amplification sequences. In addition, our preliminary data shows a high dependency on pre-analytical variables. This may resolve with high sensitivity, if the effect on signal intensity is equal across analytical and control probe sets, but it is also possible that sensitivity will be limiting. The Stanford group is working on another method for ISH amplification that may solve this problem in a parallel project (not funded by this proposal). Meanwhile, the Borowsky lab has completed optimization of IHC for the protein correlates of the OncotypeDX RNA targets, and this may permit a robust quantitative method better suited for adaptation to clinical pathology practice, as the technology for IHC is already conventional, leaving just multiplexing and multidimensional analysis for implementation development.

F. PRODUCTS: *Nothing to Report.*

G. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name	Alexander Borowsky
Project Role	Principle Investigator
Researcher Identifier	N/A
Person Months Worked	1

Funding Support	This award

Name	Richard Levenson
Project Role	Co-Investigator
Researcher Identifier	N/A
Person Months Worked	1
Funding Support	This award

Name	Hidetoshi Mori
Project Role	Technician
Researcher Identifier	N/A
Person Months Worked	10
Funding Support	This award

Conclusion

In conclusion, at the time of this first year progress report, planned work objectives are progressing on schedule. The bulk of the first year of work occurred at UC Davis, but critical peripheral work including the design and assembly of a new secondary ion mass spectrometer (not funded by this study) have been completed at Stanford. We anticipate continued progress toward the goals of this proposal in the coming years.

References and Appendices:

None.

Next-Generation Molecular Histology Using Highly Multiplexed Ion Beam Imaging (MIBI) of Breast Cancer Tissue Specimens

Borowsky Lab 2015 – 2016

For multiplex IHC: Selection and Optimization of Primary Antibodies for Target List.

We have chosen anti-human antibodies for ER, PgR, HER2, Ki67, BAG1, SMA, CK8/18, ECAD, P53, SLC7A5, NRDG1, HTF9C and CEACAM5. Testing the antibody affinity and specificity were primarily investigated by performing FFPE treated breast cancer cell lines (MCF7, T47D, BT474 and Hs578T) which are ER+/- and HER2+/-. To detect multiple target on one tissue section, we have used tyramide-based multiplex IHC. Signal detection was performed with Zeiss Laser scanning confocal microscopy (LSM710) which can separate fluorescence signals effectively by splitting a certain range of emission signals. Fig.1A shows IHC with ER and HER2 on each cell line. Fig.1B shows IHC with some of other markers (BAG1, p53 and ECAD). Other markers are also tested on these samples (data not shown).

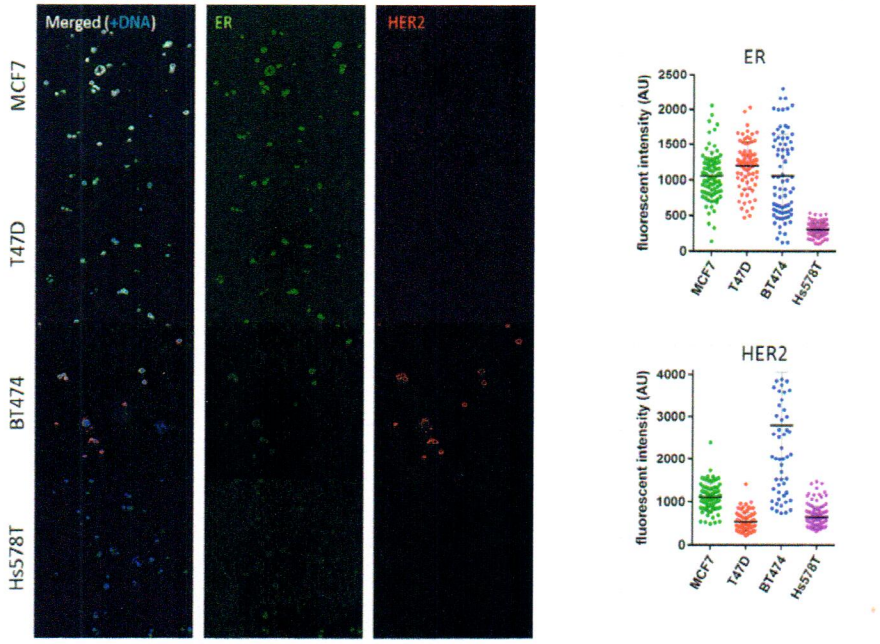

Figure1A. Quantification of Tyramide (TSA)-based multiplexed Immunohistochemistry (IHC) of ER and HER2 on tissue section of FFPE pseudo tissue with breast cancer cells (MCF7, T47D, BT474 and Hs578T).

Quantification of these images was also indicated on **Fig. 1A and B**, which shows significant variations in signal for each marker.

The procedure for TSA-based multiplex IHC needs cycles of antibody reactions, reaction between HRP and TSA probe and termination of the IHC reactions. While we have optimized the experimental conditions, we found better ways for reprobing samples (manuscript in preparation). In short, we have tested two strategies: One is to cancel the association in target protein/primary antibody/secondary antibody by chemical or microwave, and the other is to terminate the activity of HRP with hydrogen peroxide. Whereas each method has pros&cons, we identified either

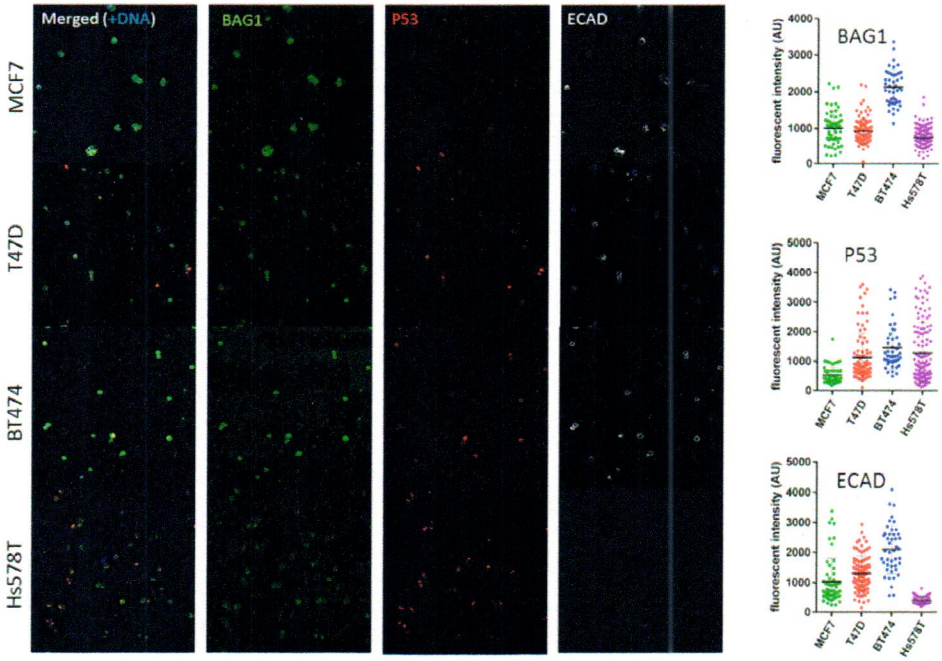

Figure1B. TSA-based multiplexed IHC of BAG1, P53 and ECAD on tissue section of FFPE pseudo tissue with breast cancer cells.

microwave or hydrogen peroxide is convenient for IHC.

The procedure for TSA-based multiplex IHC needs cycles of antibody reactions, reaction between HRP and TSA probe and termination of the IHC reactions. While we have optimized the experimental conditions, we found better ways for reprobing samples (manuscript in preparation).

In short, we have tested two strategies: One is to cancel the association in target protein/primary antibody/secondary antibody by chemical or microwave, and the other is to terminate the activity of HRP with hydrogen peroxide. Whereas each method has pros and cons, we identified either microwave or hydrogen peroxide is convenient for IHC.

After we tested these antibodies on FFPE treated breast cancer cell pellets, TSA-based multiplex IHC was performed to test if it also works for breast cancer tissue **(Fig.2).** Antibodies for ER, HER2, BAG1, P53, ECAD, SLC7A5, HTF9C, ACTA2 (SMA), NDRG, CEACAM and KRT8 were tested (PgR is not shown in the image). The limit of TSA-based multiplex IHC is 6 markers (plus DNA as 7 colors), we challenged to visualize a set of IHC4plus markers (ER, PR, HER2, Ki67, KRT8) and markers for Mammostrat (HTF9C, SLC7A5, NDRG1, P53, CEACAM5; and KRT8 to indicate tumor area) on human normal breast tissue and cancer tissue.

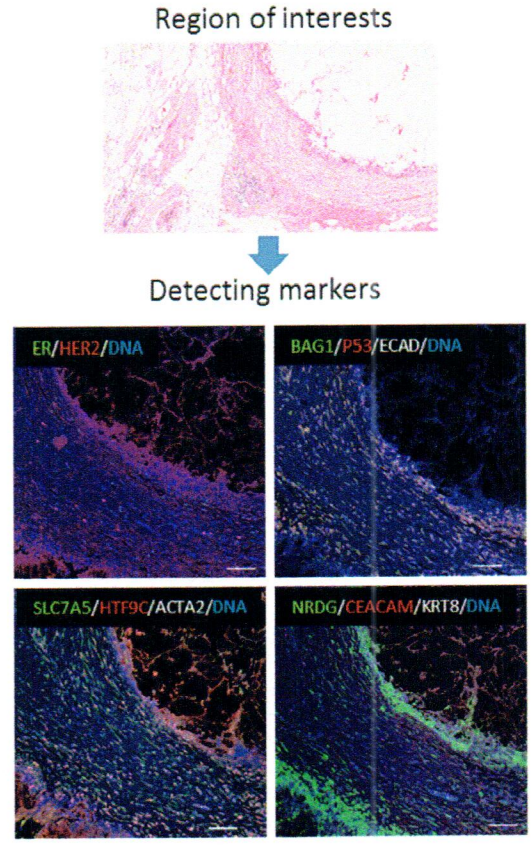

Figure 2. A test for 4-color TSA-based multiplexed IHC on human breast cancer tissues.

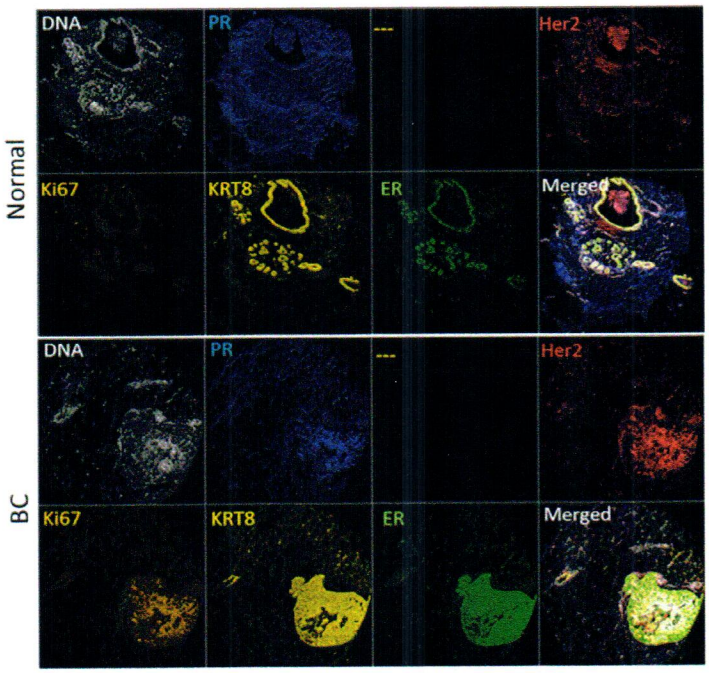

Figure3. TSA-based multiplexed IHC on normal and breast cancer (BC) tissue for IHC4 (ER, PR, Her2 and Ki67) plus KRT8.

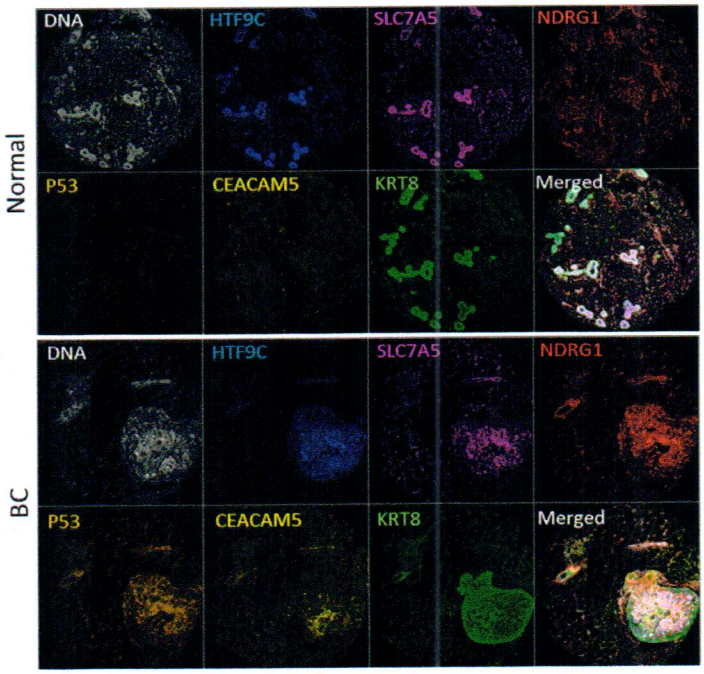

Figure4. TSA-based multiplexed IHC on normal and breast cancer (BC) tissue for Mammostrat markers (HTF9C, SLC7A5, NDRG1, P53 and CEACAM5) and KRT8.

Figures 3 and 4 Show that the optimized antibodies and protocols are suitable for simultaneous analysis of "IHC4" markers as well as the Mammostrat panel. **Figure 5** illustrates a variety of normal breast and breast cancers tested using the IHC4 panel, and demonstrate the ability of this panel to be useful in detailed tumoral complexity/heterogeneity analyses.

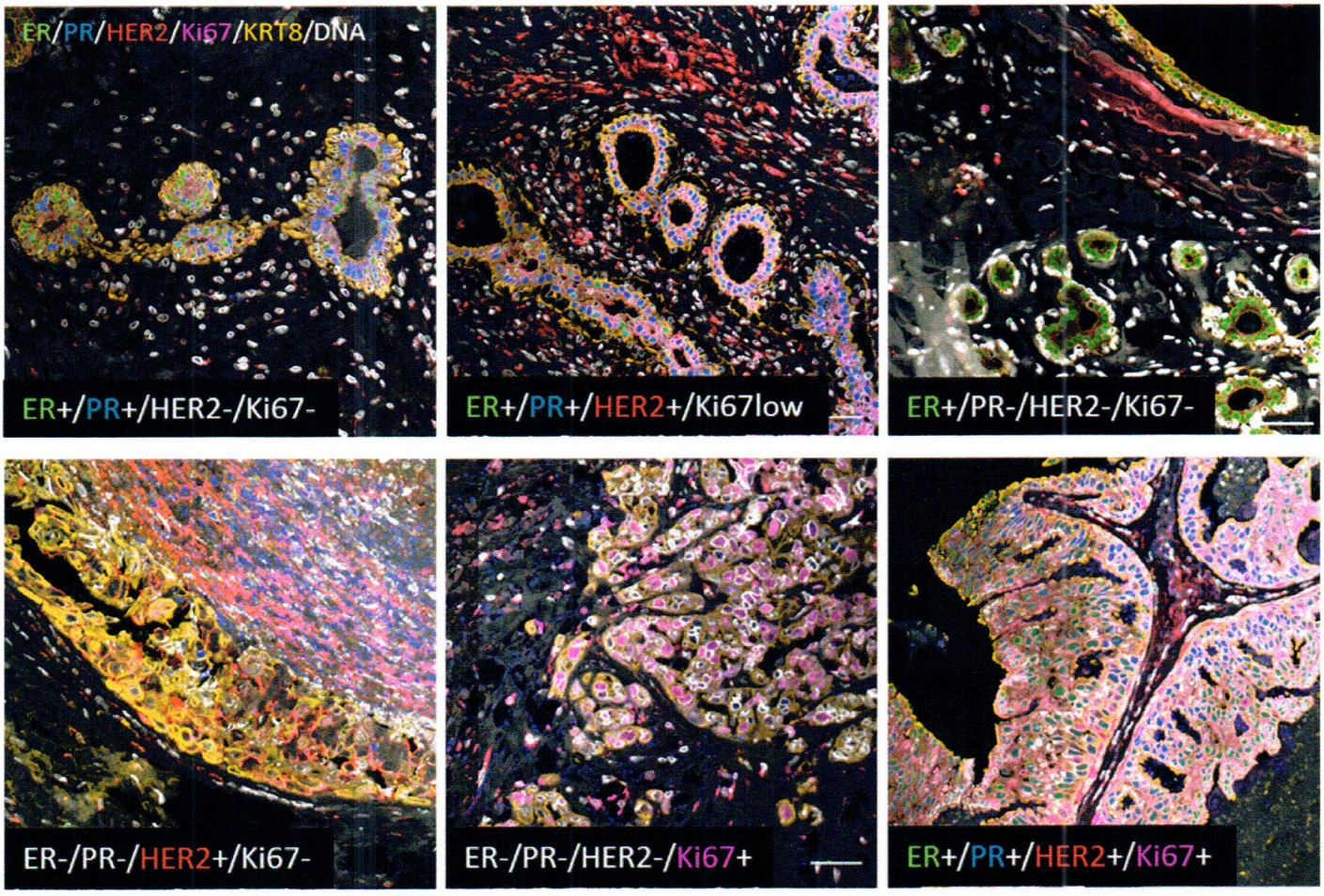

Figure 5. Heterogeneity in human breast cancer for IHC4 (ER, PR, Her2 and Ki67) markers.

For Multiplex ISH: Testing for Fluorescence Based ISH Methods.

For detecting gene expressions of target markers on breast cancer tissue samples, we have tested two different types of fluorescence in situ hybridization (FISH) probe. One is fluorescence labeled RNA FISH probe targeting Long Non-Coding region of transcript (lncRNA; Stellaris FISH probe), and the other is branching DNA based RNA FISH probes (ACD RNAscope).

To validate the specificity and quality of the lncRNA FISH probe, formalin fixed breast cancer cells were used as samples. First, we tested HER2 and ACTB expressions on HER2 positive- (BT474) and negative-cell lines (Hs578T, weak positive MDA-MB-361). As Fig.6 shows, each signal was detected specifically. And Fig.7 displays heat map of each signal spot, indicating significant differences in the expression in HER2 gene. However, the amount of ACTB

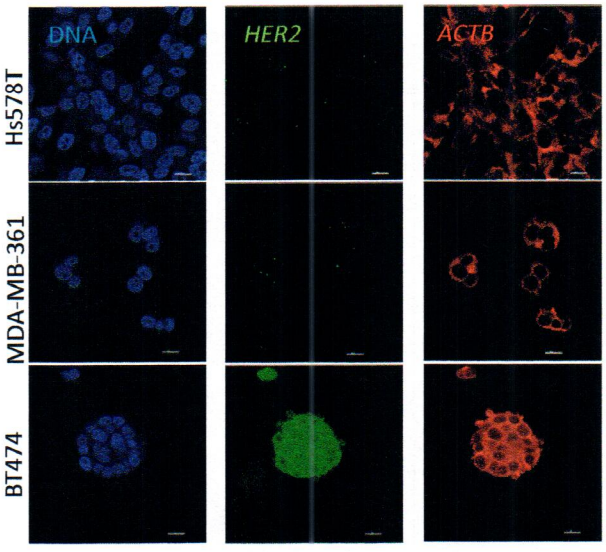

Figure6. A test for detecting *HER2* and *ACTB* gene expression by fluorescence in situ hybridization

per cell indicates the difference of ACTB gene expressions higher in Hs578T but lower in MDA-MB-361 and BT474. Therefore, we tested the other gene (TFRC) as a control gene expression (Fig.8). In comparison to ACTB, TFRC expression was almost same levels in all cell lines.

These Stellaris FISH probes were then tested on breast cancer tissue section to detect HER2, ACTB and TFRC with different fluorescence colors. Whereas TFRC and ACTB were detected as a weak signal, not all cells had these signals and no HER2 expression was detected on HER2+ tissue (Fig.9). This might be most possibly because of the RNA quality of the tissue sample.

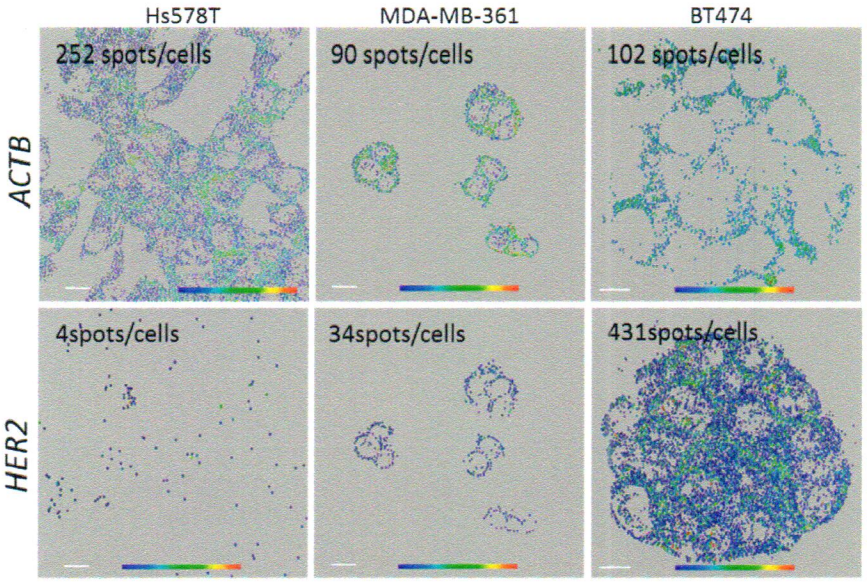

Figure7. Quantification of FISH results by IMARIS software.

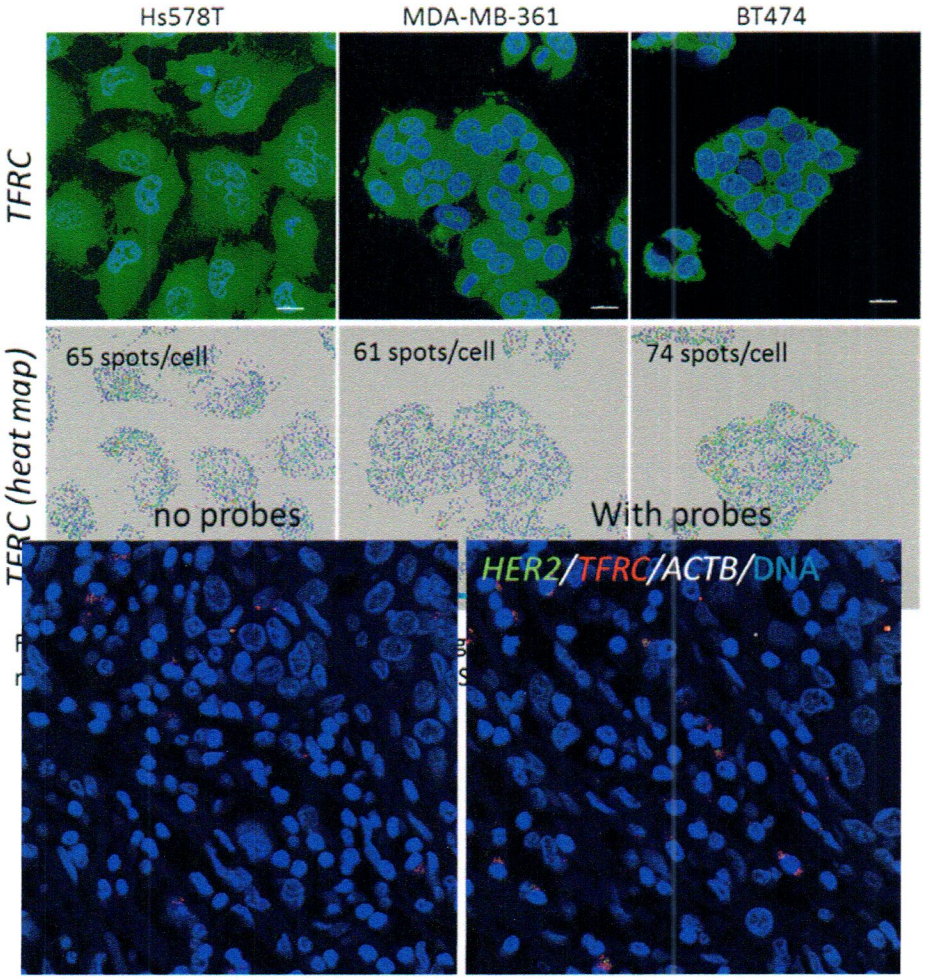

Figure 9. Testing Stellaris FISH probes on HER2+ human breast cancer tissue.

Nolan Lab 2015 - 2016

New generation of instrument

During the last funding period, we have significantly increased the capabilities of multiplexed ion beam imaging through improvements in instrumentation, reagents, and tissue staining protocols. A new time of flight mass analyzer with a modified reflectron has lowered the noise floor 4x while increasing sensitivity and mass resolution by 2x. Addition of a gold germanium liquid metal alloy ion source has improved instrument throughput by 4x, such that a 600um tissue microarray core can be imaged at 500nm resolution in 45-60min. 82 antibodies have now been validated on archival FFPE human tissue sections. These antibodies have been assembled into 40plex staining panels for analysis of the tumor immune microenvironment, and have now been tested extensively on over 200 different tissue blocks and 15 different tissue types. Improved staining protocols have now significantly reduced residual background from non-specific antibody binding, such that relatively low abundance targets (i.e. pd1, GITR) can be readily discerned.

New channels to increase single cell dimensionality

Over the past year we have significantly increased the multiplexing capability of MIBI by the introduction of 7 new channels (Figure 1a) in addition to the lanthanides as reported originally in Angelo et al nature Medicine. Two examples are shown for Au197 and I127 both conjugated to a CD3 antibody. Performance is assessed by staining peripheral blood mononuclear cells with these different anti-CD3 metal conjugates (Figure 1b). As can be seen CD4 T cells have a positive signal for both conjugates, as expected with much lower signal (background staining) for B-cells which are a negative control.

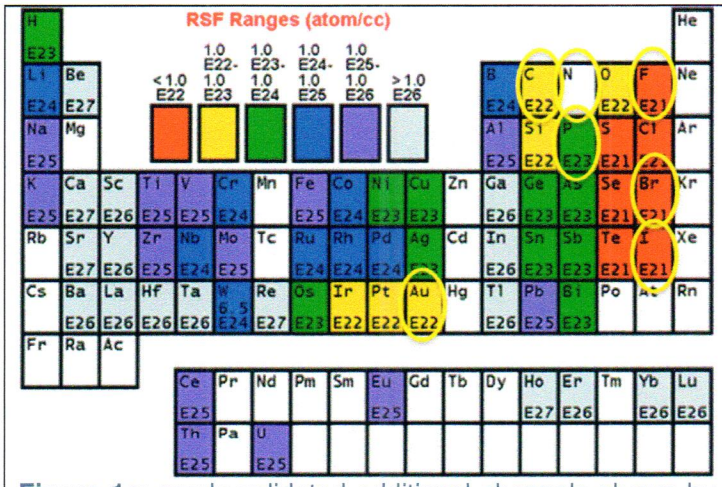

Figure 1a: newly validated additional channels shown by yellow rings

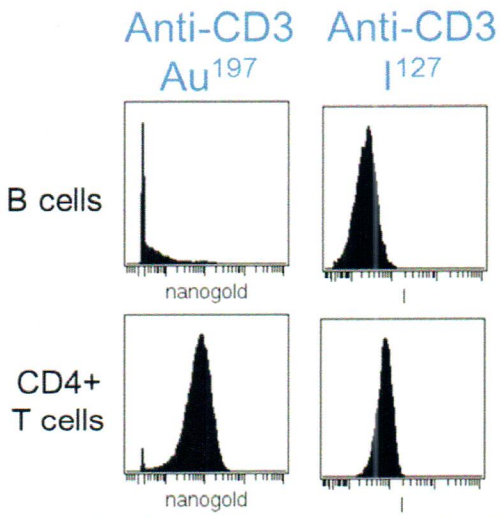

Figure 1b: Examples of new channels shown here for ^{197}Au and ^{127}I conjugated to a CD3 antibody.

Subcellular architecture

Part of our current effort is focused on studying cellular substructure. For this we are exploiting the abundance of naturally occurring isotopes such as ^{12}C, ^{13}C, ^{14}N, ^{15}N, ^{16}O, ^{18}O, ^{31}P, ^{32}S and measuring their cellular distribution. Incorporating measurements of these isotopes with antibodies will provide an unprecedented level of resolution of cellular architecture that will enable models to be built for normal and diseased cells. Although he relevance of this to cancer is clear, the application will be to many other disease states. Below we provide our preliminary experiments looking inside the nucleus, specifically at chromatin structure.

Visualization of open chromatin

In a human cell, approximately two meters of DNA are packed within a five-micron nucleus—a topological challenge solved by the cell through the hierarchical folding of DNA around histone proteins to form nucleosomes, and the compaction of nucleosomes into chromatin (Kornberg, 1974). This hierarchical packaging

sequesters inactive genomic regions and leaves biologically active regions – be they promoters, enhancers, or other regulatory elements – accessible to transcription machinery. Within the chromatin architecture further transcriptional regulation is imposed by a dynamic epigenetic code that includes DNA methylation, nucleosome positioning, histone composition, and modification, as well as transcription factors, chromatin remodelers, and non-coding RNAs. Together chromatin architecture both physical and chemical (through covalent modification with certain groups) has an essential role in determining the phenotype of a cell. However, in spite of the wealth of knowledge about the complexity of chromatin structure relatively little is known about the overall or genome-wide structure of chromatin, taking into account all the above features. Up until recently methodologies for assaying chromatin structure and composition often required tens to hundreds of millions of cells as input material, averaging out the information from cellular populations. This necessarily missed important information within cellular sub-types that could not be acquired in amounts sufficient for genome-wide chromatin analyses.

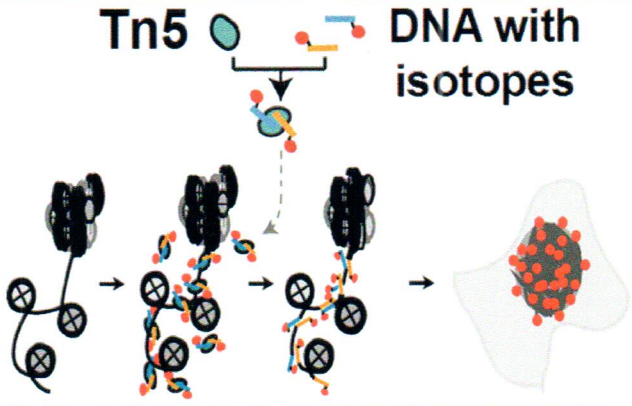

Figure 2: Super-resolution open chromatin imaging.

The assay of transposase accessible chromatin (Buenrostro et al., 2013) (ATAC-seq) uses hyperactive Tn5 transposase (Tn5) (Goryshin and Reznikoff, 1998; Adey et al., 2010) to simultaneously cut and ligate adapters for high-throughput sequencing at regions of increased accessibility. Genome-wide mapping of insertion ends by high-throughput sequencing allows for multidimensional assays of the regulatory landscape of chromatin. While this assay has provided invaluable information about the chromatin landscape, by its nature it cannot provide details about physical structure. Thus, taking advantage of ATAC-Seq, to visualize open chromatin by inserting fluorescent DNA adaptors into accessible chromatin loci with hyperactive Tn5 transposase we reasoned that by loading Tn5 transposase with fluorine-labeled adaptors instead, the inserts could enable us to image the accessibility of genome at super resolution in 3D. *Taking advantage of the finely focused primary ion beam of nanoSIMS, which allows to image single cells and tissue with sub-cellular resolution (50 nm), here we proposed to investigate the 3D organization of chromatin using super-resolution nanoSIMS imaging.*

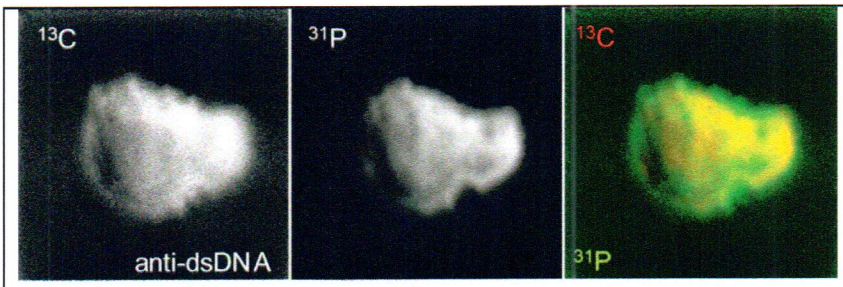

Figure 3a: an antibody to double stranded DNA validates the use of naturally occurring ^{31}P to mark DNA

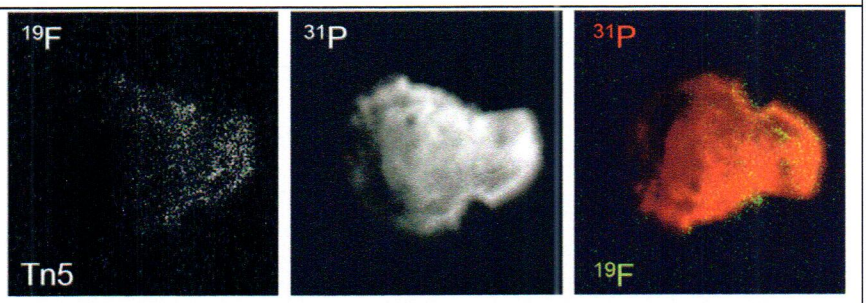

Figure 3b: Fluorine tagged Tn5 and 31P together reveal open chromatin

As a first step we determined whether ^{31}phosphorus that is the naturally occurring monoisotopic element could be used to visualize DNA. The image below shows permeabilized Jurkat cells that were incubated with a ^{13}C-conjugated antibody against DNA. Using a pseudo coloring scheme of red for ^{13}C-anti DNA and green for ^{31}P followed by a merge we can discern the DNA as yellow thereby validating the approach of using the naturally occurring isotope of phosphorus to visualize DNA (Figure 3a). In the second step of this experiment we visualized open chromatin with fluorine-labeled Tn5 as shown in the merge of figure 3b.

Next steps will be to study the molecular structure of open chromatin in depth with MIBI by examining the presence or absence of different proteins within the epigenetic landscape and ultimately building a model that can predict the architecture of open chromatin and how it changes in disease, an example of which is to

understand the role of epigenetics in T-cell exhaustion. *The resultant models of chromatin architecture will be based on subcellular data of exquisite resolution that before was unattainable.*