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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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REPORT DOCUMENTATION PAGE

We have completed the goals of the project timeline, in large part, in this first 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 1/3rd in progress. We have optimized multiplexed IF for comparison to MIBI secondary ion mass spectrometry. And we have begun to develop 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
Breast Cancer Diagnosis, Pathology, Immunophenotype, Multiplex, Morphology, RNA In Situ Hybridization, Immunohistochemistry/Immunofluorescence, Predictive Biomarkers

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INTRODUCTION:
Current breast cancer diagnosis includes predictive assays to guide therapy decisions, involving a minimum of 3 assays: 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**
PI: Alexander Borowsky

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, plans to report for the 2017 December meeting as well.

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 all proposed antibodies.

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

Dependent on Stanford.

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

Completed multiplexing using TSA method.

Year 2-3.

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

Pilot work completed, Ongoing testing for NCE period.

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

Pilot work completed, Ongoing testing for NCE period.

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. Adoption of the inForm (Perkin Elmer) software.

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

Ongoing testing for NCE period.

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

Pilot analyses completed. Ongoing testing for NCE period.

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

Completed for IHC markers, IHC markers to replace ISH completed.

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. Ongoing testing for NCE period.
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. Second MS in preparation for quantitative anlysis for algorithmic risk
stratification.

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 multiparameter data into SPADE software package for
population analysis.
In progress.
Ic. 4 (Davis) Develop cell position matrices for aim 3 evaluation.

Year 3. Ongoing testing for NCE period.
Ic. 5 (Davis and Stanford) Test display utility, and modify for user/pathology interface.

Year 3. Ongoing testing for NCE period.
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. Ongoing testing for NCE period.
Ic. 8 (Davis) Compare output scores to clinically derived conventional scores.

Year 3. Ongoing testing for NCE period.
Ic. 9 (Davis and Stanford) Report utility findings—manuscript.

Year 3. Ongoing testing for NCE period.

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.

21 targets optimized.
Iia. 3 (Davis) Prepare mass tagged ISH probes.

Year 3. Ongoing testing for NCE period.
IIa. 4 (Davis) Prepare tissue samples with mass tagged ISH probes: single label, double label and half panel and full panel.  
Completed with non-mass tagged.

IIa.5 (Stanford) nanoSIMS MIBI imaging of single, double and panel labeled samples.  
Year 3. Ongoing testing for NCE period.

IIa.6 (Stanford) Initial image analysis of MIBI images for display of quantitative ISH signals.  
N/A converted to Ab methods.

IIa.7 (Stanford) Image cell segmentation and cell distributions by ISH phenotype and cell morphology.  
N/A converted to Ab methods

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. 
Completed 21 targets quantified.

IIb.3 (Davis) Prepare matched samples used in RT-PCR for MIBI ISH. 
N/A converted to Ab methods

IIb.4 (Stanford) MIBI imaging of matched samples. 
N/A converted to Ab methods

IIb.5 (Stanford) Use MIBI image analysis tools to quantify signal intensity and distribution of the MIBI ISH. 
N/A converted to Ab methods

IIb.6 (Davis and Stanford) Prepare standard curves of RT-PCR analyte concentration v. MIBI ISH quantitation. 
N/A converted to Ab methods

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). 
N/A converted to Ab methods

IIb.11 (Davis and Stanford) Report technical applications findings—manuscript. 
N/A converted to Ab methods

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. 
N/A converted to Ab methods. Ongoing testing for NCE period.

IIc. 2 (Stanford) Compare computed average to qRT-PCR values. 
N/A converted to Ab methods. Ongoing testing for NCE period.

IIc. 3 (Stanford) Normalize values with control probes for input into algorithm. 
N/A converted to Ab methods. Ongoing testing for NCE period.

IIc. 4 (Davis) Reverse engineer an in situ Oncotype DX recurrence score by comparing to clinically derived (deidentified) score values across multiple tumors. 
In progress using mIHC via IF with inForm. Ongoing testing for NCE period.

IIc. 5 (Davis) Validate in situ recurrence score against an additional test set and define the variance parameters. 
In progress using mIHC via IF with inForm. Ongoing testing for NCE period.

IIc. 6 (Davis) Determine features associated with standard v. in situ score discrepancies. 
In progress using mIHC via IF with inForm. Ongoing testing for NCE period.
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.

Illa: 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?

Ilia. 1 (Stanford) Use CellProfiler with both MIBI IHC and MIBI ISH data to identify subsets of cells with “high scores” or “low scores”.

In progress using miHC via IF with inForm. Ongoing testing for NCE period.

Ilia. 2 (Davis) Construct score histograms of tumor cell populations to compare distributions across multiple breast cancer phenotypes.

In progress using miHC via IF with inForm. Ongoing testing for NCE period.

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

In progress using miHC via IF with inForm. Ongoing testing for NCE period.

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 reagents. 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.


G. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

| Name          | Alexander Borowsky |
Conclusion

In conclusion, at the time of this year 3 progress report, planned work objectives are largely completed, though a shift toward non-ISH methods slowed progress. 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 period of no cost extension.

References and Appendices:

None.

Overall summary of accomplishments from both groups
All the antibodies originally proposed have been optimized for multiplex immunodetection as of the reporting period. One change in aims was also completed, this was to develop IHC protein detection for the OncotypeDX mRNA set. Additional functionality of the method was also accomplished, specifically we have generated and validated new reagents for application to MIBI enabling visualization of chromatin and other nuclear structures at super resolution in single cells.

Borowsky Lab
Antibody reagents optimized.
Antibodies for all of the target proteins in the original proposal have been optimized and tested using, initially, OpalTSA and Vectra (Perkin Elmer) imaging. These include: ER, PR, HER2, Ki67, SMA(ACTA2), CK8/18, ECAD, P53, SLC7A5, NRDG1, HTF9C, CEACAM5, STK15, Survivin, CCNB1, MYBL2, MMP11, CTSL2, GRB7, GSTM1, CD68, BAG1, BCL2, SCUBE2, ACTB, GAPDH, RPLPO, GUS, and TFRC. The latter group (italics) represents the OncotypeDX mRNA list adapted to protein analysis, with additional Oncotype markers overlapping the original IHC list (ER, PR, HER2, Ki67). Reverse engineering the OncotypeDX recurrence score from quantitated multiplex protein analysis is still in progress, with analysis of a specially developed tissue array of breast cancers with OncotypeDX results. In addition to this algorithm, comparator algorithms will be applied including IHC4, IHC4+Bag1, IHC intrinsic subtype, and proliferative rate alone. We aim to apply this new method to first a retrospective set of breast cancers with long term follow up, and then to a prospective registry trial format (not funded here, but for future planned proposals).

Nolan Lab
Visualization of open chromatin
As described in our previous report we are continuing our efforts to optimize the visualization of chromatin structure in single cells. 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 at high resolution. 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. The assay of transposase accessible chromatin with high throughput sequencing (Buenrostro et al., 2013) (ATAC-seq) uses hyperactive Tn5 transposase (Tn5) (Goryshin and Reznikoff, 1998; Adey et al., 2010) loaded in vitro with DNA adaptors for high throughput sequencing, that are selectively inserted into accessible chromatin loci within living cells. Deep sequencing of adaptor-tagged sites...
reveals active regulatory elements, transcription factor binding sites, and nucleosome positions within open chromatin (Buenrostro et al., 2013).

While ATAC-Seq has provided invaluable information about the chromatin landscape, by its nature it cannot provide details about physical structure. In a follow-up study the same group reported a modified version of ATAC-Seq called transposase-accessible chromatin with visualization (ATAC-see), a transposase-mediated imaging technology that directly images the accessible genome in situ (Chen et al. Nature Methods 2016). ATAC-see utilizes hyperactive Tn5 transposase loaded with fluorophore-conjugated adaptors to insert into accessible chromatin loci. The fluorophore used was ATTO 514, a new hydrophilic fluorescent label with strong absorption, high fluorescence quantum yield, and exceptional thermal and photo-stability. 3D spatial organization of accessible DNA in situ was visualized by confocal microscopy of the ATTO514 labelled cells.

We adapted ATAC-see for MIBI and high resolution nano-SIMS. Using K562 cells as a model system, and taking advantage of ATAC-see to visualize open chromatin by fluorescence, we presented a modification of ATAC-see in which fluorinated adaptors conjugated to ATTO 514 loaded onto hyperactive Tn5 transposase can insert into accessible chromatin loci. By endowing these adaptors with a double label (ATTO514 and fluorinated nucleotides with fluorine-modified ribose in the 2’ position) open chromatin could be visualized both by fluorescence and super-resolution nano-SIMS respectively. Below The high purity of the adaptors we used (either singly or doubly labeled) are shown by electron spray ionization mass spectrometry (Figure 1).

Pilot experiments in the K562 cell line with the adaptors shown above (Figure 1a – c) demonstrated that ATAC fragment sizes using the recombinant purified hyperactive Tn5 transposase loaded with adaptors conjugated either to both ATTO514 and fluorinated oligos or to only fluorinated oligos generated very similar distributions of insert size (Figure 2) and distance from the transcriptional start site (TSS) (Figure 3) compared with unmodified Tn5-loaded adaptors. Furthermore, the genomic distribution of ATAC-seq peaks were also similar between Tn5 loaded with Atto514-fluorinated adaptors and Nextera Tn5 (that uses unmodified adaptors) (Figure 4). Thus, Tn5 bearing ATTO514 and fluorinated tags does not affect transposase activity.
Our modified ATAC-see experiments revealed the 3D spatial organization of accessible DNA in situ. After incubation with Tn5 loaded with modified adaptors, the resulting high resolution nano-SIMS images showed that the accessible chromatin is heterogeneously distributed throughout the nucleus (fluorinated signals) and is distinct from endogenous phosphorus which demarcates DNA. Thus, the signal shows the integration of fluorinated adaptors into accessible genomic DNA.

**Additional technological advances for reagent generation**

i) Reasoning that ATTO514 has a similar chemical structure to fluorescein and oregon green we evaluated an antibody that recognizes these fluorophores as also being able to recognize ATTO514. Indeed, we successfully validated an anti-FITC antibody as a reagent to recognize ATTO514. This could be valuable for amplifying the ATAC-see signal further and a new reagent for mass cytometry.

ii) Generation of $^{19}$F-trans-activating (tracr)RNA:dCas9 probes. So far, we have generated major satellite (MajSat) sgRNA which recognizes the repetitive sequences found in the pericentromeres. This will provide insight into chromosomal packaging and spindle attachment adding another dimension to the study of nuclear architecture. Since our CRISPR/Cas9 system works well for the visualization of repetitive sequences we can also visualize other nuclear organelles and loci such as, centromere DNA, telomere DNA, ribosomal DNA or Muc4 and Muc1 genes. To date, we have visualized pericentromeres, centromeres and telomeres in mouse cells (MEF cells) and telomeres in human cells (Jurkat cells).

iii) Generation of halogen-labeled antibodies to further increase the number of parameters to measure per single cell with nanoSIMS or MIBI. We have successfully labelled antibodies with nucleotides synthesized with halogens which can then be a readout for multiplex imaging (Figure 5).

iv) Generation of monoclonal antibodies using “heavy” arginine. By culturing hybridomas in media containing heavy arginine labeled with $^{13}$C, the resultant antibody will be another reagent for MIBI and nanoSIMS.

![Figure 4: Genomic distribution of ATAC-seq peaks were similar among ATTO 514-fluorinated adaptor loaded Tn5 and Nextera Tn5](image)

![Figure 5: ATTO514-Fluorine-Tn5 (open chromatin) signals inversely correlate with endogenous phosphorus (DNA) as expected](image)

![Figure 6: Antibodies conjugated with halogen-labeled oligos can be analyzed by MIBI to reveal chromatin structure.](image)
Conclusions
The tools we have developed now enable us to integrate imaging and epigenomics to provide a general and scalable approach for characterizing the spatio-temoral architecture of gene control under normal and disease conditions.