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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

PRINCIPAL INVESTIGATOR: Garry P. Nolan

CONTRACTING ORGANIZATION: Stanford University
Palo Alto, CA 94304-1212

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14. ABSTRACT 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 (Clariant) 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.					
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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 (Clariant) 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

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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? 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. Please see Figure 1 Appendix I.

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.

1a. 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.

- *1a.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 (de-identified) using conventional formalin fixed and paraffin embedded tissue blocks will be used.
 - **Completed.**
- *1a.2* (Davis) Optimize titers using conventional immunohistochemistry.
 - **Completed for 10 of 13 proposed antibodies. 3 additional in progress.**
- *1a.3* (Davis) Prepare mass tagged primary antibodies.
 - Year 2 planned.
- *1a.4* (Davis) Prepare tissue samples with mass tagged antibodies: single label, double label and complete panel (13 label).
 - Year 2-3.
- *1a.5* (Stanford) NanoSIMS MIBI imaging of single, double and panel labeled samples.
 - We have been actively conjugating and validating antibody probes for immunohistochemical analysis of normal and neoplastic breast tissue. Antibodies targeting 45 different proteins have undergone preliminary testing and validation against positive and negative control tissue specimens with known expression patterns, including Pan Keratin, dsDNA, Nuclear Pore Complex, CD45, PD-1, CA9, Galectin, CD8, CD44, CD24, Epcam, Her2, CD20, ER, PD-L1, E-cadherin, Ki67, pAKT, CD19, MUC1, CD163, CD68, CD4, CD3, Beta

Catenin, FOXA1, Collagen 1, H3K4me2, Keratin 5, H3K9me2, Keratin 6, Pan-Actin, Keratin 7, H3K18ac, Keratin 10, H3K9ac, Keratin 14, H3K27me3, Vimentin, H2BK5ac, N-Cadherin, HH3, GATA3, pAKT, and pCREB. SLC7A5, NRDG1, HTF9C, CEACAM5, and SMA are currently being procured from the manufacturer and will be tested as well within the next few months. These antibodies have been successfully used in multiplexed panels with twenty-five simultaneous protein targets. Slide substrates for optimal sample conductance and tissue adherence have also been determined.

- Finally, the quantitative accuracy of MIBI has been compared to a US Food and Drug Administration (FDA)-approved quantitative image analysis (QIA) workflow for determining the staining intensity of scanned IHC tissue sections. QIA was used to quantify ER α nuclear expression in a cohort of nine breast tumors. Manually annotated tumor-containing regions were subsequently analyzed using an automated algorithm optimized for determining immunoperoxidase nuclear staining intensity. The resultant data included mean intensity and an overall H-score (a scale for quantifying the intensity of expression of a protein in IHC staining). Linear regression analysis comparing mean ER α nuclear staining intensity by MIBI or IHC demonstrated robust agreement between the two methods ($r = 0.99$, $P < 0.00001$). We calculated cutoff points for MIBI staining intensity with the resultant linear equation ($MIBI = 0.064 + 0.0073 \times IHC$) using the respective values for negative, 1+, 2+ and 3+ employed by QIA; these cutoff points were used to calculate an overall H-score, which also demonstrated robust agreement ($r = 0.99$, $P < 0.00001$). The strong correlation between H-scores derived from each method suggests that MIBI not only captured the mean overall staining intensity but also was able to accurately capture the biological variability of ER α expression. This implies that the true distribution of staining intensity was valid and accurately recapitulated.
- New instrument developed and nearly ready for shipment to Stanford next month. Initial Images shown in Figure 1, Appendix II.
- **Pilot work completed, test samples Year 2.**
- *1a.6* (Stanford) Initial image analysis of MIBI images for display of categorical and quantitative signals.
 - **Pilot work completed, test samples begin Year 2.**
- *1a.7* (Stanford) Image cell segmentation and cell distributions by 13x immunophenotype and cell morphology.
 - Secondly, we are continuing to develop and optimize image segmentation algorithms that divide tissue into subcellular regions of interest (i.e. nuclei, cytoplasm, membrane). To facilitate this, we are optimizing antibody marker channels specific for each region of interest. Histone H3 and dsDNA are being used as nuclear markers; lamin A/C and nuclear pore complex are perinuclear markers which are anticipated to assist in dividing clumped nuclei; actin and tubulin are being optimized for membrane segmentation.
 - Though this is not expected to be ready until Year 3, due to the recent development of alternative approaches for high dimensional imaging in the Nolan laboratory (CODEX, under separate funding) we have already had the opportunity to initiate development of high dimensional imaging stratification. Appendix II, Figure 2 shows example data.

1b. Measure standard curves for each analyte against western blots using cell lines and tumor samples. Compare quantitation dynamic ranges to conventional IHC.

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

- **Completed.**
- *lb.2* (Davis) Conduct quantitative western blot analysis for cell/ tissue quantitative protein determination for each antibody.
 - **Completed.** Please see Figure 2 Appendix I
- *lb.3* (Davis) Prepare matched samples used in western blots (Figure 2 appendix I) for conventional and MIBI IHC.
 - **Completed.** Please see Figure 3 Appendix I
- *lb.4* (Davis) Conduct conventional IHC.
 - **Completed.**
- *lb.5* (Davis) Use Aperio image analysis tools to quantify signal intensity and distribution of conventional IHC.
 - **Pilot testing completed, re-optimized using IMARIS image analysis. Additional tool sets under evaluation.** Please see Figure 4, Appendix I
- *lb.6* (Stanford) MIBI imaging of matched samples.
 - **Year 2.**
- *lb.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.**
- *lb.8* (Davis) Prepare standard curves of western quantified analyte concentration v. conventional IHC quantitation.
 - **In progress.**
- *lb.9* (Davis and Stanford) Prepare standard curves of western quantified analyte concentration v. MIBI IHC.
 - **Year 2.**
- *lb.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.**
- *lb. 11* (Davis and Stanford) Report technical applications findings—manuscript.
 - **Year 2-3.**

lc. Automate IHC4 + BAG1 score, and “Mammostrat” score using one slide 13 marker quantitative image. Continue development of the analysis software.

- *lc.1* (Stanford) Utilize cytokeratin and/or ECAD channels to segment epithelium from stroma.
 - **Completed.** Please see Figure 5 Appendix I
- *lc.2* (Stanford) CellProfiler segmentation using hematoxylin channel (aluminum peak) or addition of dsDNA antibody if needed (Davis prep, Stanford analysis).
 - **Completed.**
- *lc.3* (Stanford) Import segmented multiparameter data into SPADE software package for population analysis.
 - **Completed. Updated algorithm to Vortex, which we implemented to overcome deficiencies in SPADE. See Appendix II, Figure 3.**
- *lc.4* (Davis) Develop cell position matrices for aim 3 evaluations.
 - **Year 2.**
- *lc.5* (Davis and Stanford) Test display utility, and modify for user/pathology interface.
 - **Year 3.**
- *lc.6* (Davis) Use standard curve quantified (ER, HER2) and categorical percentages (PR, KI67) to provide input for IHC4 score algorithm.
 - **Pilot methods completed. Evaluation measurements Year 2.**
- *lc.7* (Davis) Use standard curves and categorical percentages to provide input for Mammostrat score algorithm.

- **Year 3.**
- *lc.8* (Davis) Compare output scores to clinically derived conventional scores.
 - **Year 3.**
- *lc.9* (Davis and Stanford) Report utility findings—manuscript.
 - **Year 3.**

Aim II: MIBI OncotypeDX 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)

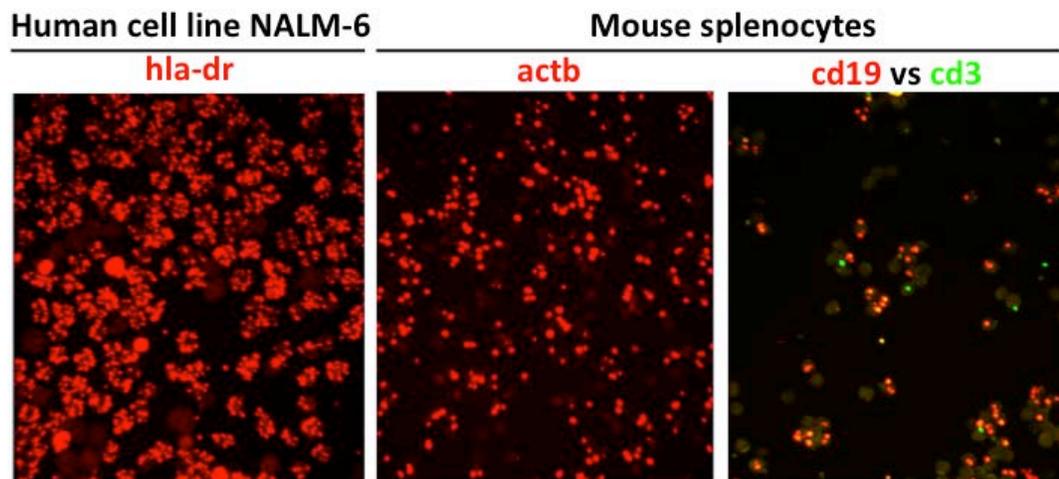
IIa. Compare hybridization results for mass tagged probe designs from both collaborating companies (ACD and Biosearch). Develop hybridization conditions for mixing probe types.

- *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.
 - **In progress. 5 of 21 targets optimized.**
- *IIa.3* (Davis) Prepare mass tagged ISH probes.
 - **Year 2.**
- *IIa.4* (Davis) Prepare tissue samples with mass tagged ISH probes: single label, double label and half panel and full panel.
 - **Year 2.**
- *IIa.5* (Stanford) NanoSIMS MIBI imaging of single, double and panel labeled samples.
 - **Year 2.**
- *IIa.6* (Stanford) Initial image analysis of MIBI images for display of quantitative ISH signals.
 - **Year 2.**
- *IIa.7* (Stanford) Image cell segmentation and cell distributions by ISH phenotype and cell morphology.
 - **Year 2-3.**

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.** Please see Figure 3 Appendix I
- *IIb.2* (Davis) Conduct quantitative RT-PCR analysis for cell/ tissue quantitative mRNA determination for each transcript.
 - **In Progress. 12 of 21 targets quantified.**
- *IIb.3* (Davis) Prepare matched samples used in RT-PCR for MIBI ISH.
 - **Year 2.**
 - **The Stanford group has recently published (Nature Methods, Frei et al 2016) the PLAYR technique for high multiplexing of RNA targets in a manner commensurate with isotopic analysis such as CyTOF. This is a prelude to work with tissue.** PLAYR uses the concept of proximity ligation to detect individual transcripts in single cells, and is compatible with immunostaining. Pairs of DNA oligonucleotide probes (probe pairs) are designed

to hybridize to two adjacent regions of target transcripts in fixed and permeabilized cells. Each probe in a pair is composed of two regions with distinct function. The role of the first region is to selectively hybridize to its cognate target RNA sequence. The second region, separated from the first by a short spacer, acts as template for the binding and circularization of two additional oligonucleotides (termed backbone and insert). When hybridized to two adjacent probes the backbone and insert oligonucleotides form a single-stranded DNA circle that can be ligated. The ligated, closed circle is then amplified through rolling circle amplification by phi29 polymerase initiated by the 3' OH of one of the probes in a pair. As phi29 continues to polymerize, it creates a linear molecule that contains hundreds of concatenated complementary copies of the original circle. Then, using a labeled oligonucleotide that is complementary to the insert region of the amplicon, one can detect any given probe pair through binding to the amplified product. For analysis by flow cytometry fluorescently labeled oligonucleotides are used for detection. Alternatively, metal-conjugated oligonucleotides enable mass cytometric analyses using a CyTOF instrument. Example stainings are seen below.



- *IIb.4* (Stanford) MIBI imaging of matched samples.
 - **Year 2.**
- *IIb.5* (Stanford) Use MIBI image analysis tools to quantify signal intensity and distribution of the MIBI ISH.
 - **Year 2-3.**
- *IIb.6* (Davis and Stanford) Prepare standard curves of RT-PCR analyte concentration v. MIBI ISH quantitation.
 - **Year 2-3.**
- *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 2-3.**
- *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 OncotypeDX mRNA *in situ* to OncotypeDX 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* OncotypeDX recurrence score by comparing to clinically derived (de-identified) score values across multiple tumors.
 - **Year 2-3.**
- *IIc.* (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 micro-anatomic 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 parameters, but molecularly defined populations proximity and relationship to the tumor shape and intersection with the surrounding tissue structures.

Year 3.

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.*

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

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C. How were the results disseminated to communities of interest? *Nothing to Report.*

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

Outlined in part A above.

E. CHANGES/PROBLEMS: So far, the project is progressing without major problems. Several minor issues, such as choice of image analyses software have resulted in minor changes to our plans, but nothing significant. One major difficulty has been obtaining test ISH probes from some of the industry providers. The Material Transfer Agreements (MTAs) have taken longer than anticipated to finalize.

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	11
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:

Appendix I Figures from UC Davis.
Appendix II Figures from Stanford

Immunohistochemistry and mass spectrometry for highly multiplexed cellular molecular imaging.

- Levenson RM, Borowsky AD, Angelo M. *Lab Invest.* 2015 Apr;95(4):397-405. doi: 10.1038/labinvest.2015.2. Epub 2015 Mar 2. Review. PMID: 25730370

Multiplexed ion beam imaging of human breast tumors.

- Angelo M, Bendall SC, Finck R, Hale MB, Hitzman C, Borowsky AD, Levenson RM, Lowe JB, Liu SD, Zhao S, Natkunam Y, Nolan GP. *Nat Med.* 2014 Apr;20(4):436-42. doi: 10.1038/nm.3488. Epub 2014 Mar 2. PMID: 24584119

Figure 1. The following set of images, obtained using the new MIBI device designed by Dr. Michael Angelo and Dr. Sean Bendall, and imaged in the Angelo lab at Stanford, demonstrates the multiplexing capabilities of the MIBI. This is an 18-plex image.

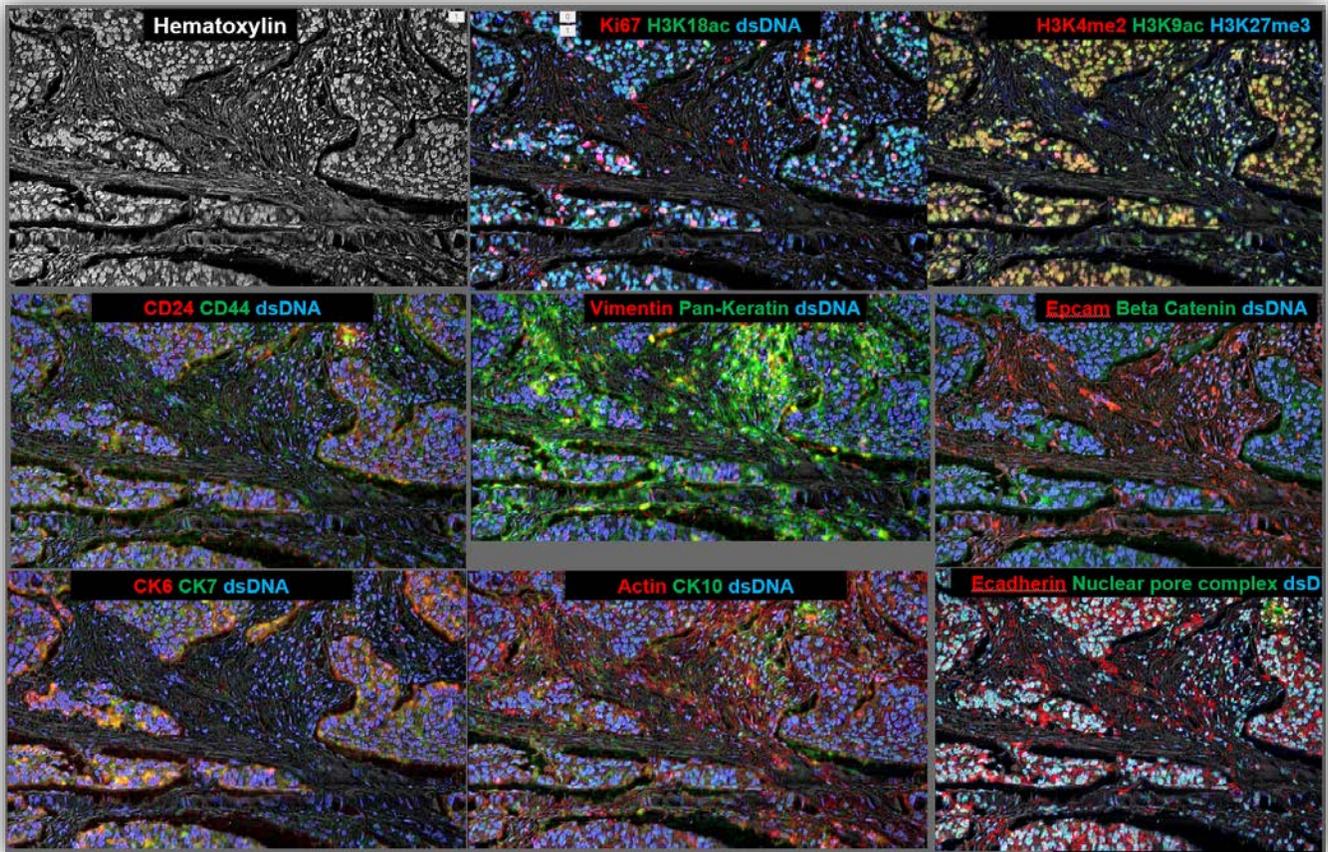


Figure 2. – CODEX analysis of mouse spleen cryosections co-stained for 28 antigens.

(A), (B) Three levels of magnification of 28 color CODEX staining of normal spleen. Three collated images on the top left correspond to the legend of antibody renderings per cycle, gross morphology photograph of MRL/lpr (left) and normal (right) spleen embedded in O.C.T. block prior to sectioning and montage of antibody renderings in all 14 CODEX cycles at high resolution. Red color corresponds to antibodies rendered by extension with dUTP-Cy5, green – dCTP-Cy3. All images are derived from a single scan with a 20x air objective of an area covered by 47 tiled fields. **(C)** staining of MRL/lpr spleen sections with same panel as in (A). Due to much larger area of the MRL/lpr spleen cross-section two characteristic regions together occupying same area as the normal spleen sample were imaged and jointly quantified. **(D)** Comparing cytometric data obtained by regular fluorescent flow cytometry (top row) with quantification from segmented CODEX images (bottom row) **(E)** Top two panels show heatmaps depicting the likelihood probability of a cell type to be within a vicinity of another cell type within a normal (top panel) spleen or MRL/lpr (middle panel) spleen. Bottom panel is a fraction bar blot showing changes in percentages of each cell type computationally identified in CODEX spleen data. Asterisk marks the DN T-cells vastly exceeding the normal numbers in MRL/lpr spleen.

Thus, we have developed the means, when MIBI imaging is ready, to appropriately segment high dimensional images.

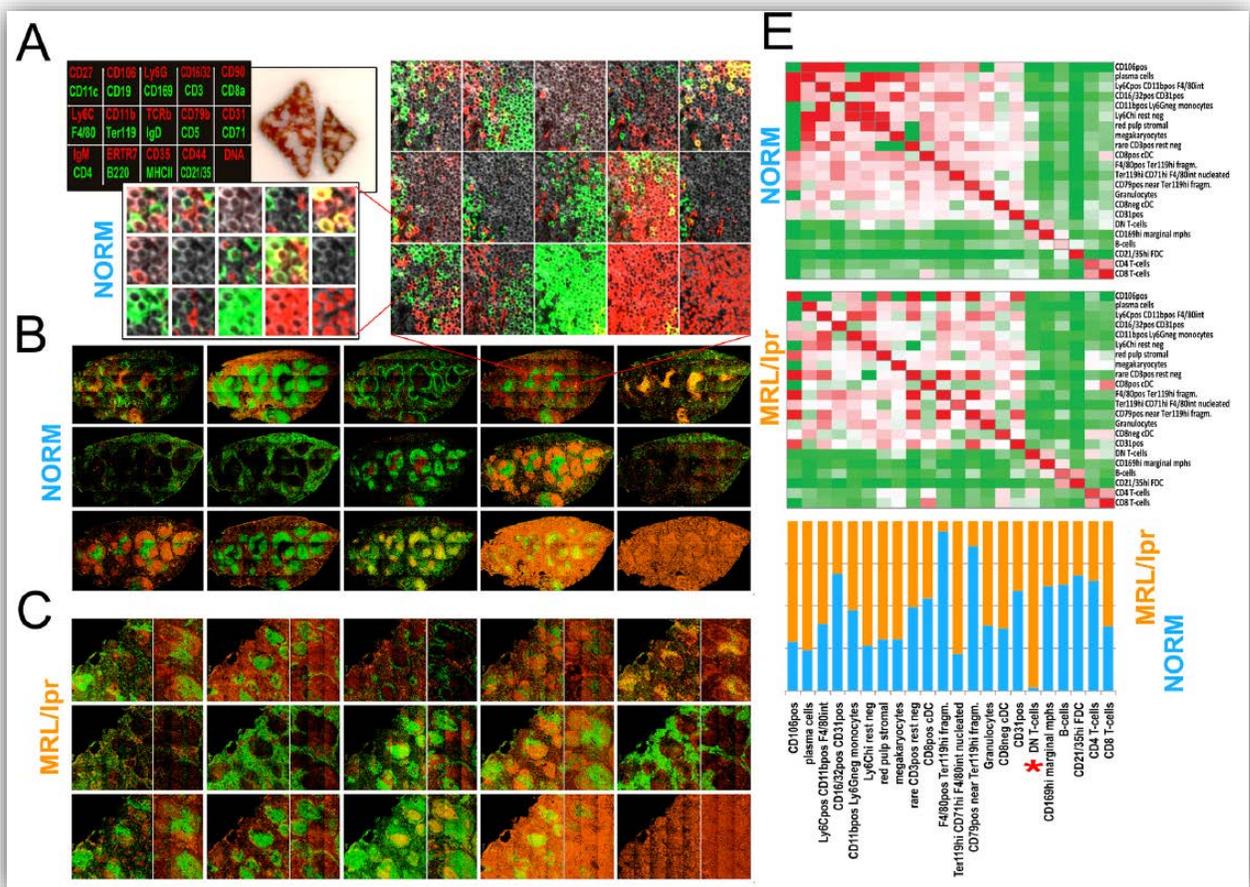


Figure 3. CODEX analysis of mouse spleen cells co-stained for 22 antigens.

(A) Montage of a fragment of imaging field of the 11 cycles of CODEX used to render the staining of mouse cells with 22 antibodies. TCEP cycles were omitted from the montage. First cycle corresponds to top left and last to bottom right. Right most bottom image shows cells pseudo-colored according to expression of all 22 antigens. Each antigen/cycle corresponds to a color from spectrum palette. Under each field of view is a biaxial corresponding to co-expression analysis of the antigens of the antigens measured in this cycle in all cells **(B-C)** Divisive marker trees using Vortex derived data for spleen (B) and bone marrow (C) produced by X-shift analysis of 22-dimensional CODEX dataset. Callouts contains biaxial plots for characteristic antigens for selected population identified in X-shift analysis of 22-dimensional CODEX data. This is a preliminary run for data analysis by imaging.

