THE NIH COMMON FUND
2nd ANNUAL SINGLE CELL ANALYSIS INVESTIGATORS MEETING
APRIL 21-22, 2014 ■ NEUROSCIENCE CENTER, CONFERENCE ROOM C & D ■ ROCKVILLE, MD

Meeting materials and details are available online:
https://nihsinglecellmeeting2014.eventbrite.com
...or scan with a QR code reader on a mobile device.

MONDAY, APRIL 21, 2014

7:30 a.m. Registration Check-In

8:00 a.m. Welcome/Opening Remarks
Thomas Insel, Director of the National Institute of Mental Health (NIMH)
Roderic Pettigrew, Director of the National Institute of Biomedical Imaging and Bioengineering (NIBIB)

8:10 a.m. Single Cell Analysis Program Overview
Andrea Beckel-Mitchener, Program Co-Coordinator, NIMH
Richard Conroy, Program Co-Coordinator, NIBIB

8:20 a.m. Session 1 – Single cell analysis in a complex dynamic environment

Keynote Address
How the Nanomechanics by Which Macrophages Pick-Up Their Prey is Adversely Affected by Few Common Drugs
Viola Vogel, ETH Zurich Swiss Federal Institute of Technology, Switzerland

8:50 a.m. Novel Tools for Monitoring the Innate Immune Signaling Network at Single Cell Level
Sergi Regot, Covert Lab, Stanford University

9:10 a.m. Near-Field Laser Ablation Sampling for Single Cell Biomolecule Mass Spectrometry
Kermit Murray, Louisiana State University

9:30 a.m. Microwell Arrays for High Throughput Transcriptomics and Proteomics
Peter Sims, Columbia University

9:50 a.m. Single Cell in situ RNA Profiling by Sequential Hybridization
Long Cai, California Institute of Technology

10:10 a.m. BREAK

10:30 a.m. **Session 2 – What are the current technical limits to identifying and characterizing a cell?**

Keynote Address
Spatially-Resolved Proteomic Mapping of Living Cells Using Engineered Peroxidase Reporters
Alice Ting, Massachusetts Institute of Technology

11:00 a.m. Temporal and Spatial Analyses of the of Neural Stem Cells Transcriptome in situ
Rui Sousa-Neves, Case Western Reserve University

11:20 a.m. Fluorescent Probes for Quantitation of Secretory Protein Levels in Single Live Cells
Erik Snapp, Albert Einstein College of Medicine

11:40 a.m. Monitoring T Cell Activation by Single Molecule Fish and Flow Cytometry
Sanjay Tyagi, Rutgers University

12:00 p.m. The Development of a Fyn FRET Biosensor by Directed Evolution for Single Cell Imaging
Yingxiao Peter Wang, University of California San Diego

12:20 p.m. **Lunch on your own**

1:45 p.m. **Session 3— Phenotypic & Genotypic characterizations of complex populations of individual cells**

1:45 p.m. Highly Multiplexed Subcellular RNA Sequencing in situ
Je Hyuk Lee, Wyss Institute and Harvard University

2:05 p.m. Individual CAR+ T Cells Recycle Effector Functions by Conjugating to Multiple Tumor Cells
Navin Varadarajan, University of Houston

2:25 p.m. **Lightning Round I (8 Talks)**
5 minutes and 1 slide each

Nanowell-Based Technologies for Single-Cell Analysis
J. Christopher Love, Massachusetts Institute of Technology

Optical Force Based Detection and Characterization of Disease in Mammalian Cells
Sean Hart, LumaCyte, LLC

Mapping pH on the Surface of Cancer Cells
Yana Reshetnyak and Oleg Andreev, University of Rhode Island

Solving the Puzzle of Cell Heterogeneity vs Disease Phenotype with Supercells
Wolfgang Losert, University of Maryland

Harvesting Single-Cell Transciptomes in Three-Dimensional Tissues
Deirdre Meldrum, Arizona State University

MultiOmyx™ Based Identification of Intestinal Progenitor Cell Landscapes
Michael Gerdes, GE Global Research Center

Progress in the Use of Patchclamp and RNA-Seq in the Evaluation of the Heterogeneity of Single-Cells
James Knowles, University of Southern California

Highly Multiplexed Quantification and Localization of Targeted RNA Transcripts in situ
HoSuk Lee, University of California San Diego

3:05 p.m. BREAK

3:20 p.m. Whole Exome Sequencing of Circulating Tumor Cells Provides a Window into Metastatic Cancer
Viktor Adalsteinsson, J. Chris Love Lab, Koch Institute at MIT & Broad Institute

3:40 p.m. Barcoding Thousands of Single Cells in a Single Tube by Droplet Microfluidics
Allon Klein, Kirschner Lab, Harvard University

4:00 p.m. Lightning Round II (7 talks)
5 minutes and 1 slide each

High-Throughput Robotic Analysis of Integrated Neuronal Phenotypes
Hongkui Zeng, Allen Institute for Brain Science

Quantitative Single Cell Analysis of Patient-Derived Cancer Stem Cells Identifies Unique Chemotherapy Response Signatures
Michael Masterman-Smith, University of California Los Angeles

Single Cell Genome Sequencing Reveals Clonal Stasis and Diversity in Breast Cancer
Nicholas Navin, University of Texas MD Anderson Cancer Center

Single Cell Whole Genome Amplification
Mark Franklin, GE Healthcare

Nano-well Assisted Patterning of Cells for High-Throughput Screening
Brittany Thomas, J. Chris Love Lab, Massachusetts Institute of Technology

PCR-Activated Cell Sorting
Adam Abate, University of California San Francisco

Towards Transcriptome Sequencing of 10,000 Single Adult Human Neurons
Kun Zhang, University of California San Diego

4:35 p.m. Wrap-Up

4:45 p.m. Day 1 – General Meeting Adjourns

6:00 p.m. - 9:00 p.m. Poster Session
Brookside Conference Room
Bethesda North Marriot Hotel and Conference Center
5701 Marinelli Road Bethesda, MD 20852

TUESDAY, APRIL 22, 2014

7:30 a.m. Registration Check-In

8:00 a.m. Recap on Day 1 and Remarks for Day 2
Yong Yao, SCAP Workgroup Team Lead, NIMH

8:10 a.m. Session 4 – Using multi-scale models and integrated, quantitative measurements to study function and disease

Keynote Address
Single Cell RNA-Seq Dissection of Kidney Development
Steve Potter, University of Cincinnati

8:40 a.m. Instrumentation Development for the Simultaneous Atomic Force Microscopy, Nuclear Magnetic Resonance Spectroscopy, and Fluorescence Microscopy of Single Cells
Charilaos Mousoulis, Neu Lab, Purdue University

9:00 a.m. Transcriptome Analysis of Cells in their Natural Microenvironment Reveals the Constraints on Generation and Regulation of Functional Plasticity
James Eberwine, University of Pennsylvania

9:20 a.m. Histo Mosaic: A Novel Diagnostic Technique to Detect Genetic Mutations in Tissue Slices
Frank Cheng-Chung Lee, Kartalov Lab, University of Southern California

9:40 a.m. BREAK

10:10 a.m. Session 5 – Challenges in longitudinal single cell analysis during development

Keynote Address
Induced Pluripotent Stem Cells and the Impact of Genomic Variation on Psychiatric Disorders
Flora Vaccarino, Yale University

10:40 a.m. Assembly and Use of Dual-View Inverted Plane Illumination Microscopy for Rapid, Spatially Isotropic Four-Dimensional Imaging
Hari Shroff, Section on High Resolution Optical Imaging, NIBIB/NIH

11:00 a.m. Integration of Single Cell Surface Phenotype, Function, and Transcriptome to Study the Proliferative and Homing Capacity of Human Mucosal T-Cells
Todd Gierahn, J. Chris Love Lab, Koch Institute at MIT

11:20 a.m. Common Analysis of Reference RNA at Single Cell Levels
Junhyong Kim, University of Pennsylvania

11:50 a.m. Wrap-Up

12:00 p.m. Day 2 – General Meeting Adjourns

SPEAKER ABSTRACTS

1. How the Nanomechanics by Which Macrophages Pick-Up Their Prey is Adversely Affected by Few Common Drugs

Viola Vogel

Department of Health Sciences and Technology, ETH Zurich, 8093 Zurich, Switzerland.

Antibiotics have saved millions of lives, but what comes next? Many strategies have been applied to fight bacterial infections, primarily designed to either kill bacteria via antibiotics or more recently to prevent their adhesion to surfaces and host tissues. Little attention though has been given to ask how these strategies might affect the ability of our immune cells to fight bacterial infections. We thus employed single cell assays to study the nanomechanics by which macrophages pick up their prey, and how these processes are affected by pharmaceutical drugs. To clear pathogens from host tissues or biomaterial surfaces, macrophages have to break a large cluster of adhesive bonds by which bacteria hold on to surfaces or tissue fibers. Single cell assays and nanotech tools were exploited here to analyze the single-molecule nanomechanics how various bacterial adhesins work and the mechanobiological processes that enable macrophages to lift-off bacteria from surfaces. We will then discuss specific examples how novel insights into such nanomechanical aspects suddenly reveal adverse and unanticipated side effects of common antibacterial drugs as they impair the ability of our immune cells to fight infections. What has escaped general attention, for example, is the finding that some drugs that are currently developed to prevent bacterial adhesion, also reduce the rate by which E. coli can be picked up by macrophages. Also some antibiotics can hinder the efficiency of macrophages to clear pathogens. Research into the mechanobiological aspects of bacteria and immune cells is thus not only scientifically rewarding, but might impact our ability to deal with infections.

2. Novel Tools for Monitoring the Innate Immune Signaling Network at Single Cell Level

Markus Willard Covert, Keara Lane, Jacob Hughey, Sergi Regot, Miriam Gutschow, Silvia Carrasco

Stanford University, Stanford, California

To understand the dynamics of the innate immune signaling network in single cells is a fundamental goal of immunology. Our lab studies how cells decode complex environmental information by measuring the single-cell responses of NF-κB to combinations of stimuli and time-dependent stimuli (Nature, 2010; Science Signaling 2009). Although these and similar approaches have been extremely useful in characterizing phenotypic heterogeneity within a population of cells (also in studying p53, for example), the conclusions that can be drawn from them are limited by the relatively low number of measureable outputs.

Here, we describe an expansion of the scope of live-cell dynamic imaging of the immune system, with new technologies that increase the number of measureable outputs. In particular, we have created a library of constructs and cells that enable monitoring of a variety of factors, encompassing multiple parallel signaling pathways. Moreover, to understand how network dynamics control gene expression, we developed a method to correlate the dynamics of transcription factors with the dynamics of endogenous gene expression in single cells, by integrating recently developed techniques for RNA FISH with our live cell imaging technology. Taken together, we have achieved a more detailed and system-level understanding of how environmental information is encoded in signaling network dynamics, and produced some new technology which we anticipate will be useful for the broader scientific community.

3. Near-field Laser Ablation Sampling for Single Cell Biomolecule Mass Spectrometry

Kermit K. Murray, Suman Ghorai, Chinthaka Seneviratne

Louisiana State University, Department of Chemistry, Baton Rouge, Louisiana

Mass spectrometry is one of the primary analysis techniques for cell biochemistry, but there are technological barriers in sampling scale that must be overcome for it to be used to its full potential with single cells. Mass spectrometry imaging techniques are currently limited to small molecules at high spatial resolution and large molecules at relatively modest spatial resolution. The goal of this research is to atomic force microscopy with laser ablation sampling for sub-micrometer sampling of large biomolecules from single cells and tissue. The laser ablation sample transfer system uses an AFM stage to hold a gold-coated silicon tip with a 30 nm radius at a distance of 10 nm from the sample surface. The metal tip acts as an antenna for the electromagnetic radiation and enables the ablation of the sample with a spot size much smaller than a laser focused with a conventional lens system. A 355 nm Nd:YAG laser was focused onto the gold-coated silicon needle at an angle 70º from normal reliably results in the removal of material from a 1 to 2 µm diameter and 200 nm deep spot. This corresponds a few ng of ablated material, which can be captured on a metal surface for MALDI analysis or at the tip of a nanocapillary for electrospray analysis. Nanoscale laser ablation sampling developed in this project will have applications not only in mass spectrometry but also in microfluidics. Laser ablation sampling with droplet capture will provide a new method for spatially resolved sampling into a microfluidic device. It will also allow a separation step to be added to mass spectrometry imaging that will enable the imaging of minor biomolecule components of tissue.

4. Microwell Arrays for High Throughput Transcriptomics and Proteomics

Peter A. Sims, Gregory Vieira, Sayantan Bose

Department of Systems Biology, Columbia University Medical Center, New York, New York

We are developing new tools for RNA and protein expression profiling that combine microfluidics, single molecule fluorescence microscopy, and next-generation sequencing to facilitate sensitive detection and identification of RNA transcripts and proteins. Our technologies take advantage of reversibly sealable microwell arrays fabricated in polydimethylsiloxane (PDMS), which can be chemically functionalized to capture proteins and nucleic acids. For transcriptomics, we have developed an approach to large-scale single cell RNA-Seq with on-chip amplification. For proteomics, we are working to identify individual protein molecules without the use of protein-specific affinity reagents. We use the microwells in our device to capture peptides resulting from tryptic digestion of protein molecules on a glass surface. With amino acid-specific fluorescent labels and endopeptidases, we can obtain course-grained sequence information from peptides at the single molecule level. Our ultimate goal is to develop a technique that is sensitive enough for unbiased identification of individual protein molecules from single cells.

5. Single Cell in situ RNA Profiling by Sequential Hybridization

Long Cai, Eric Lubeck, Ahmet. Coskun, Timur Zhiyentayev, Mubhij Ahmed

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California

We have recently demonstrated a technology using sequential hybridization and single molecule FISH to multiplex a large number of mRNA molecules directly in single cells in complex tissue samples. mRNAs in cells are barcoded by sequential rounds of hybridization, imaging, and probe stripping. The number of barcodes available with this approach scales as F^N, where F is the number of distinct fluorophores and N is the number of hybridization rounds. We call this method seqFISH and it is conceptually akin to “sequencing” mRNAs directly in cells by FISH.

6. Spatially-Resolved Proteomic Mapping of Living Cells Using Engineered Peroxidase Reporters

Alice Ting

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts

Microscopy and mass spectrometry (MS)-based proteomics are complementary techniques: the former provides spatiotemporal information in living cells, but only for a handful of recombinant proteins at a time, while the latter can detect thousands of endogenous proteins simultaneously, but only in lysed samples. In this talk, I will describe a new technology that combines the strengths of microscopy and MS by generating spatially and temporally-resolved proteomic maps of endogenous proteins within living cells. The method relies on a genetically targetable peroxidase enzyme that biotinylates nearby proteins, which are subsequently identified by MS. We used this approach to identify 495 proteins within the human mitochondrial matrix and 127 proteins within the mitochondrial intermembrane space (IMS), including 50 proteins that have never before been assigned to mitochondria. The labeling was exceptionally specific, able to distinguish between inner membrane proteins facing the matrix versus the IMS. Our catalog also revised the sub-mitochondrial localizations for several well-studied proteins, with the new assignments confirmed by electron microscopy.

7. Temporal and Spatial Analyses of the of Neural Stem Cells Transcriptome in situ

Rui Sousa-Neves[1], Thomas Atta-Fosu[2], J. Sebastian Chahda[3], Nathan Stopczynski[3], Weihong Guo[2], Claudia M. Mizutani[3]

[1] Dept. of Genetics and Genome Sciences, School of Medicine; [2] Dept. of Mathematics, [3] Dept. of Biology, Case Western Reserve University, Cleveland, Ohio

During development, progenitor neural stem cells self-renew and differentiate by deploying a complex series of transcription activators and repressors. The tight genetic control of these two cell states is highly conserved across the animal kingdom and warrants that tissues reach a relatively fixed final number of cell types, and at the same time, prevents over-proliferation of progenitors, which may lead to cancer. Although self-renewal and differentiation are associated to global transcriptome changes, the modulation of these cell states can only be fully understood at the single cell level in intact tissues. However, in vitro, cells tend to display abnormal and unpredictable behaviors. Together, these facts highlight the urgent need to develop single cell tools that preserve tissue complexity. To that end, our goal is to combine three different technologies that allow us to: (1) trace cell lineages within intact developing tissue that are labeled according to their the birth order, (2) assess active transcriptional states of multiple genes by detecting nascent transcripts in a combinatorial barcoding system, and (3) remove the expression of key genes implicated in tumorigenesis within the labeled cell lineages. Here we show that different neural cell lineages can be labeled by a color code that reports the age of cells and the phenotypes associated to the knockdown of genes that affect regulation of neural stem cells. We also show advances in high resolution detection of 7 to 15 nascent transcripts using bar-coding. Together, these tools provide the ideal experimental conditions to follow cell changes over time, selectively induce abnormal cellular choices, analyze and model growth patterns and monitor variations in the transcriptome in intact tissues. Further improvements of these tools have the potential to bring the resolution of spatial and temporal analyses of the transcriptome to several dozen if not hundreds of genes simultaneously, and apply to different tissues and organisms in which such lineage tracing analyses can be performed.

8. Fluorescent Probes for Quantitation of Secretory Protein Levels in Single Live Cells

Erik L. Snapp[2] *and Matthew Levy*[1]

[1]*Department of Biochemistry,* [2]*Department of Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, New York*

Secretory proteins are often robust markers of changes in disease-relevant cellular states including ER stress and metastasis. The absence of technologies for detecting specific luminal secretory proteins in live cells represents a major gap in cell imaging tools. Our goal is to develop and deliver highly sensitive reporters that can detect differences in the expression of diagnostic secretory proteins within a population of live cells. To do this, we will combine three existing technologies to create a new class of imaging tools, STABs (**S**ecretory **T**argeting **A**ptamer **B**eacons). As proof of concept we will focus on developing reagents which when internalized by cells signal the presence of vascular endothelial growth factor (VEGF). Successful results with this secreted protein will be extended to the UPR-induced endoplasmic reticulum proteins Ero1 and ERdj4. Theoretically, up to four distinct fluorescent dyes can be paired with unique aptamers to report on the expression of four different proteins. We envision the probe technology will have utility for basic research and rapid clinical analysis of tissue samples.

9. Monitoring T Cell Activation by Single Molecule Fish and Flow Cytometry

Yuri Bushkin, Felix Radford, Richard Pine, Alfred Lardizabal, Bonita Mangura, Maria Laura Gennaro, Sanjay Tyagi

Public Health Research Institute, New Jersey Medical School, Rutgers University, Newark, New Jersey, United States of America

Pathogens in host body fluids are usually detected by microbiological cultures or by in vitro amplification of their nucleic acids. Alternatively, either humoral (antibody) or cell-based (T cell-mediated) immune responses elicited in the host are exploited to identify the presence of a pathogen. In current assays peripheral blood mononuclear cells are stimulated with pathogen-derived peptides ex vivo and the activation of antigen-specific T cells is measured by the cytokines secreted by bulk cell population.

We are developing an approach in which activated T cells are profiled by monitoring the induction of mRNAs that occurs within individual cells and by the surface markers that distinguish between different T cell subtypes. These multiplex gene expression and phenotypic profiles can not only reveal the presence of pathogens but also hold the promise of distinguishing between different stages of the disease, for example, between latent and active infection.

For the detection of mRNA synthesis occurring upon activation, we use single molecule FISH in combination with flow cytometry. Developed earlier in our own laboratory, single molecule FISH (sm-FISH) is a powerful technique that employs about 50 oligonucleotide probes which simultaneously bind to the same mRNA target and produce a bright fluorescent spot for each mRNA molecules. For the detection of surface markers we use conventional immunofluorescence.

Our results show that the adoption of sm-FISH in flow cytometry yields a sensitivity of 5-10 RNA molecules/cell. We found that while some cytokine proteins persist in cells from prior in vivo stimulations, there is no remnant of mRNAs from those events. Thus the mRNA signals that we detect stem from mRNAs synthesized de novo upon ex vivo stimulation, indicating that mRNA based assays will have better signal to background ratios. We demonstrate multiplex detection of pairs of cytokine mRNAs in single cells. We have applied this approach for the detection of *Mycobacterium tuberculosis* specific T cells in PBMC isolated from patients suffering from active pulmonary tuberculosis and those that have been exposed to certain common viruses.

10. The Development of a Fyn FRET Biosensor by Directed Evolution for Single Cell Imaging

Yingxiao Peter Wang[1], *Mingxing Ouyang*[1], *Jing Liang*[2], *Huimin Zhao*[2]

[1]*Department of Bioengineering, UCSD, San Diego, California;* [2]*Department of Chemical and Biomolecular Engineering, UIUC, Champaign, Illinois*

Genetically-encoded biosensors based on fluorescence proteins (FPs) and fluorescence resonance energy transfer (FRET) have enabled the specific targeting and visualization of signaling events in live cells with high spatiotemporal resolutions. Single-molecule FRET biosensors, consisting of a donor and an acceptor FP fused together with two molecular domains capable of interacting with each other, have been successfully developed to monitor the activity of a variety of signaling molecules, including tyrosine/serine/threonine kinases. However, several weaknesses of these biosensors have hindered their broader application: (1) the dynamic range of these biosensors is generally limited; (2) the biosensors may interfere with and perturbed by endogenous signaling molecules at certain subcellular locations. At the current stage, the optimization of these FP-based biosensors is rather semi-rational and labor intensive. We have a developed a general high-throughput screening (HTS) method based on directed evolution to develop sensitive and specific FRET biosensors. We have first developed a yeast library and screened for a mutated binding domain for phosphorylated peptide sequence. When this mutated binding domain and the peptide sequence are connected by a linker and then concatenated in between a pair of FRET FPs, a drastic increase in sensitivity can be achieved. Further work is conducted to improve the specificity of the mutated biosensor via directed evolution. The results indicate that the optimized Fyn FRET biosensor developed can specifically respond to the stimulation by Fyn *in vitro*, but not by its close family member Src or other kinases. The mutation of the consensus binding domain and tyrosine residue in the biosensor also eliminate the FRET response of the biosensor upon stimulation *in vitro*. Similar results can be observed in single live cells, with the knockout of Fyn family having eliminated the FRET response which can be restored by the reconstitution of only Fyn, but not Src, Yes nor a kinase-dead mutant of Fyn. These results clearly indicate that we can systematically develop a sensitive and specific FRET biosensor utilizing directed evolution and high throughput screening. While the results provide the proof-of-concept for this systematic approach of engineering biosensors, this HTS method should be generalized for the development of, in principle, any FP-based biosensor to detect posttranslational modifications. We believe that this will advance the development of genetically-encoded biosensors and impact significantly on live cell imaging and cell biology in general.

11. Highly Multiplexed Subcellular RNA Sequencing in situ

Je Hyuk Lee, Evan R. Daugharthy*, Jonathan Scheiman, Reza Kalhor, Thomas C. Ferrante Joyce L. Yang, Richard Terry, Sauveur S. F. Jeanty, Chao Li, Ryoji Amamoto[2], Derek T. Peters[2], Brian M. Turczyk, Adam H. Marblestone, Samuel A. Inverso, Amy Bernard[3], Prashant Mali, Xavier Rios, John Aach, George M. Church*

Wyss Institute; Dept. of Genetics, Harvard Medical School, Boston, Massachusetts; [2] Dept. of Stem Cell and Regenerative Biology, Harvard University, Boston, Massachusetts; [3] Allen Institute for Brain Science, Seattle, Washington

Pairing transcriptome-wide RNA sequencing with *in situ* localization could considerably advance our understanding of the spatial aspects of gene regulation. Here we describe fluorescent *in situ* RNA sequencing (FISSEQ), in which stably cross-linked cDNA amplicons are sequenced within a biological sample. Using 30-base reads from 8,742 genes *in situ*, we examined RNA expression and localization in primary fibroblasts during wound healing *in vitro* with a median per-base error rate of 0.64%, detecting predominantly fibroblast markers, such as fibronectin (*FN1*), collagens (*COL1A1*, *COL1A2*, *COL3A1*), matrix metallopeptidases and inhibitors (*MMP2*, *TIMP1*), osteonectin (*SPARC*), stanniocalcin (*STC1*), and the bone morphogenesis-associated TGF-induced protein (*TGFBI*). FISSEQ correlated with Illumina RNA-seq with Pearson's *r* between 0.52 to 0.69 ($p < 10^{-16}$). Against gene expression arrays (BeadChip, Illumina), Pearson's *r* was as high as 0.73 ($p < 10^{-16}$) among moderately expressed genes. When migrating and contact inhibited cells were compared in a wound healing assay, 12 genes showed significant differences in gene expression ($p<0.05$ and >5-fold), eight of which were associated with the ECM-receptor-cytoskeleton interaction during cell migration, including *GID4*, *FHDC1*, *PRPF40A*, *LMO7*, and *WNK1*. We also tracked development-associated alternative splicing of *FN1* across different regions within the sample, as well as targeted sequencing of 5' UTR and/or RNA barcodes *in situ*.

Beyond transcriptome profiling, our platform facilitates numerous future applications. While highly parallel targeted detection *in situ* is challenging due to overlapping of signals, our partition sequencing method solves this problem. By sequencing the transcriptome and targeted variants simultaneously, tumor tissues can be analyzed for gene expression and evolving mutations *in situ*. Our platform also enables detection of RNA barcodes from any integrated or epichromosomal vectors. A 20-base sequence barcode can uniquely label up to 10^{12} cells, which is more than sufficient to label the nearly 100 billion neurons in the human brain for connectome studies. By fusing membrane proteins to high-affinity RNA binding peptides, it should also be possible to track a large number of synaptic pathways *in situ*. Similar to the history of next-generation sequencing, we expect rapid advancements in read length, sequencing depth and coverage, and library preparation (i.e. fragmentation, ribosomal RNA depletion, targeted sequencing) over the next several years. We are currently adapting FISSEQ to well-established next generation sequencers, increasing our throughput by up to four orders of magnitude. These advances will enable testing multiple hypotheses experimentally *in vivo*, dramatically reducing the cost of functional analysis per genetic variant.

12. Individual CAR+ T Cells Recycle Effector Functions by Conjugating to Multiple Tumor Cells

Navin Varadarajan[1], Ivan Liadi[1], Harjeet Singh[2], Nicolas Rey-Villamizar, Gabrielle Romain, Amin Merouane[1], Partow Kebriaei[2], Helen Huls[2], Peng Qiu[3], Badrinath Roysam[1], Laurence J.N. Cooper[2]

[1] University of Houston, Houston, Texas; [2] The University of Texas MD Anderson Cancer Center, Houston, Texas; [3] Georgia Institute of Technology and Emory University, Atlanta

Clinical grade T cells genetically modified to express a CD19-specific chimeric antigen receptor (CAR) for the investigational treatment of B-cell malignancies comprise a heterogeneous population of cells. The ability of administered T cells to participate in serial killing of tumor cells and avoid activation-induced cell death (AICD) is a predictor of therapeutic success. We developed Timelapse Imaging Microscopy In Nanowell Grids (TIMING) to dynamically analyze thousands of interactions between T cells and tumor cells. Engineered CD19-specific CAR^+ T cells launch fully-competent anti-tumor responses as defined by polarized motility, serial-killing, IFN-γ secretion and protection from AICD. They appear functionally tuned to respond to cell density; at elevated tumor cell numbers, individual CAR^+ T cells facilitated by their motility and polarization instigate simultaneous contacts for serial-killing while avoiding AICD. When tumor cells are limiting, T cells prolong contact with single targets, but subsequently undergo apoptosis. These data may help explain clinical observations in which the proliferation and subsequent numeric decline of administered CD19-specific T cells is adaptively controlled to match tumor bioburden, and can guide the selection of donors as well as the manufacturing schema used to generate a clinical product.

13. Whole Exome Sequencing of Circulating Tumor Cells Provides a Window into Metastatic Cancer

Viktor A. Adalsteinsson[1,4,10], Jens G. Lohr[1,2,3,10], Kristian Cibulskis[1,10], Atish D Choudhury[1,2,3], Mara Rosenberg[1], Peter Cruz-Gordillo[1], Joshua Francis[1,2], ChengZhong Zhang[1,2], Alex K. Shalek[5], Rahul Satija[1], John T. Trombetta[1], Diana Lu[1], Naren Tallapragada[4], Narmin Tahirova[4], Sora Kim[1], Brendan Blumenstiel[1], Carrie Sougnez[1], Alarice Lowe[6], Daniel Auclair[1], Eliezer M. Van Allen[1,2,3], Mari Nakabayashi[2,3], Rosina T. Lis[2], Gwo-Shu M. Lee[2], Tiantian Li[2], Matthew S. Chabot[2], Amy Ly[7], Mary-Ellen Taplin[2,3], Thomas E. Clancy[2,3,6], Massimo Loda[1,2,3,6], Aviv Regev[1,8,9], Matthew Meyerson[1,2,3], William C. Hahn[1,2,3,6], Philip W. Kantoff[2,3], Todd R. Golub[1,2,3,9], Gad Getz[1,7], Jesse S. Boehm[1]*, J. Christopher Love[1,4]**

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(10) These authors contributed equally to this work.

Comprehensive analyses of cancer genomes promise to inform prognoses and precise cancer treatments. A major barrier, however, is inaccessibility of metastatic tissue. A potential solution is to characterize circulating tumor cells (CTCs), but this requires overcoming multiple hurdles. Here, we report an integrated process to isolate, qualify, and sequence whole exomes of CTCs with high fidelity, using a census-based sequencing strategy. Power calculations suggest that mapping of >99.995% of the standard exome is possible in CTCs. We validated our process in two prostate cancer patients including one for whom we sequenced CTCs, a lymph node metastasis, and nine cores of the primary tumor. 51 of 73 CTC mutations (70%) were observed in matched tissue. Moreover, we identified 10 early-trunk and 56 metastatic-trunk mutations in the non-CTC tumor samples and found 90% and 73% of these, respectively, in CTC exomes. This study establishes a foundation for CTC genomics in the clinic.

14. Barcoding Thousands of Single Cells in a Single Tube by Droplet Microfluidics

Allon M Klein[1], Linas Mazutis[2]*, Ilke Akartuna[2]*, David Weitz[2], and Marc W Kirschner[1]*

** Equal contributors; [1]Dept. of Systems Biology, Harvard Medical School, Boston, Massachusetts; [2]School of Engineering and Applied Sciences, Harvard University, Cambridge, Massachusetts*

Among the central goals of single cell analysis are the identification of rare cell states, as well as cell sub-populations and their population hierarchy. For this goal, a system must be able to profile a representative sample of the target cell population, preferably consisting of hundreds or thousands of cells; it should capture multiple measurements per cell such as mRNA, protein levels and protein modifications; it should provide sensitivity and accuracy in these measurements; and it should be performed in tissues, or with cells obtained from tissues for immediate analysis. There is a difficult trade-off between analyzing many cells, and many cellular components per cell. A few methods address the accessible “middle ground” of assaying up to 100 cells for a wide but targeted panel of components, but this scale is not sufficient for many of the goals of single cell analysis.

We describe our progress in developing tools for profiling mRNA transcripts in at least 1,000 cells per run, with the aim of simultaneously profiling the concentration of tens of proteins at a next stage of method development. For this purpose, we have developed a pipeline for delivering more than 10^5 unique DNA barcodes to individual cells in a single tube using droplet microfluidic technology. Single cell collection and processing is rapid (less than one hour for thousands of cells) and requires significantly smaller amount of reagents when compared to other approaches. After barcoding, the material from all cells is combined for bulk processing, a step that greatly simplifies sample processing. The sensitivity of our method is currently limited mainly by the depth of sequencing, which is likely to improve with advancement of Illumina sequencers. Our presentation reviews technical innovations, assays of accuracy and sensitivity, and developmental milestones we have achieved to date.

15. Single Cell RNA-Seq Dissection of Kidney Development

S. Steven Potter[1], Eric W. Brunskill[1], Joo-Seop Park[2],Eunah Chung[2], Feng Chen[3], Bliss Magella[1]

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We have used a single cell RNA-seq strategy to dissect the molecular mechanisms of early kidney development. At several stages of kidney development histologically uniform populations of cells give rise to multiple distinct lineages. Single cell analysis allows us to define cellular level heterogeneities that presage distinct developmental decisions. By performing single cell studies at three stages of kidney development we were able to create an atlas of the gene expression patterns in different cell type lineages that drive nephron formation. Several interesting findings emerged. We provide a global view of the extensively polarized gene expression already present in the renal vesicle, the first epithelial precursor of the nephron. We also define quite unexpected RNA processing patterns. For example Hox read through transcripts can be spliced to produce intergenic homeobox swaps. We also identify a surprising level of exonic noncoding transcription, with RNA-seq reads restricted to a small subset of the coding region, often the 3' end. Perhaps most interesting, there was an unexpected pattern of precocious expression of differentiation markers. In particular, at early developmental times single cells often expressed genes related to several developmental pathways. For example, a single renal vesicle cell could simultaneously express genes associated with differentiated podoctyes, proximal tubules, and distal tubules. This provides powerful evidence that organogenesis involves a process of multilineage priming, followed by the combined actions of gene repression, turning off the genes of most possible lineages, and the activation of progressive numbers of genes associated with the chosen developmental direction.

16. Instrumentation Development for the Simultaneous Atomic Force Microscopy, Nuclear Magnetic Resonance Spectroscopy, and Fluorescence Microscopy of Single Cells

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Investigation of chemical and physical characteristics at the single-cell level can provide direct, quantitative observations of cellular properties and function with minimal variability. We have developed a tool for the simultaneous acquisition of multiple measurements in single cells including stiffness, adhesion, and chemical content. Single cell measures are attained by the conjunction of atomic force microscopy (AFM) with nuclear magnetic resonance (NMR) technologies, which is enabled by nanofabricated AFM/NMR hybrid cantilever probes. AFM is employed for the non-destructive measurement of cellular stiffness and adhesion by monitoring the cantilever's deflection as a spherical tip comes into contact with the cell. In addition to the AFM experiments, the cantilever allows for simultaneous NMR analysis through an embedded coil for the serial transmission of magnetic pulses and reception of resonance responses of the cell (Figure 1a). The probes were designed and fabricated as novel multi-turn, planar microcoils at the free end of the cantilevers (Figure 1b). The cantilever and cantilever holder have been designed to allow electrical connection to the radiofrequency instrumentation (either tabletop setup or commercial NMR/MRI spectrometer). A spherical tip of 27 μm diameter is manually attached to the cantilever (Figure 1c), enabling the capability of liquid imaging with a spring constant of approximately 0.25 N/m measured in air. A custom instrumentation setup was developed with the pulses and resonance frequencies controlled and recorded, respectively, using LabVIEW. Two different routes of simultaneous NMR acquisition are being investigated. The first one is based on the use of rare-earth neodymium magnets in a configuration that provides a 0.5 Tesla static magnetic field (i.e. 21.4 MHz proton frequency). The magnet assembly is coupled with an epifluorescence wide-field microscope along with the AFM probe head and instrumentation, providing an additional channel of real-time fluorescence or optical imaging and tracking of the observed cells (Figure 1d). In the second implementation, the NMR and AFM acquisition will take place in the wide bore of a small-animal MRI, which provides a static field of 7 Tesla (i.e. 300 MHz proton frequency). For this purpose, the same mounting assembly (AFM scanner and cantilever holder) can be used with a different sample stage. The AFM functionality of the microscope-coupled hybrid AFM/NMR is illustrated in Figure 2 with the mapping of topography and compressive moduli in a 30 μm thick bovine cartilage section, a representative three-dimensional microenvironment with distinct spatial heterogeneity in matrix and cellular composition.

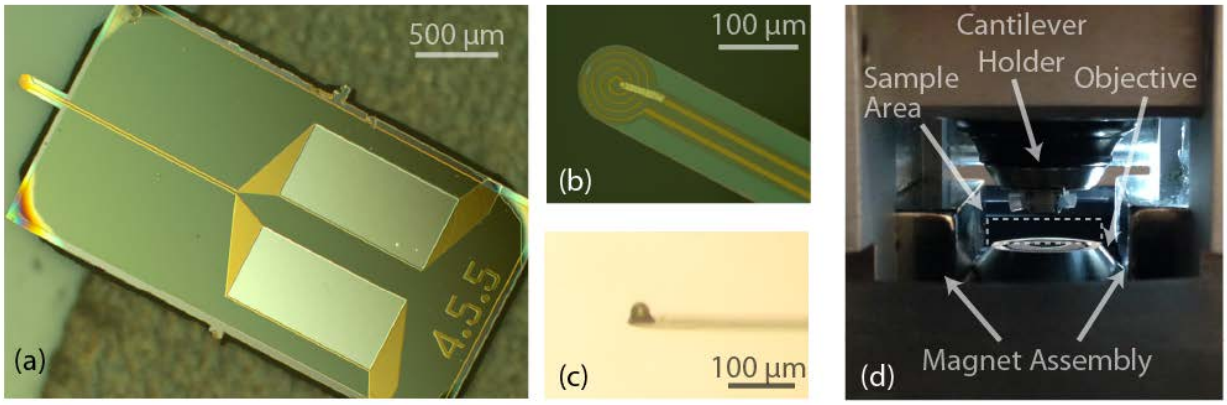

Figure 1. Probe structure and detail of the fluorescence microscopy-enabled tabletop AFM/NMR instrumentation. a) AFM/NMR hybrid cantilever probe with a 4-turn coil, b) detail of the 4-turn coil at the free end of the cantilever that enables NMR function, c) cantilever after the attachment of 27 micron spherical glass tip, and d) detail of the assembly around the sample area which enables the NMR, AFM and simultaneous fluorescence and optical imaging of the sample.

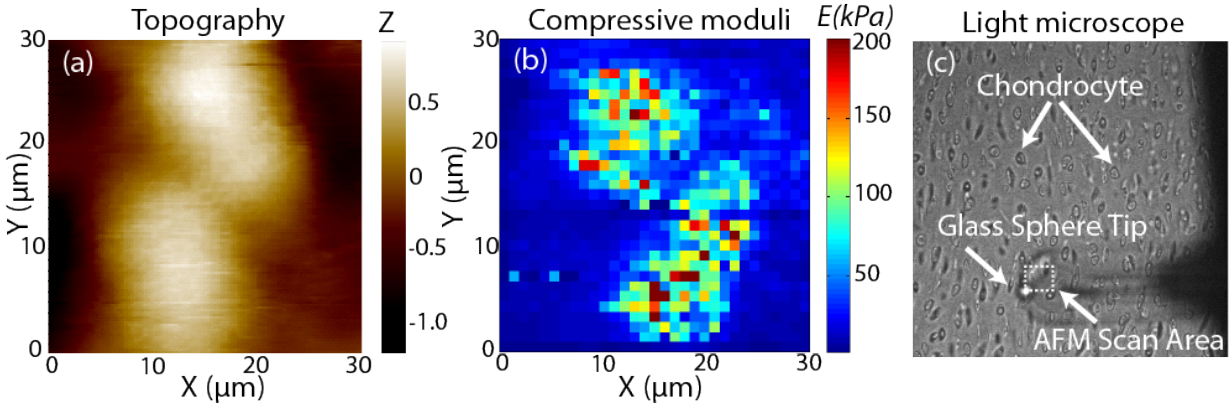

Figure 2. Combined AFM and light microscopy of a 30 µm thick bovine cartilage section. (a) Topography image by contact mode AFM, (b) corresponding compressive modulus map by force volume mode AFM, (c) light microscopy image illustrating the AFM scan area with the cantilever suspending on top. By a close examination of the light microscopy image, we can determine that there is a chondrocyte at the left side of the AFM scan area and another two at the right side, below the surface, which makes the compressive moduli at both sides significantly lower than that of the center.

17. Transcriptome Analysis of Cells in their Natural Microenvironment Reveals the Constraints on Generation and Regulation of Functional Plasticity

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The cellular microenvironment frames the functioning of cells so that they can act in coordinated systems. This coordination is reflected in identifiable iterative functional responses of cells to stimuli, which in turn suggests that the cells transcriptome may be similarly responsive. We have used the newly developed Transcriptome *In Vivo* Analysis methodology to assess the transcriptional landscape of cells in the live brain slice preparation where many of the normal neuronal connections are intact. This minimally invasive approach permits capture of the mRNA complement of cells through the light-induced activation of a RNA capture moiety. Initial data from these studies have provided fundamental insights into cellular phenotype and the constraints on transcriptional plasticity imposed by systems level coordination of cellular function. Data will be presented showing that while gross cellular morphology is similar between cells the systems input associates with transcriptome variability. If one makes the analogy to which came first “the chicken or the egg”, then one interpretation of these data is that systems develop in context of the available post-mitotic cells and are able to mold them only in context of the total “transcriptome potential” (all of the genes that ***can*** be expressed within a cell not simply those that are) of that particular cellular phenotype, thereby enabling while constraining the functioning of the circuit. In other words circuits develop and are maintained only as long as the total transcriptional potential of the constituent cells can be variably modulated. The flexibility and use of multiplexible TIVA approaches to address cell biological questions will be highlighted. These and other data provide a framework for investigating and understanding the role of the “connectome” in controlling, modulating and shaping the functionality of it’s constituent cells.

18. Histo Mosaic: A Novel Diagnostic Technique to Detect Genetic Mutations in Tissue Slices

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Current cancer diagnostic methods for tissue samples have high throughput but low sensitivity (immunohistochemistry (IHC), in-situ hybridization (ISH), bulk PCR), or high sensitivity but very low throughput (laser capture microdissection (LCM)). We have developed a novel technique for tissue analysis, called HistoMosaic that uniquely combines high throughput and high sensitivity. It leverages semiconductor technology to build millions of in-situ wells on tissue slides, and then runs PCR in each well in parallel. The result is a potentially high-resolution genomic map of the tissue down to single cell resolution depending on the size of the wells. The compartmentalization of tissue directly on glass slide assures that rare mutations are not drowned out by wild-types and spatial information remains intact. We have demonstrated the ability to perform PCR on wild-type KRAS DNA native in tissue on top of formalin fixed paraffin embedded slide. We also proved that the assay is able to distinguish spiked mutant and wild-type templates at 1 to 100 fold difference in PCR reactions performed in micro-wells. This technique could be extended to reverse-transcriptase PCR to create map of gene expression as well. Because the technique uses FFPE tissue slides, we can perform retroactive studies using patient histories and access archival sample to understand mutation influence on outcomes.

19. Induced Pluripotent Stem Cells and the Impact of Genomic Variation on Psychiatric Disorders

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The central nervous system contains the most diverse cell population of the human body both because of the extraordinary ability of neural stem cells to differentiate into a variety of neural cell types and the likely occurrence of somatic mosaicism during neurodevelopment. Thus, the study of the brain and its disorders exemplifies a very fruitful application of single cell technologies; yet, it also presents unique challenges. The low/uneven coverage when amplifying the genomes of single cells is an obstacle to the discovery of somatic genomic variants between sister cells. With regards to single cell transcriptome analyses, neural cells contain a complex variety of low abundance RNAs that are generally hard to detect. A potential alternative is to derive clonally amplified cell populations from single cells. Induced pluripotent stem cell (iPSC) lines derive from single somatic cells, amplifying the unique genome of their cell of origin and allowing the discovery of low frequency somatic mutations in the parental somatic cell population. We have been using iPSC lines derived from human skin fibroblasts to investigate the extent of somatic genomic variation in the skin of normal individuals. We obtained whole genome and whole transcriptome data from several iPSC lines and studied copy number variation (CNV) and single nucleotide variation (SNV) of multiple iPSC lines with respect to their somatic cells of origin. While each iPSC line manifests 2-4 CNVs apparently absent from the originating fibroblasts at the population level, at least 50% of these events are preexisting as low frequency somatic variants (15%-0.3% frequency) in the original fibroblasts cell population. Conversely, at least 30% of normal skin fibroblasts harbor a CNV. Furthermore, preliminary studies suggest that a single iPSC line and thus a single fibroblast cell in children of school age carries an average of 200 somatic SNVs validated in the cells of origin. Thus, there is a large extent of genomic variation in normal skin fibroblasts, which is surprising, as somatic mosaicism has mostly been linked to aging or disorders such as cancer.

To study the effect of *de novo* and somatic genomic variation (acquired post-zygotically in somatic cells or acquired during in vitro cell divisions) on neuronal differentiation and function, we generated cerebral cortical organoids from iPSC lines derived from 15 individuals in 5 families encompassing probands with autism and macrocephaly to recapitulate telencephalic neurodevelopment *in vitro*. This model allows the direct study of gene expression and function as neural cells divide and differentiate. Network analysis was used to combine genomes, transcriptomes and cellular phenotypes. The network analysis and biological validations suggest that cells from probands have a shorter cell cycle and a significant imbalance in inhibitory/excitatory neurons in early brain development driven by a set of key transcription factors. Current analyses focus on causative upstream factors that may illuminate the pathophysiology of neurodevelopmental disorders of complex etiology.

20. Assembly and Use of Dual-View Inverted Plane Illumination Microscopy for Rapid, Spatially Isotropic Four-Dimensional Imaging

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In line with the goal of our grant to translate prototype technologies into mature and stable systems that can be used in typical biology labs, we describe the construction and use of a compact dual-view inverted selective plane illumination microscope (diSPIM) for time-lapse, volumetric (4D) imaging of living samples at subcellular resolution. Our protocol covers assembly of the diSPIM from commercially available parts (including fiber-coupled excitation), procedures for aligning optics and verifying system performance, use of freely available software for system control and post-processing operations on raw data, and sample preparation. Unlike existing light sheet microscopy protocols, our method does not require the sample to be embedded in agarose; instead, samples are prepared conventionally on glass coverslips. For tissue culture cells or small embryos (such as *C. elegans*) successful implementation of the protocol results in isotropic resolution and acquisition speeds up to several volumes per second. Assembling and verifying diSPIM performance takes ~6 days, sample preparation and data acquisition up to 5 days, and post-processing 3-8 hours depending on the size of the data.

21. Integration of Single Cell Surface Phenotype, Function, and Transcriptome to Study the Proliferative and Homing Capacity of Human Mucosal T-Cells

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Mucosal immune cells play a prominent role in preventing many infectious diseases but can cause severe inflammatory diseases if they become dysregulated. Despite their importance in a litany of human diseases, little is known about their biology, primarily because the small number of cells that can be sampled from these surfaces limits the number and types of assays that can be used to study them. To remove this bottleneck, we have established an experimental pipeline for the analysis of mucosal immune cells using arrays of nanowells. The nanowells efficiently capture and retain single cells, enabling detailed and iterative analysis of the same live cells. Currently, the pipeline entails 16-color cytometry followed by cell activation and measurement of four secreted cytokines. Using this pipeline, we have identified unique subsets of T cells highly enriched in the human cervix. These mucosal T cell subsets demonstrate higher cytokine secretion capacity than other differentiated T cell in the same tissue. We are currently adding a proliferation assay and an end point single cell transcriptome analysis to enable in depth analysis of mucosal samples. With this pipeline, we will study the homing markers and the proliferative capacity of these cells to better understand the mechanisms that recruit and retain T cells in the female genital tract.

22. Common Analysis of Reference RNA at Single Cell Levels

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Recently there has been an explosion of technical developments and publications for single cell transcriptome sequencing. While methodological options have grown, the technical characteristics of various methods and the expected precision of single cell profiling is still not clear. Here, we employ three different sequencing methods on replicate single cell-level dilution samples of Human Brain Reference RNA (Life Technology) and Universal Human Reference RNA (Agilent) to assess the bias and variance of single cell transcriptome sequencing. We find that different sequencing methods exhibit specific biases but nevertheless the overall information accuracy of all examined methods is high. We discuss issues of accuracy, sensitivity, and bias in detail.

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1. Towards Transcriptome Sequencing of 10,000 Single Adult Human Neurons

Kun Zhang

Dept. of Bioengineering, University of California at San Diego

One major focus of the SCAP-T San Diego U01 center is to produce 10,000 transcriptome data sets of single neurons for unbiased classification of neuronal cell types in the human adult brain. A primary goal in the first two years of our project was to establish a robust and scalable pipeline for single-neuron transcriptome sequencing. To this end we have evaluated multiple strategies for single cell isolation from human post-mortem brains and various methods for single-cell transcriptome sequencing. We have optimized a production pipeline based on a combination of neuronal nuclei preparation and sorting, cDNA generation and amplification with SmartSeq in Fluidigm C1 microfluidic chips, sequencing library preparation with Tn5 transposase and Illumina sequencing. We have achieved a detection sensitivity close to single molecule, and are generating consistent data at a current throughput of ~500 neurons per month. Various aspects of pipeline optimization, quality control and preliminary analysis of 1,000 single neurons will be presented.

2. Harvesting Single-Cell Transciptomes in Three-Dimensional Tissues

Deirdre R. Meldrum, Shih-Hui (Joseph) Chao, Thai Tran, Laimonas Kelbauskas, Weimin Gao, David Richardson, Hong Wang, William Langenbach, Dean Smith

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Despite major advancements in single-cell technologies, the field has suffered from the lack of multiplexed methods to analyze large numbers of single cells *in situ* to deliver significant clinical impact. We are developing an innovative integrated system of nanometer-resolution 2-photon laser lysis (2PLL) and microfluidics to analyze single-cell heterogeneity *in situ*. Users can simply load a sub-millimeter live 3D tissue into a microfabricated trap in a microfluidic channel, select the target cells in the tissue based on their locations and morphology, and the system will then harvest total mRNA cell-by-cell in one hour. The system will correlate the mRNA expression levels of individual cells and their *in situ* coordinates within the tissue to reconstruct a 3D map of gene expression at the single-cell level. The system will be compatible with existing transcriptomic analyses, such as high-throughput qRT-PCR and RNA sequencing. We will present the preliminary results and validation on using the 2PLL system to harvest and quantify single-cell mRNA transcripts from 3D cell clusters of the MFC10 cell line in microfluidic channels. Specifically, we have 1) developed a streamlined process to select a cell cluster of interest and load it onto a microfluidic chip, 2) fine-tuned the 2PLL to precisely lyse target single cells in a 3D cluster without damaging the neighboring cells, 3) optimized conditions for harvesting and detecting mRNA transcripts, and 4) interfaced the system with the Fluidigm BioMark to perform high-content qRT-PCR at the single-cell level. Under the R21 project, we have established conditions for analyzing single-cells that are lysed by a 2PLL system at the tissue level. Our long-term goal is to establish a high-content instrument to harvest single-cell DNA/RNA/protein in heterogeneous live tissues for downstream analyses.

3. MultiOmyx™ based identification of intestinal progenitor cell landscapes

Michael Gerdes[1], Eliot McKinley[2], Kenneth Lau[2], Alberto Santamaria- Pang[1], Dirk Padfield[1], Vidya Kamath[1], Keyur Desai[1], Sarah Zhong[1], Joseph Roland[2], Frank Revetta[2], Jeff Franklin[2], Robert Coffey[2]

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Investigations into the mouse intestinal crypt stem cell environment have uncovered a panoply of cellular markers that identify different progenitor populations that are quiescent, partially quiescent, or quickly cycling. These progenitor cells provide homeostatic and damage response functions to the rapidly renewing intestinal epithelium. The interrelationship of stem cells expressing various combinations of markers is an active area of research and has led to competing models of stem cell dynamics in the crypt. By crossing Lgr5-EGFP- to Lrig1-Apple-reporter mice, we generated a model marking both the rapidly cycling Lgr5 (Barker et al. 2007) and the largely quiescent Lrig1 (Powell et al. 2012) stem cell populations. Uniquely with this approach, we can use antibodies for EGFP or Apple to reveal transcriptional output from Lgr5 or Lrig1 loci, respectively. These results can be compared directly to antibody staining against these native proteins to help determine how these genes are regulated post-transcriptionally. Using this double reporter mouse, we are employing single cell analysis with MultiOmyx of FFPE sections of the normal intestinal epithelium to correlate Lrig1- and Lgr5-positive stem cells with other known intestinal stem cell markers. This progenitor cell landscape is being assessed with respect to the proliferation and differentiation status within this environment using a comprehensive panel of 25 cellular markers made possible by MultiOmyx and iterative sequential staining and dye inactivation between staining rounds. Clustering of marker expression patterns will be used to evaluate and define the different cell populations. In the process of this analysis, we are maximizing the accuracy and reproducibility of cell segmentation and quantification by optimizing data analysis and collection parameters. The cell segmentation algorithms were improved using machine-learning approaches derived from user defined training sets. In a second set of studies in human colon carcinoma, 9 markers were used to define a co-expression map of cells undergoing EMT. Strikingly, altered intracellular localization and expression levels of PLAC8 in colon tumors is directly linked to cells undergoing atypical EMT, whereby CDH3 is up-regulated rather than CDH2. At the leading edge of tumors, we detected co-expression of PLAC8, CDH3, and Vimentin. The dynamic nature of marker expression changes associated with EMT is revealed by this approach (Li et al. 2014).

4. Quantification of Protein Levels in Single Cells

Brian E. Chen, Chiu-An Lo, Ibrahim Kays, Farida Emran, Tsung-Jung Lin, Vedrana Cvetkovska

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Accurate measurement of the amount of specific protein a cell produces is important for investigating basic molecular processes of the cell. The current methods for determining protein amounts have poor cellular resolution and are inherently destructive to cells, limiting the accuracy and relevance of the measurements. We have developed a technique that allows for quantitation of protein levels in single living cells. This Protein Quantitation Ratioing (PQR) technique uses a genetic tag that produces a stoichiometric ratio of a fluorescent protein reporter and the protein of interest during protein translation. The fluorescence intensity (i.e., brightness of the cell) is directly proportional to the number of molecules produced of the protein of interest, and thus is used to determine the relative protein amount within the cell. Using quantitative imaging and electrophysiology, we demonstrate that PQR can produce stoichiometric separations and linear relationships between different genes. Using the circadian system, we demonstrate cyclical changes in fluorescence in small lateral ventral neurons in the *Drosophila* brain. We use genome editing techniques to insert Protein Quantitation Reporters into endogenous genomic loci in three different genomes for quantitation of endogenous protein levels. Fluorescence quantification of endogenous RPL13A protein levels in a cell can be used to normalize across experimental and optical conditions, such as spherical aberrations, optical distortions, and imaging depths during *in vivo* imaging. The Protein Quantitation Ratioing technique allows for measurements of endogenous or exogenous protein amounts in single living cells, to relate cellular phenotypes as a function of protein concentrations.

5. Tattletales and T-Bow: Multiplex Fluorescent Protein Biosensors To Measure and Make Better Serial Killers

George McNamara and Laurence J.N. Cooper

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We do single cell killing assays on nanowell chips imaging thousands of human cytolytic CD8+ T-cells, each with one or more target cells. We now identify kills by fluorescent markers and morphology changes. We want to go beyond killing to measure multiple parameters in our live cells, in real time. We aim to generate sets of sequence specific Transcription Activators-Like Effectors - fluorescent protein fusion proteins (TALE-FPs) with cognate synthetic tandem repeat array landing sites, introduce these sets into our human CD8 T-cells using our Sleeping Beauty transposon system (same workflow for introducing chimeric antigen receptors for our clinical cAR T-cell therapy), and perform timelapse imaging of the localized reporters in our live T-cells in our single cell killing assay. We aim to report in live T-cells, in real time, (i) the activity of five T-cell activation responsive promoters, and (ii) biosensors for five molecules (ex. ATP/ADP, glucose, lactate, NADH/NAD^+, pyruvate.

Our key concept is that localizing with TALE (or nuclease dead Cas9) on its own landing site array we get better signal-t0noise ratio and dark volume for more reporters. Robinett (1996) established that a single 256 element landing site is sufficient to image GFP-LacI-nls as a bright dot.

The promoter → TALE-FPs bound to each of their cognate landing site array, will produce a rainbow of subcellular dots, “T-Bow”, to report on critical genes involved in T-cell activation, killing and memory. We will be able to use promoters of critical genes, ex. Interferon-gamma, Granzyme B, Perforin, CD28 and CTLA-4, or specific T-cell response elements. Initial response elements include hypoxia inducible factor (8xHRE), NF-KappaB, NFAT, STAT, and T-bet/Eomesodermin.

Our first TALE-Biosensors will be to the primary metabolites of the cell. With any of over 160 FP biosensors available by “plug and play” (see Newman 2011 *Chem Rev* and Frommer 2014 http://biosensor.dpb.carnegiescience.edu/biosensors), many other combinations of biosensors can be introduced into our T-cells and target cells.

Our preferred Sleeping Beauty transposase transfection method results in stochastic copy number of introduced genes. We anticipate this will enable us to go beyond conventional DNA based “bar codes” (that require lysis and sequencing of cells) to have unique live cell “dot codes” for every clone in both out T-cells and tumor cells. Combined with Cas9 and TALEN gene knockouts and homologous recombination gene replacement, we have a powerful platform to measure and rewire our T-cells to make better serial killers.

6. Development of a Prototype System (Microscope and Software) For Automated Live Cell Imaging And Exhaustive Single-Cell Lineage Analysis

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Single-cell lineage analysis is a research technique that characterizes cells at single cell resolution by performing cell tracking and creating lineage maps. This analysis has made a major breakthrough in developmental and cell biology, since a chronological record of events could identify the progenitor of a cell in question. Further, the record provides critical information on 4W & H (when, what, which cells, where and how) to address why the progeny has different feature(s). Single-cell lineage tracking is, however, known as an extremely laborious and time-consuming process and has been widely assumed that this type of analysis is hard to become a routine research tool. We therefore developed a prototype system, which is composed of a made-to-order microscope and a series of new software, for automated live cell imaging and single-cell lineage tracking. This system has dramatically reduced the processing time of single-cell lineage analysis, paving a way to make the single cell lineage analysis available as a routine laboratory tool that could reveal novel characteristics of mammalian cells.

Made-to-order microscope. (1) Microscope stage holds an 8-well chamber x2, allowing simultaneous comparison of 16 different conditions (creates up to 16 independent live cell imaging movies at real-time). (2) Live cell imaging can continue ~500 hours by acquiring images every 5-10 min and covering ~50,000 cell (creates 1-3 Tb data). (3) The Microscope is controlled by MetaMorph (commercially available software) installed on a Windows computer and image files created by MetaMorph are automatically transferred to a Macintosh computer.

Software (Macintosh) for the automated live cell imaging movie creation and the exhaustive single cell tracking. (1) C/C++/Objective-C is used. (2) The software transfers imaging files crated by MateaMorph (Windows) to a Macintosh computer. Then, the software assigns file names, creates focused image, adjusts image contrast and generates movies (up to 16 moves). (3) The software automatically identifies cells in images and performs an exhaustive single cell tracking. (4) The tracking controller unit of the software allows monitoring status of auto-cell tracking, verifying the results of auto-tracking and cratering cell lineage database. (5) A Data Analysis Program allows to generate cell lineage maps, performs statistical and other data analyses.

We analyzed HeLa cells and revealed that the culture is composed of highly heterogeneous cells. Furthermore, some cells show cell division abnormalities. Other cells, including primary cell analyses have been under way. We are going to expand our study to differentiating and iPS cells.

7. Single Cell in situ RNA Profiling by Sequential Hybridization

(Pg. 13)

Long Cai, Eric Lubeck, Ahmet. Coskun, Timur Zhiyentayev, Mubhij Ahmed

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We have recently demonstrated a technology using sequential hybridization and single molecule FISH to multiplex a large number of mRNA molecules directly in single cells in complex tissue samples. mRNAs in cells are barcoded by sequential rounds of hybridization, imaging, and probe stripping. The number of barcodes available with this approach scales as F^N, where F is the number of distinct fluorophores and N is the number of hybridization rounds. We call this method seqFISH and it is conceptually akin to “sequencing” mRNAs directly in cells by FISH.

8. Learn about the NIH Common Fund

Mai-Kim Norman and Ravi Basavappa

Office of Strategic Coordination, Office of the Director, National Institutes of Health, Bethesda, Maryland

Visual presentation only - please visit commonfund.nih.gov/ for more information.

9. Assembly and Use of Dual-View Inverted Plane Illumination Microscopy for Rapid, Spatially Isotropic Four-Dimensional Imaging

(Pg. 29)

Hari Shroff[1], Abhishek Kumar[1], Yicong Wu[1], Ryan Christensen[1], Panagiotis Chandris[1], William Gandler[2], Evan McCreedy[2], Alexandra Bokinsky[2], Daniel A. Colón-Ramos[3], Zhirong Bao[4], Matthew McAuliffe[2], Gary Rondeau[5]

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In line with the goal of our grant to translate prototype technologies into mature and stable systems that can be used in typical biology labs, we describe the construction and use of a compact dual-view inverted selective plane illumination microscope (diSPIM) for time-lapse, volumetric (4D) imaging of living samples at subcellular resolution. Our protocol covers assembly of the diSPIM from commercially available parts (including fiber-coupled excitation), procedures for aligning optics and verifying system performance, use of freely available software for system control and post-processing operations on raw data, and sample preparation. Unlike existing light sheet microscopy protocols, our method does not require the sample to be embedded in agarose; instead, samples are prepared conventionally on glass coverslips. For tissue culture cells or small embryos (such as *C. elegans*) successful implementation of the protocol results in isotropic resolution and acquisition speeds up to several volumes per second. Assembling and verifying diSPIM performance takes ~6 days, sample preparation and data acquisition up to 5 days, and post-processing 3-8 hours depending on the size of the data.

10. Analysis of Epigenetic States at Selected Genomic Loci with Single Cell Resolution

Daniel Mar, Karol Bomsztyk, Oleg Denisenko

University of Washington, Seattle, Washington

Epigenetic mechanisms, including transitions in chromatin structure and histone/DNA modifications, govern gene expression in a cell specific manner. To facilitate epigenetic studies, we have previously introduced Fast chromatin immuno-precipitation, Fast ChIP, and microplate-based Matrix ChIP assays that greatly improved sensitivity, reproducibility and throughput of the method, while decreasing cost and time per sample. Computation tools for data analysis and storage were also developed. However, these and other currently available approaches for chromatin analysis yield averaged epigenetic states for cells in a sample, therefore have limited application to study tissues composed of multiple cell types. To further facilitate epigenetic studies *in vivo* we have developed a novel method for chromatin analysis at individual genes with single cell resolution.

This method is a two-component proximity assay, where a fluorogenic substrate is tethered to the gene via biotinylated DNA probe, while the cognate enzyme is bound to the protein of interest as an antibody conjugate. Given close proximity, interaction between these components produces fluorescent signal visualized and quantified under microscope. We have previously demonstrated feasibility of this idea in a budding yeast model system. Two reagents were generated and tested, i) fluorescein diacetate (FDA), covalently attached to streptavidin, and ii) porcine liver esterase conjugated to secondary antibodies. With these reagents and biotinylated probes to different loci, we were able to detect a DNA binding protein (Rap1p) at loci that were previously defined as its targets, while no signal was detected at a control locus. These observations served as a proof of principle, and allowed for application of the assay to a more complex system, such as mammalian cells. In these studies we focused on DNA methylation analysis because of the established role of this modification in orchestrating gene expression in health and disease. Human renal cell cultures were used, HK2 and HEK293. With reagents tested in yeast and biotinylated probes to loci with high (centromeres) and low (GAPDH) levels of DNA methylation, we demonstrate that this assay allows for detection of DNA methylation present at a locus of interest in a single cell. For quantitative analysis we used Texas Red labeled antibodies that recognize both, FDA and fluorescein, thus allowing for normalization of green signal (fluorescein), and providing an estimate of DNA methylation density at a locus. For method validation, we used conventional ChIP assay. Applicability of this approach to FFPE specimens was also tested. Method challenges, limits and alternative routes are discussed.

11. Nanowell-Based Technologies for Single-Cell Analysis

J. Christopher Love

Koch Institute at MIT, Cambridge, Massachusetts

Many immune-mediated diseases—infectious diseases like HIV and autoimmune diseases like multiple sclerosis or diabetes—mediate pathology in specific tissues, yet most of our knowledge about them has resulted from studying cells circulating in blood. Increasing evidence suggests, however, that the biology of diseases in affected tissues can vary substantially from that in the blood, and understanding these differences may be critical to improve patient care. The significant heterogeneities among cells resident in tissues necessitates characterizing such samples with single-cell resolution, but existing technologies routinely employed by clinical immunologists (flow cytometry, ELISpot) require an excess of cells to use for analysis. Their inefficiencies can hinder the ability to pursue science understanding the human biology of diseases and treatments in tissues because biopsies yield very few cells.

In this talk, I will present advances on the development of this technology for characterizing sparse samples such as lymphocytes from tissue and tumor biopsies. Examples of antigen-specific screening of B cells from colon tissue from HIV-infected patients, and functional characterization of tumor-infiltrating T cells will be described. I will also discuss protocols for efficient retrieval and quality control of DNA libraries from single cells for sequencing. These examples will emphasize the translation of this technology from proof-of-concept towards clinical application in infectious disease and cancer.

12. Optical Force Based Detection and Characterization of Disease in Mammalian Cells

Sean J. Hart[1], Colin G. Hebert[2], and Alex Terray[2]

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There is a compelling need to develop instrumentation capable of characterizing and sorting cells for medical research and disease detection that are sensitive, selective, automated, and cost effective. Our research seeks to develop laser based separations that do not rely on labels such as antibodies or fluorescent molecules for cell detection. Rather, we utilize inherent differences in optical force, which arise from variations in particle size, shape, refractive index, or morphology, as a means of separating and characterizing particles. Optical forces occur when photons reflect and refract through a transparent particle and impart momentum. In our system, individual cells are analyzed automatically inside a microfluidic device using a near-infrared laser beam that exerts a physical force on the cells, which is then measured. The magnitude of the force on each cell is related to the intrinsic properties of the cell and varies across cell types and for differing diseases. Data and results for several applications will be presented including cell differentiation, viral infection of cells, phagocytosis of bacteria, apoptosis, and detection of cancerous cells. These applications will be discussed in the context of automated label-free cell analysis and sorting.

13. Mapping pH on the Surface of Cancer Cells

Yana K. Reshetnyak, Michael Anderson, and Oleg A. Andreev

Physics Department, University of Rhode Island, Kingston, Rhode Island

The acidity is associated with development of various pathological states such as solid tumors, ischemic stroke, neurotrauma, epileptic seizure, inflammation, infection, wounds, cystic fibrosis and others. Normal cell could be distinguished from highly glycolytic cell (for example, metastatic cancer cell) by transmembrane pH gradient and value of pH at surface of plasma membrane. We are developing novel tool to map pH at the extracellular and intracellular surfaces of individual cell in highly heterogeneous environment of cells *in vivo*. The tool would allow opening an opportunity to contribute in understanding of diseases progression and development of approaches of pH-based image-guided intervention. Our strategy is based on use of peptides of pHLIP® (pH Low Insertion Peptide) family. pHLIPs are water-soluble membrane peptides, which insert and fold in lipid bilayer of membrane only at slightly acidic conditions. Since the equilibrium is strongly shifted toward membrane inserted form at low pH, pHLIP injected into blood, circulates in body and accumulates in acidic tissue of tumors, site of inflammatory arthritis and ischemic regions. Here we used pH sensitive dye SNARF conjugated to pHLIP. SNARF-pHLIP inserts in membrane and tethers dye to membrane surface and serves as pH probe on cell surface. Cancer cells due to their predominately glycolytic metabolism produce significant amount of acid, which intensively pump out. Due to the limitation in diffusion we expect that some pH gradient will be established: pH near the cell surface will be lower than that in bulk solution. We performed experiments using cancer cells spheroids and found that pH was lower on surface when bulk solution pH was 7.0-7.4. However, when pH of bulk solution was below 6.5 the difference in pH disappeared. Using D-glucose or deoxy-glucose (non-metabolized glucose analog) it was possible to decrease or increase a pH-surface, correspondingly. These data confirm that pH on surface is lower than in bulk solution and it is caused by elevated glycolysis of cancer cells.

14. Solving the Puzzle of Cell Heterogeneity vs Disease Phenotype with Supercells

Wolfgang Losert, Julián Candia, Jayanth R. Banavar

Department of Physics, University of Maryland College Park

Cell heterogeneity and the inherent complexity due to the interplay of multiple molecular processes within the cell pose difficult challenges for current single-cell biology. Here, we introduce a novel approach that identifies a disease phenotype from multiparameter single-cell measurements, which is based on a cell-averaging procedure combined with a standard machine learning classification scheme. We are able to assess the optimal tradeoff between the number of single cells averaged and the number of measurements needed to capture phenotypic differences between healthy and diseased patients, as well as between different diseases that are difficult to diagnose otherwise. We validate our approach on two kinds of single-cell datasets, addressing the diagnosis of a premature aging disorder using images of cell nuclei, as well as the phenotypes of two non-infectious uveitides (the ocular manifestations of Behçet's disease and sarcoidosis) based on multicolor flow cytometry. [1,2] Beyond these specific examples, the approach proposed here to measuring multidimensional disease phenotypes is applicable to datasets generated by other kinds of current, state-of-the-art and forthcoming single-cell technologies, such as multidimensional mass cytometry, single-cell gene expression, and single-cell full genome sequencing techniques. We are very interested in applying our quantitative approach to analyze the tradeoff between the number of cells analyzed and the number of parameters measured to the emerging techniques presented at this meeting.

15. Progress in the Use of Patchclamp and RNA-Seq in the Evaluation of the Heterogeneity of Single-Cells

James A. Knowles, Ming-Yi Lin, Reymundo Dominguez, William J. Mack, Jae Mun Kim, Oleg V. Evgrafov, Robert H. Chow

University of Southern California, Los Angles, California

We have been performing whole-cell patch clamping followed by cytoplasmic extraction in brain and spinal cord slices both from human adult and fetal tissues. The range of tissue includes human adult neocortex from temporal, parietal, and frontal lobes; and fetal brain and spinal cord from gestation age of 10-20 weeks. Healthy cortical pyramidal neurons from layers II-V for adult tissue, subplate and cortical neurons for fetal brain and motor neurons for fetal spinal cord, were identified with infrared Dodt gradient system and standard whole-cell recording was performed. Cells were patch clamped in current-clamp mode for measuring spontaneous membrane potential change, followed by a series of current injections for triggered action potentials and in voltage-clamp mode for measuring currents from voltage-gated ion channels. Following recording, the cytoplasmic content from the soma was collected into the pipette with a strong negative pressure (-200 to -250 mmHg). The content was expelled into a PCR tube by breaking the pipette tip and a positive pressure (100 mmHg) and flash frozen with liquid N2. To date, we have collected 350 cells and this is increasing by approximately 80 cells a month.

While we have previously performed single-cell RNA-seq on neurons (Qiu et al., 2012), further examination of that data indicated that the Clontech SMARTer Ultra Low Input RNA was not accurately amplifying transcripts with low expression levels. Consequently, we have switched to using a modified SPIA reaction of NuGEN Ovation RNAseq V2 kit for cDNA Synthesis, followed by library construction using modifications of the Lucigen NxSeq kit. Our protocol does not contain any PCR amplification, which is known to be bias-prone. Instead, it uses linear amplification, which we think provides a more uniform amplification. These reads were then mapped and assigned to genes using our own RNA-Seq pipeline, now known as GT-FAR (Genome- and Transcriptome-Free Analysis of RNA-Seq). GT-FAR collects and evaluates multiple pre- and post-mapping quality metrics allowing the elimination of low quality reads in their entirety, or trims out the low quality bases. It then checks and removes any sequencing library adaptor bases from reads. The linear correlation of the number of aligned reads across all ~50,000 gene models from replicate libraries made from reference RNA is quite reproducible with average r2 = 0.95, 0.80, and 0.52 at the 100, 10, 1-cell levels, respectively. This method also has the advantage of detecting non-poly-A RNA.

16. Highly Multiplexed Quantification and Localization of Targeted RNA Transcripts in situ

Kun Zhang[1], HoSuk Lee[2], Matthew Cai[1], Ho Lim Fung[1]

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Being able to analyze the gene expression of a cell population with single-cell resolution and localization is critical for understanding the heterogeneity of structured tissues such as the cortex, tumors, and the developing embryo. Currently, the ability to characterize the transcriptome of single cells *in situ* is limited to probing a handful of RNA targets [1]. Advances in single-cell sequencing techniques have allowed the sequencing of the full transcriptome of isolated cells, but cannot retain spatial information. Fluorescent in situ sequencing (FISSEQ) is a novel technology that can fill this gap by quantifying the transcript abundance and localization simultaneously [2].

We are developing a targeted FISSEQ method that can selectively detect and localize 500+ transcripts *in situ*. Similar to exome sequencing, the motivation is to focus the acquisition of information to a subset of most informative transcripts. Due to the limited number of features that can be generated and detected within the volume of a cell, restricting the detection to pre-defined sets of transcripts is particularly appealing. To this end we leverage the multiplex capability and high specificity of padlock probes to detect targets of interest. Padlock probe sets can be designed to detect tens of thousands of targets *in vitro* [3] and each padlock probe can be tagged with a unique DNA barcode for hybridization-based decoding, which has a very quick reaction kinetics and can be performed isothermally[4]. Preliminary results on in situ rolony generation, in situ padlock capture and hybridization-based decoding will be presented.

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17. High-Throughput Robotic Analysis of Integrated Neuronal Phenotypes

Hongkui Zeng[1], Craig R. Forest[2], Ed Boyden[3]

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The cells of the brain exhibit a diversity of expressed genes, morphologies, and electrophysiological properties, and have come to be grouped into "cell types" that are distinguished by one or more of these characteristics. These properties can undergo adaptive changes with development, plasticity and disease. However, there is no one-to-one correspondence between cell type-defining expressed genes, morphological characteristics, and electrophysiological properties, and no unified taxonomy of brain cells. We have been developing the autopatcher system to enable integrative phenotyping of single cells in the brain – whole-cell patch clamp cellular recording in vivo allowing detailed electrophysiological characterization, dye infusion for morphological visualization, and extraction of cell contents for transcriptomic analysis. We have made significant progress in parameter and algorithm optimization, pressure control, miniaturization, deep brain autopatching, and autopatching in awake animals. We have also been developing new systems for multi-cell autopatching, 2-photon guided autopatching, pipette autoswapping, and auto-craniotomy.
For integrated neuronal phenotyping, we have been developing a series of standardized paradigms for electrophysiological, morphological and transcriptomic characterizations. A standard stimulation protocol for biophysical and intrinsic firing properties coupled with stringent QC criteria, as well as a systematic measurement of visual response properties, has been applied to in vivo whole-cell patching of layer 2/3 and 4 visual cortical neurons. The development of a standardized morphological reconstruction pipeline for biocytin-labeled neurons is near completion. This pipeline includes optimized staining protocol to visualize biocytin labeling in thick sections, automated bright-field microscopy to collect highest-resolution image stacks, and semi-automated reconstruction of neuronal morphology from these image stacks. To generate high-quality single-cell transcriptomic dataset, we have established single-cell RT-qPCR and RNA-seq protocols in fluorescently-labeled, FACS-sorted adult mouse cortical neurons, and are applying these protocols to cellular contents extracted from whole-cell patching. Fluidigm high-throughput RT-qPCR with hundreds of cortical layer-specific or interneuron subtype-specific marker genes has been validated for single neurons with high degree of specificity and quantifiability. We have also tested and optimized several single-cell RNA-seq methods, including SMARTer and Cel-Seq, on multiple cortical neuronal types. Clustering analyses of both RT-qPCR and RNA-seq data demonstrate that cortical neurons can be clearly classified based on transcriptomics, and cell-type signature genes can be identified. Overall, with significant progress in all these areas, we are well-poised to launch systematic and integrated investigations of individual neurons in the complex neural circuits.

18. Quantitative Single Cell Analysis of Patient-Derived Cancer Stem Cells Identifies Unique Chemotherapy Response Signatures

Michael D. Masterman-Smith[2], Jing Sun, Nicholas A. Graham, Jack Mottahedeh, Ed H. Panosyan, Dan R. Laks, Sung Hyun Lim, Araceli Nunez, William H. Yong, Thomas G. Graeber, Harley I. Kornblum

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Microfluidic tools that permit quantitative immunocytometry of microscopic patient samples have the capacity to transform diagnostics and treatment of human tumors. Using a microscale microfluidic 'chip' assay platform that enables high resolution single cell analyses, we demonstrate a systems-oriented approach to quantify the expression and activation of multiple signaling proteins in a complex and rare cell population, tumor-initiating cancer stem cells (CSCs) grown from human brain tumors. A panel of fifteen human brain CSC lines was profiled in parallel to identify multiparameter single cell signatures of EGFR-PTEN-Akt-TORC1 signaling in 14,865 total cells (mean=991 cells/sample) which correlate with response to EGFR inhibition and provide indicators of malignancy.

To parse the 89,190 multiparameter measurements, we utilized bioinformatic self-organizing maps (SOMs) and hierarchical clustering to identify two distinct clusters. One cluster was characterized by increased EGFR expression ($p = 0.0003$) with borderline significant decreased Akt and TORC1 activation. The second cluster had significantly lower EGFR expression with borderline significant higher Akt and TORC1 activation. The distinction between these clusters was further elucidated by physical measurements of the cells. High EGFR expressing cells were significantly smaller than high Akt and TORC1 activated cells ($p < 0.00025$). Concurrently, the nuclear area of high EGFR expressing cells were significantly smaller ($p < 0.00025$).

Off-chip sphere growth assays in a subpopulation of lines indicate cells from the high EGFR expressing cluster have lower LC_{50} profiles when exposed to the EGFR blocker erlotinib and have lower proliferative indices than cells with activated Akt-TORC1 signaling. The identification of a potential chemoresponsive phenotype in human-derived brain CSCs is an extremely promising finding for potential patient stratification and clinical targeting of these cells. Though multiparameter companion diagnostics for soft tissue CSCs remains a significant challenge, these data suggest patient-derived CSCs overexpressing EGFR and lacking downstream activation may be effectively targeted by EGFR inhibition. This proof-of-concept study showcases the utility of microfluidic chip-based platforms and quantitative multiparameter single cell analysis to move human CSC models into clinically useful directions.

19. Single Cell Genome Sequencing Reveals Clonal Stasis and Diversity in Breast Cancer

Nicholas E. Navin[1,2,3], Yong Wang[1], Jill Waters[1], Marco Leung[1,2], Anna Unruh[1], Whijae Roh[1], Ken Chen[3], Paul Scheet[2,4], Selina Vattathil[2,4], Han Liang[3], Asha Multani[1], Hong Zhang[5], Rui Zhao[6], Franziska Michor[6], Funda Meric-Bernstam[7]

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Sequencing studies of breast tumor cohorts have identified many prevalent mutations, but provide limited insight into the genomic diversity within tumors. Here, we developed a whole-genome and exome single-cell sequencing approach called Nuc-Seq that utilizes G2/M nuclei to achieve 91% mean coverage breadth. We applied this method to sequence single normal and tumor nuclei from an estrogen-receptor positive breast cancer and a triple-negative ductal carcinoma. In parallel, we performed single cell copy number profiling. Our data show that aneuploid rearrangements occurred early in tumor evolution and remained highly stable as the tumor masses clonally expanded. In contrast, point mutations evolved gradually, generating extensive clonal diversity. Many of the diverse mutations were shown to occur at low frequencies (<10%) in the tumor mass by targeted single-molecule sequencing. Using mathematical modeling we found that the triple-negative tumor cells had an increased mutation rate (13.3X) while the ER+ tumor cells did not. These findings have important implications for the diagnosis, therapeutic treatment and evolution of chemoresistance in breast cancer.

20. Single Cell Whole Genome Amplification

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The analysis of genomes at the single cell level offers unprecedented biological insights in diverse fields such as cancer research, immunology, and microbiology. To enable single cell genomics, a technology for amplification of genomic DNA is required that provides utmost sensitivity, accuracy and robustness.

We have previously developed a method of whole genome amplification (WGA) using multiple strand displacement amplification (MDA) by Phi29 DNA polymerase. This single subunit, proofreading DNA polymerase has excellent processivity and possesses strand displacement properties that enable the high-fidelity amplification of input DNA using random hexamer primers. Phosphorothioate modification of the primers prevents degradation by the DNA polymerase and dramatically stimulates reaction kinetics. This system is commercially available as the GenomiPhiTM DNA Amplification kit.

The project presented here builds on the merits of conventional MDA to develop methods to recover and amplify DNA from single cells of bacterial and mammalian origin. Quality of the output DNA in terms of genome coverage, uniformity of amplification, and error rate is critical to obtain useful single cell data in various applications. Microarray analysis and next-generation sequencing are used to validate our novel single cell MDA protocols and formulations.

A protocol has been developed to eliminate contaminating DNA from whole genome amplification reagents. Using a random-primed amplification method utilising cleaned reagents we have amplified genomic DNA from as little as a single bacterium. Single cell whole genome amplification and whole genome DNA sequencing results suggest that the new methods/formulations provide improved sensitivity and coverage with reduced amplification bias.

21. Nano-well Assisted Patterning of Cells for High-Throughput Screening

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Control over the spatial patterning of cells is important for the study of cell behavior in a variety of simulated biological environments. Important applications of cell patterning include tissue design, monitoring cell-cell interactions, investigating cell-microenvironment interactions, and tracking cell migration or proliferation in response to stimuli. One of the main advantages afforded by cell-based arrays as compared to biomolecular arrays is the ability to read out dynamic phenotypic responses of patterned cells, allowing for the assessment of complex biological responses. For the design of assays that require the study of cellular behavior, such as intracellular signaling or cell-to-cell communication, living cell arrays can provide controlled and high-throughput systems in which to study the effect of stimuli on cell fate and phenotype. Existing approaches for creating living cell based arrays rely on complicated surface modifications, require special equipment, and lack flexibility for designing high throughput living cell-based screens. In order to address this, we have developed a simple yet efficient cell patterning method. Nano-well assisted cell patterning (NWAP) relies on mechanical disruption of cells on a glass slide by the nano-well device in regions contacting the slide. Glass slides are first functionalized with cell surface-specific antibodies or non-specific adhesion molecules, such as poly-l-lysine, and then uniformly coated with cells of interest. Upon contact and removal of the nano-well device, cell patterns are formed by mechanical disruption. The key advantage of this technique is the ability to spatially register cell patterns with cognate nano-wells that can contain an environmental signal of interest, such as a single cell secreting a potential therapeutic. This allows for high-throughput measurements of interactions of patterned cells with discrete microenvironments. We show that NWAP can be used to create patterns of different sizes and cell densities, allowing for the precise control over patterning conditions. Finally, we use NWAP to create a high throughput screen to identify neutralizing antibodies against HIV. Key design parameters, including virus infectivity on glass, virus infection kinetics, antibody production kinetics, and neutralization signal readout were optimized to allow for rapid identification of neutralizing antibodies produced by single CHO cells loaded into nanowells. This, in combination with single antibody cell cloning, should allow for the identification of novel therapeutically relevant neutralizing antibodies against HIV.

22. PCR-Activated Cell Sorting

Adam Abate

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Fluorescence-Activated Cell Sorting (FACS) has universally impacted the biological sciences by allowing ultrahigh-throughput analysis and sorting of single cells. However, a significant limitation of FACS is that it can only robustly differentiate between cells using antibodies, precluding FACS analysis when antibodies are not available, such as for analyzing heterogeneous tumor cells or uncultivable viruses and microbes. We have developed a new ultrahigh throughput sorting technology called PCR-Activated Cell Sorting (PACS) that overcomes these limitations. In contrast to FACS, PACS allows ultrahigh-throughput analysis and sorting of single cells based on their nucleic acids. This is broadly useful throughout biology especially in instances in which specific antibodies are difficult or impossible to obtain. We are currently using PACS for analysis and sorting of uncultivable microbes and viruses existing in natural, diverse populations. We are also using the technology for analyzing and sorting cancer cells. In addition to enabling new studies not possible with FACS, PACS simplifies conventional cell sorting workflows by obviating the need for antibody labeling.

23. Analysis of Heterogeneity in Single-Cell Neural Progenitor Populations with Pathway Overdispersion

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A key promise of single-cell analysis is its ability to identify subpopulations comprising complex tissues and cell mixtures. Towards that aim we developed a computational approach for iterative identification and interpretation of heterogeneity within groups of cells assayed using single-cell RNA-seq. We apply our approach to characterize subtypes of neuronal progenitor cells found at mid-embryonic stage in the mouse brain, identifying multiple groupings of progenitor cells based on independent aspects of their transcriptional state.

The transcriptional state of a cell reflects a variety of biological processes, including persistent regulatory configuration traditionally associated with a cell type, transient processes such as cell cycle stages, local metabolic demands, or extracellular interactions. The presented approach aims to decompose this complex transcriptional signature by identifying known or newly-discovered gene sets that are linked to statistically significant heterogeneity within the measured collection of cells. Examination of literature-derived or annotated gene sets also provides important clues about likely functional interpretation of the detected heterogeneity, allowing further investigations to identify cell groups and differential expression signatures relative to the biological questions being investigated.

The method relies on a mixed-model approach to accommodate high levels of technical noise, quality differences between individual cells, as well as intrinsic biological noise. Individual error models are derived for each cell, and are used to normalize expression variance estimates for different genes, weigh the contribution of specific measurements in identifying new gene sets and deriving gene set principal component patterns. The resulting summaries are typically able to capture multiple concurrent aspects of cell variability.

In analyzing neural progenitor cells purified from E13.5 mouse brains, our approach points at a major axis of variability separating three major subsets of cells corresponding to gradual commitment to the neuronal fate: from apical progenitor to young committed neuronal cells. Alternate aspects of variation identify tangentially-migrating cells, as well as subsets of cells defined by differences in the cholesterol metabolism. We show that the identified neuronal progenitor subsets are tightly linked with the microanatomical organization of mouse ventricular regions.

24. Fully-Automated Sequential Patch Clamp Recordings In-Vivo and Progress Towards Miniaturization

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Patch clamp recording is the gold-standard for measuring single ion channel currents, synaptic input, and whole cell currents in neurons. However, patch clamp recordings are still something of an art form requiring great skill to record from only a few cells per day. Kodandaramiah et. al. recently developed a robot and neuron detection algorithm to autonomously find and record from neurons in-vivo.

We report extending the autopatching robot to obtain multiple, sequential recordings without any human interaction. Conventionally, once a recording attempt has been completed, a replacement pipette is prepared and inserted manually into the preamplifier headstage. Due to the small size of the pipettes and the silver electrode wire threaded into them, this process requires dexterous skill and attention. The pipette robot we have developed consists of a pipette insertion mechanism that replaces a used pipette while simultaneously threading the 250 µm silver wire into the pipette. We demonstrate the transition between a highly sensitive and laborsome experimental protocol to a mechanistic, repeatable, autonomous, and scalable system. Initial tests of the system show the ability to cycle through the 20-pipette cartridge and repeatably position them over a crainotomy in 8 week old male C57B/6 mice for autopatching.

We are also working to reduce the size and cost of the mechanical actuation system and pipettes to increase the number of possible simultaneous recordings in-vivo. Piezoelectric actuation systems used currently with the autopatcher are too large to be used on an awake, freely moving animal, and prohibit recording from dense array of pipettes for multiple simultaneous recordings. Our solution uses a piezo squiggle motor (27x13x7.5 mm) capable of 0.5 µm resolution with a 20 gram load and has a much smaller form factor than the standard piezo motor. We have also developed and tested fused silica micropipettes with a 350 µm outer diameter, which significantly increases the number of pipettes than can be co-located in one brain region and significantly reduces tissue damage due displaced tissue volume of larger pipettes, especially when targeting deep brain structures. We are also collaborating with Reid Harrison (Intan) to develop a chip-based intracellular amplifier that reduces the cost of patch clamping electronics by 2 orders of magnitude. These amplifiers show low noise and high resolution results in vitro and in-vivo and could revolutionize the field of patch clamping.

25. Micro-Environmental Control Techniques for Time-Lapse Imaging

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Bioptechs, Inc.

Technical Advances in microscopy, imaging, manipulation, chemistry, and photonics have created a rich environment for live-cell experimentation. In order to utilize these technical developments it is necessary to maintain the viability of the specimens in an environment that is also compatible with the associated optics. Experiments are longer, specimens more complex, and the sensitivity to thermally induced Z axis drift is more critical than ever. Therefore, new micro-environmental control technologies have to be employed to keep pace with todays research needs. In this presentation I will describe the current and future technologies for micro-environmental control.

26. Missing Link

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Single cell analysis is an extraordinary opportunity to learn about the biology of selected cells or cell types in vitro as well as in vivo, but also to challenge the current scientific standards and classic cannons for their sustainability in response to new technologies.

Working for many years with single cells separated in vitro from blood, bone marrow, epithelial, and tumor tissues, we have learned that the nomenclature used for identification of those single cells was only the conventional name given by classification authors to identify classes or genera of cells or tissues which undergo live cycles, maturation and differentiation processes, or are damaged (e.g., malignant alteration) and show apoptotic changes. The standard names are only modal synthesis of visual signals conveniently given appropriate names. Otherwise, they present a wide array of cells which differ metabolically, genetically and biochemically, if not so much morphologically – a full range of differences that may complicate any single cell analysis. This is particularly true for tumor cells which, after every division, recover into two different – similar, but not identical – cells with their own pattern of further growth.

The understanding of this spatiotemporal moment is a ***missing link*** that must be considered in any single cell analysis. But, is there something what can be used as a continuum, a chain that preserves its characteristics and can be used as an additional parameter if morphology and pathology are giving only modal value of visual signals?

We believe that intracellular molecular kinetics is one of such links. Defined as spatiotemporal measurements of the kinetics of accumulation of nano-particles as a result of bioactive protein catalysis of artificial substrates, could be a new method for testing small molecules for their effect on tumor cell metabolism and for possible metabolic management of antitumor medications.

We will present a concept and model for spaciotemporal measurements in a single, morphologically classified cell.

27. Live Single Cell Functional Phenotyping in Droplet Nano-Liter Reactors

Tania Konry

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While single cell heterogeneity is present in all biological systems, most studies cannot address it due to technical limitations. Here we describe a nano-liter droplet microfluidic-based approach for stimulation and monitoring of surfaceand secreted markers of live single immune dendritic cells (DCs) as well as monitoring the live T cell/DC interaction. This nano-liter in vivo simulating microenvironment allows delivering various stimuli reagents to each cell and appropriate gas exchanges which are necessary to ensure functionality and viability of encapsulated cells. Labeling bioassay and microsphere sensors were integrated into nano-liter reaction volume of the droplet to monitor live single cell surface markers and secretion analysis in the time-dependent fashion. Thus live cell stimulation, secretion and surface monitoring can be obtained simultaneously in distinct microenvironment, which previously was possible using complicated and multi-step in vitro and in vivo live-cell microscopy, together with immunological studies of the outcome secretion of cellular function.

28. Deconstructing the Dynamic Transcriptional Program of Pluripotent Stem Cells

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Pluripotent stem cells (PSCs) are capable of dynamic interconversion between distinct substates, but the regulatory circuits specifying these states and enabling transitions between them are not well understood. We set out to address this question and map the landscape of gene expression variability in PSCs by performing single-cell expression profiling using QPCR, RNA-Seq, single-molecule FISH, and quantitative immunofluorescence on PSCs under different chemical and genetic perturbations. We find that signaling factors and developmental regulators show highly variable expression in PSCs, with expression states for variable regulatory factors being coupled together, persisting through multiple cell divisions, and influencing target gene expression. Expression variability can be influenced by perturbation of signaling pathways and chromatin regulators. Strikingly, either removal of mature miRNAs or pharmacologic blockage of external signaling pathways drives PSCs into a low-noise ground state characterized by more uniform gene expression, increased self-renewal efficiency, and a distinct chromatin state. We find that this effect is mediated by modulation of opposing miRNA families on the *c-myc* / *Lin28* / let-7 axis. These findings illuminate the causes of non-genetic heterogeneity in PSCs and their consequences for cellular decision-making. The approach taken here provides a digital view of complex transcriptional programs, allows for their deconvolution into component modules and study of the interplay between them, and can readily be extended to other systems.

29. A Genetically Encoded Blue-to-Red Fluorescent Timer (Ft) Enables Brain Activity Mapping at Multiple Time-Points

Kiriana Cowansage & Mark Mayford

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To date, relatively few methodologies have been developed to permit studies of functional changes in neuronal activity that occur over extended periods of time. Our laboratory has developed and characterized a novel inducible transgenic mouse expressing a blue-to-red fluorescent timer (Subach et al, 2009) under control of the *c-fos* immediate early gene promoter. Characterization of this mouse indicates that FT is strongly expressed throughout the brain and can be behaviorally induced in its blue form after 48h off doxycycline (dox). Following induction by cellular activity, FT transitions to red within 48h and continues to emit measurable fluorescence for more than 100h in total. Here, we ask if this activity mapping system can be used to distinguish circuit-level differences between two episodes of brain activity triggered by exposure to perceptually different contexts. FT mice pre-exposed to two feature-rich spatial environments (Box A and Box B) were later tagged off dox with an 8-minute exposure to either Box A (day 1) followed by Box B (day 5), or Box A (day 1) followed by Box A (day 5). We find preliminary evidence that the pattern of neurons activated in CA1 of the hippocampus after a first exposure to Box A differ significantly from those activated four days later by exposure to Box B. These results suggest that the FT mouse can serve as a useful tool for studying circuit dynamics within behaviorally relevant neural networks over longer time intervals and broader anatomical regions than was previously possible.

30. Assessment of Neuron Heterogeneity in the Brain by Single-Cell Mass Spectrometry

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Single-cell mass spectrometry (MS) brings together advanced technologies to extend bioanalysis to volume-limited samples, opening new possibilities to investigate states of health and disease. We and others have developed analytical technologies to access the metabolome, proteome, and genome in single cells. Our combined experiences with mass spectrometry underscore that this technology is particularly powerful in addressing the chemical complexity of samples, but the success of measurements in the single-cell realm require careful and systematic handling of the specimen, microprobe sampling of just picoliters-femtoliters of volumes, as well as mass spectrometric detection with exceptional sensitivity.

We recently adapted capillary electrophoresis (CE) to single-cell MS and systematically explored the platform's utility to address neuronal heterogeneity in the brain. Singe identified neurons of 50–50 μm diameter were isolated from the central nervous system of Aplysia californica (sea slug), the model for the learning of memory, and measured using the CE-MS platform. The technology was quantitative to help quantify endogenous compounds in individual neurons, and it afforded <300 amol detection limit, which was sufficiently sensitive to profile the metabolome of the central nervous system. Here we discuss the technology and demonstrate its broad-range success at surveying hundreds of signals, mostly metabolites and small peptides, in 80+ individual neurons belonging to 10 different neuron types. The data demonstrate that neurons of different genotype have drastically different metabolic composition, but it also reveals that neurons of the same genotype can exhibit important metabolic differences. Unsupervised multivariate analysis of the data revealed that B1 and B2 neurons, which share locational, function, and morphological similarity of the central nervous system, have different metabolic composition and respond differently to extrinsic effect. Specifically, culturing caused the chemical composition of B1 and B2 neurons to become indistinguishable by our platform, indicating that these neurons regulate their metabolomes differently. The combined results establish that MS combined with single-cell sampling and high-efficiency chemical separation is a viable approach to monitor biochemical changes in single isolated neurons of the brain.

During these efforts, we have identified and solved a number of technological and methodological challenges to advance single-cell metabolomics. To this end, we present here our single-cell MS technology, the corresponding protocols, and explore new ways to hyphenate the platform to different types of mass spectrometers, anticipating that these advances will help mature single-cell CE-MS into a broadly utilized platform.

31. Microfluidic Perfusion Based Single Cell-Scale Respirometric Analyzer

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Significant progress has been made in the study of single cell genomics and proteomics indicating heterogeneity of cells within a population. However, to obtain a complete understanding of single cell processes complimentary matching studies of individual cell functions is needed, with cell energy metabolism being among the most critical cell functions. For this reason, development of a viable single cell respirometric analyzer can play a critical role in the growing area of single cell analysis.

Respirometry is based on real-time measurement of oxygen consumption rates in the fluid surrounding cells or tissues. Stepwise employment of various modulators of mitochondria functions, such as respiratory substrates, specific inhibitors of respiratory chain enzymes, oxidative phosphorylation uncoupler, and modulators of mitochondria transporters enables to characterize defects in mitochondrial membrane structure, functional activity of individual mitochondria respiratory and transport enzymes and the complex integration of mitochondria energy system. Respirometric analysis has potential to help unravel metabolically rare cells, which could be initiators of certain pathological transformations. The gold standard platforms for metabolic assays today are instruments based on amperometric and fluorimetric measurements of oxygen consumption rates as many as thousands and millions of cells.

Our group has been developing nano and micro pipettes with the objective of incorporating these pipettes as key components of single cell analysis tools. The goal of the present project is develop the capability to carry out dynamic respirometric measurements on individual cells, either one at a time or concurrently in an array configuration on many cells in order to characterize heterogeneity of cellular energy metabolism as well as to obtain average respiration behavior in cell population. Our development relies on the use of double-barrel pipette to perfuse individual cells that may be pre-positioned on a substrate or naturally distributed on surfaces of tissue samples. The open end of the double-barrel pipette is placed above a cell and the fluid carrying oxygen and other molecules and ions in one of the pipette barrels is forced to flow toward the cell, while the fluid is also forced to flow away from the cell taking the remaining oxygen and other analytes at the same rate in the other pipette barrel. The fiber optics oxygen sensor set in the other pipette barrel reads the oxygen signal.

We envision that this approach can be extended to the measurement of other cellular analytes through incorporation of corresponding sensors along with the oxygen sensor.

32. Real-Time Bioinformatics for Rapid Genotyping and De Novo Sequencing

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Knowing the disease-causing agent in infectious diseases, which contribute greatly to morbidity and mortality in the United States, and its antimicrobial susceptibility is essential for positive outcomes. Speed of discovery is critical because mortality rates in sepsis and other infectious diseases increase rapidly from time of infection. As classical clinical assays give only limited information, whole genome sequencing is becoming an increasingly important tool to identify human pathogens, to understand the infection history of disease outbreaks, and to determine the molecular mechanisms of pathogenicity including possible antibiotic resistance.

Single-cell genomic (SCG) approaches provide an alternative to the time-consuming approach of culturing pathogens and SCG is particularly relevant if pathogens cannot be brought into culture, which is the case for the vast majority of microbes. SCG with bacteria also provides information about associated phages, and these approaches can be applied to microbial eukaryotes (e.g., pathogenic fungi) that have smaller, less complex genomes than multicellular taxa. Currently, bioinformatic analysis of sequence data is performed only after sequencing of the DNA library is completed. To facilitate rapid clinical response against pathogens identified from specimens obtained as single cells or mixed species samples after cell sorting, we will perform the computational analysis simultaneously with sequencing reactions. Preliminary data, with partial reads of an multiple displacement amplified (MDA) single cell genome library from *Escherichia coli* demonstrate the feasibility of our approach. The state-of-the-art assembly strategy for single-cell data is based on iterative assembly with increasing *k*-mer (short runs of nucleotides) length and a majority of frequent *k*-mers can be derived from partial reads while controlling the false discovery rate.

33. Single Cell Proximity-Based Analysis Reveals Molecular Regulators Within the Bone Marrow Niche

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Interactions between heterologous cells are critical determinants of tissue development and adult tissue function. In the context of stem/progenitor cell niches, extrinsic molecular signals governing quiescence and self-renewal are essential for maintenance of cellular homeostasis. The discovery of niche-derived mediators of cell interactions has relied upon the manipulation of cells or genes based on previously hypothesized functional relationships. Here we report an approach biased only by the anatomic proximity of hematopoietic stem/progenitor cells (HSPC) to a putative niche cell as the criterion for molecular analysis. Comparative RNA-Seq profiling of single endosteal mesenchymal cells immediately proximal to transplanted HSPCs and those located further away revealed that HSPC-proximal cells have a distinct genome-wide transcriptional signature, highly enriched for genes previously implicated in HSPC niche function. Gene products of transcripts preferentially expressed in HSPC-proximal cells enabled an antibody-based method for prospectively isolating a comparable population and identified interleukin18 and Embigin as regulating quiescence and localization of hematopoietic progenitors, respectively. Proximity-based single cell analysis may be a generally applicable strategy for identifying niche cells and clarifying the molecular basis of heterologous cell interactions in-vivo.

34. Single Cell Transfection with Single Molecule Precision Using a Nanopore

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Synthetic biology demands tools capable of precisely modifying a cell's genetic code and modulating gene expression to create a predictable phenotype. Thus, a method for conveying a biologically relevant number of distinct bio-molecules into a cell is required. Yet, bulk gene delivery methods, such as electroporation and lipofection, require more than a million copies of an expression vector per cell. Therefore, they are grossly inefficient, resulting in low viability that is likely due to nonspecific and non-uniform delivery. On the other hand, single cell transfection tools are reportedly highly specific and promote high viability, but they rely on the stochastic processes for delivery of an average dose. Here, we present the development of a single cell electroporation system using a synthetic nanopore. It is not only highly specific and very efficient, but also transfects with single molecule resolution at low voltage (1 V) with minimal perturbation to the cell membrane—no other method offers such capabilities. The linchpin supporting this novel gene delivery method is a nanometer-diameter pore sputtered through a silicon nitride membrane <30 nm thick with a cross-section comparable to the DNA double helix or the bending radius of a plasmid. The silicon chip supporting a silicon nitride membrane with a pore through it was embedded in the multi-level microfluidic device that provides direct fluidic, electrical and optical access. A distinctive blockade in the open pore electrolytic current develops when a charged nucleic acid molecule immersed in electrolyte is impelled across the membrane through the pore by an applied electric field. This same electric field can also be used to deliver a nucleic acid molecule into a cell via electroporation. Due to the sub-nanometer control exercised in the fabrication of a nanopore, the electric field is essentially focused to a region around the nanopore, thereby minimizing disruption to the cell membrane during electroporation and so promoting high cell viability. To leverage the electric field for electroporation, cells can be positioned in close proximity to the pore using optical tweezers. Using this system, we demonstrate that 1. the blockades in the pore current corresponded to the number of molecules transfecting a cell; 2. single cells transfected with circular plasmids forcing the expression of DsRed fluoresced in red for 90 hours post transfection; and 3. when siRNAs targeting GFP were transfected into single cells that constitutively express both GFP and DsRed, these cells lost green fluorescence while fluoresced in red.

35. The Dynamics and Regulators of Cell Fate Decisions Are Revealed By Pseudo-Temporal Ordering of Single Cells

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Defining the transcriptional dynamics of a temporal process such as cell differentiation is challenging owing to the high variability in gene expression between individual cells. Time-series gene expression analyses of bulk cells have difficulty distinguishing early and late phases of a transcriptional cascade or identifying rare sub-populations of cells, and single-cell proteomic methods rely on *a priori* knowledge of key distinguishing markers[1]. Here we describe Monocle, an unsupervised algorithm that increases the temporal resolution of transcriptome dynamics using single-cell RNA-Seq data collected at multiple time points. Applied to the differentiation of primary human myoblasts, Monocle revealed switch-like changes in expression of key regulatory factors, sequential waves of gene regulation, and expression of regulators that were not known to have a role in differentiation. We validated some of these predicted regulators in a loss-of function screen. Monocle can in principle be used to recover single-cell gene expression kinetics from a wide array of cellular processes, including differentiation, proliferation and oncogenic transformation.

36. Insights Into Retinal Development Gained Through Comparative Multi-Species Single Cell Transcriptomics

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All adult tissues are composed of specialized populations of cells that play precise roles in the function of that tissue. In the central nervous system (CNS), there are a seemingly endless variety of neurons that wire into specific circuits and respond to specific stimuli. In order for the cells of the CNS to develop and connect properly, progenitor cells must make coordinated decisions about what specific cell fates to generate and then execute the downstream genetic programs with temporal and spatial precision to allow those cells to differentiate properly and connect at the right place and time. Characterizing the gene networks that underlie the developmental transitions leading to neuronal diversity is a fundamental goal of systems neuroscience. However, the cellular complexity of the nervous system makes this a daunting task. The retina is a widely used model system of CNS development due to its relatively simple layered structure, conserved neuronal birth order and easily identifiable mature cell types. Within the retina, the ganglion cells are the final output neurons. Since these ganglion cells are a diverse population and comprise only a small percentage of the total retinal cells (~2%), a detailed characterization of the gene networks that control their cell fate specification has encountered similar complexity-based obstacles as in the CNS. To overcome these issues, my lab employs single cell transcriptome analyses of ganglion cells isolated from multiple developmental times. In addition to using different times, we also isolate cells from the retinas of mouse, zebrafish and chicken. Specifically, we use transgenic mice and fish harboring fluorescent reporters to pinpoint our cells. In the chicken, we isolate cells based on size and then use a PCR-based screening method to identify the retinal ganglion cells for further profiling. We have transcriptionally profiled dozens of cells from each organism and are currently examining the similarities and differences among the cells. To validate the single cell results we perform in situ hybridizations on both tissue sections and dissociated retinal cells. We routinely see a very high correlation between the single cell profiling and subsequent in situ based experiments. In the future we will generate mutations based on our single cell results and examine the consequences on retinal development, both at a whole tissue level and at a single cell level. By comparing the single cell transcriptomes from multiple species, we are uncovering the conserved gene networks operating in individual developing ganglion cells.

37. A Phenotypic Signature for Pancreatic Cancer Metastasis

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Ninety percent of cancer patient deaths are associated with cancer metastasis. In particular, genomic analysis of pancreatic cancer suggests no consensus molecular signatures specific to metastasis. Here, we investigated the possibility that pancreatic cancer cells that had successfully metastasized to the liver displayed distinct physical properties from those in the primary tumor. We developed a comprehensive morphological analysis, visually-aided morpho-phenotyping recognition (VAMPIRE) approach to classify irregular cellular and nuclear shapes using a limited number of common shape modes through eigenshape decomposition and clustering approaches. Using an automated high-throughput microscopy assay, approximately 39,000 cells were analyzed from 13 previously sequenced patient-derived pancreatic cancer cell lines. Our results show that the lack of cell/nuclear morphological heterogeneity is a highly predictive feature of metastatic pancreatic cancer cells. This phenotypic signature for metastasis is further established among a cohort of 10 breast cancer cell lines. Our results also provide evidence that cancer metastasis is a selection process for the most common cell biophysical features. Our findings indicate that consideration of cell heterogeneity in phenotypes in primary tumors is key to decipher the molecular signatures specific to metastases as opposed to the global analysis of cancer cell populations.

38. Single-probe Sampling and Ionization Technique for Mass Spectrometry Analysis of Single Cells: Development and Applications

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University of Oklahoma

Single cell mass spectrometry (MS) has the potential to completely change the paradigm of biological sampling for molecular analysis, and the impact of this technical advancement is impossible to underestimate. To realize the potential of this method, new and superior analytical methodology must be developed to execute the MS analysis at the single cell level of resolution with minimal perturbation of the *in vivo* condition. We have developed a novel MS method for single cell analysis using a new technology: the Single-probe (SP) device. The SP is a multifunctional unit capable of both microscale sampling and MS ionization. Due to its extremely small tip size, the SP can be inserted into individual living eukaryotic cells allowing for single cell MS analysis to be performed in real-time under ambient conditions (room temperature and atmospheric pressure). The SP is fabricated by embedding a fused silica capillary and a nano-electrospray (nano-ESI) emitter inside of a laser-pulled dual-bore quartz needle. For single cell sampling, the SP is inserted into the cell, and the embedded silica capillary transports sampling solvent to the tip of SP. The cellular component species are immediately drawn and ionized via the nano-ESI emitter for MS detection in real-time. We have validated our novel single cell analysis method through a diverse series of experiments, including the detection of different small drug-like molecules inside of living cells. The utilization of our novel SP technique for *in-situ* and real-time analysis of single cells will broadly impact both biological and pharmaceutical sciences.

39. Single Cell Analysis Platforms for Bridging Phenotypic Response to Genotype

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A growing body of evidence supports the presence of cancer "stem-like" cells (CSC) in many cancers. In the CSC model only a limited subset of the heterogeneous population actually retains the ability initiate new tumors, grow, and metastasize. Understanding and analyzing this heterogeneity is important to improve our basic understanding of cancer biology. The ability to target specific cell subtypes has great potential for cancer therapeutics. Unfortunately, tumor cell heterogeneity is difficult to study. Traditional research tools, such as petri dishes and bulk PCR, are insufficient as they create data that is reported as averages over large numbers of cells, ignoring underlying cellular heterogeneity. Recently, single cell genomic tools (e.g., Fluidigm® platform) have been introduced; however, these systems cannot easily culture cells long term and perform biological assays. As a result, it is still poorly understood how genotype manifests in phenotypic responses and what genetic changes lead to a particular phenotype.

We report multiple microfluidic platforms developed for robust single cell capture, long-term clonal culture of heterogeneous single cells for drug screening and differentiation study, and selective cell retrieval for further phenotypic and genotypic analysis. High-throughput single-cell drug screening chip is designed to screen drug responsiveness (or drug resistance) and/or monitor development of cancer. This platform allows high-throughput screening of >1,000 assays from single experiment. Single-cell sphere formation chip is a derivative from the single cell drug-screening chip, allowing suspension cell culture by non-adherent surface coating. This platform grants orders of magnitude higher throughput of forming single cell derived spheres than conventional methods. Single-cell migration chip allowing single cell chemotaxis on chip is designed to study cancer cell motility and chemo-attraction of cells in an array of multiple migration channels. Ratio controlled cell-cell interaction chip precisely controlling the number and type of interacting cells is designed to understand cell-cell interaction and its effect on cancer cell proliferation, differentiation and drug resistance. Finally, single-cell retrieval technique will be introduced to selectively release the target single cells from novel CNT/metal modified surfaces within the microfluidic devices with high precision by focused laser exposure. This allows to characterize single cell genotype after observing, in assays, the phenotypic responses of the cells.

40. Temporal and Spatial Analyses of the of Neural Stem Cells Transcriptome in Situ

(Pg. 15)

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During development, progenitor neural stem cells self-renew and differentiate by deploying a complex series of transcription activators and repressors. The tight genetic control of these two cell states is highly conserved across the animal kingdom and warrants that tissues reach a relatively fixed final number of cell types, and at the same time, prevents over-proliferation of progenitors, which may lead to cancer. Although self-renewal and differentiation are associated to global transcriptome changes, the modulation of these cell states can only be fully understood at the single cell level in intact tissues. However, in vitro, cells tend to display abnormal and unpredictable behaviors. Together, these facts highlight the urgent need to develop single cell tools that preserve tissue complexity. To that end, our goal is to combine three different technologies that allow us to: (1) trace cell lineages within intact developing tissue that are labeled according to their the birth order, (2) assess active transcriptional states of multiple genes by detecting nascent transcripts in a combinatorial barcoding system, and (3) remove the expression of key genes implicated in tumorigenesis within the labeled cell lineages. Here we show that different neural cell lineages can be labeled by a color code that reports the age of cells and the phenotypes associated to the knockdown of genes that affect regulation of neural stem cells. We also show advances in high resolution detection of 7 to 15 nascent transcripts using bar-coding. Together, these tools provide the ideal experimental conditions to follow cell changes over time, selectively induce abnormal cellular choices, analyze and model growth patterns and monitor variations in the transcriptome in intact tissues. Further improvements of these tools have the potential to bring the resolution of spatial and temporal analyses of the transcriptome to several dozen if not hundreds of genes simultaneously, and apply to different tissues and organisms in which such lineage tracing analyses can be performed.

41. The Genotype-Tissue Expression Project

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Genome-wide association studies have identified thousands of novel loci for common diseases, but most are not associated with protein-coding changes and the mechanisms underlying the disease susceptibility remain unknown. The careful examination of gene expression and its relationship to genetic variation has thus become a critical next step in the elucidation of the genetic basis of common disease. Cell context is a key determinant of gene regulation, but to date, the challenge of collecting large numbers of diverse tissues in humans has largely precluded such studies outside of a few easily sampled cell types.

The Genotype-Tissue Expression (GTEx) project will create a public atlas of human gene expression and its relationship with genetic variation in multiple reference tissues, and an associated tissue bank to allow external investigators to perform additional assays on the samples. After a pilot period was completed in January 2013, the resource is scaling up to include approximately 900 post-mortem donors by the end of 2015. Nearly 30 tissues on average are collected from each donor, and each sample undergoes expert pathology review and gene expression analysis by deep RNA-Seq. Results indicate high quality nucleic acids from a wide range of tissues that yield robust gene expression profiles. Genome-wide analysis to detect cis-eQTLs, and to evaluate allele and tissue-specific expression patterns, has shown to validate known eQTLs and reveal novel ones. Preliminary data suggest that the GTEx resource will be a powerful tool to unravel patterns of genetic variation and gene regulation across diverse human tissue types.

42. The NIH Common Fund Single Cell Analysis Program: Research Opportunities to Define Cell "States" and Cell-To-Cell Variation

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Many biological experiments are performed on groups of cells under the assumption that all cells of a particular "type" are identical. However, recent evidence from studies of single cells reveals that individual cells within the same population may differ dramatically, and that these differences can have important consequences for the health and function of the entire population. Experimental approaches that examine only population-level characteristics can obscure these crucial differences. New approaches to single cell analyses are needed to uncover fundamental biological principles and ultimately improve the detection, diagnosis, and treatment of disease.

Responding to this demand, the NIH Common Fund Single Cell Analysis Program was initiated in 2012 and supports: 1) centers examining the transcriptional signatures of individual human cells in order to analyze cell-to-cell heterogeneity and to define specific cell types or "states" in a given population; 2) individual projects focusing on early-stage development of highly innovative tools and technologies that substantially improve the capabilities of single cell analysis; and 3) individual projects accelerating translation of promising technologies for single cell analysis from prototype into practice. The program intends to evolve through new funding announcements over the next several years. More information about the program is available at http://commonfund.nih.gov/Singlecell.

KEYNOTE SPEAKER BIOSKETCHES

Steve Potter

Steve Potter is a Professor of Developmental Biology, Cincinnati Children's Medical Center. He received his undergraduate degree from UCLA, PhD from University of North Carolina, and postdoctoral training at Harvard Medical School. Early accomplishments include showing that mitochondrial DNA is maternally inherited (1974), showing that the moderately repetitive fraction of DNA is made of transposable elements (1979), and publishing one of the early gene targeted mice (1991). We have recently focused on the use of single cell transcriptional profiling to study the mechanisms of early organogenesis. As members of the GUDMAP consortium we have helped to create an atlas of gene expression patterns in the developing kidney. This began with laser capture microdissection of developmental compartments, such as the renal vesicle, coupled with microarray gene expression profiling. We then used cell type specific transgenic-GFP mice and FACS to carry out RNA-seq gene expression analysis of specific cell types. Current efforts focus on microfluidics/robotics RNA-seq analysis of single cells from multiple developing systems, including the kidney, gut, lung and face. We find that single cell studies can create gene expression profile blueprints that define lineage decisions and drive developmental progression from progenitor to differentiated cell type.

Alice Ting

Alice Ting was born in Taiwan and emigrated with her family to the U.S. at age three. She grew up in Dallas, Texas, with two younger brothers. At age 15, Alice moved to Denton, Texas, to complete her last two years of high school at the Texas Academy of Math and Science. There, she developed a strong interest in organic chemistry, first through coursework, then through tutoring the subject, then through research in the laboratory of Prof. Roderick Bates.

Alice received her undergraduate degree in chemistry from Harvard. She started working in the laboratory of Prof. E. J. Corey as a freshman, and remained there until her graduation in 1996. In the Corey lab, Alice worked on the development of catalysts for asymmetric dihydroxylation and on the cloning of lanosterol synthase genes. As a Harvard undergraduate, Alice also served as a TA for Stuart Schreiber's Chemistry 27 course. These experiences cemented her interest in biological chemistry.

As a graduate student with Prof. Peter Schultz, first at Berkeley from 1996-1999, then at Scripps from 1999-2000, Alice worked on unnatural amino acid mutagenesis, single molecule imaging, and kinase engineering by phage display. After graduation in 2000, Alice moved across the street to UCSD, to complete a post-doc with Prof. Roger Tsien. There, her work led to the development of fluorescent reporters for imaging kinase activities in living cells.

Alice Ting has been a faculty member in the MIT Chemistry Department since 2002. Her work has been on the development of probes and reporters for live cell imaging. To simultaneously harness the power of genetics and the power of chemistry, Alice's lab has exploited enzymes that act on both protein and small-molecule substrates. Her lab has developed new technologies for imaging protein trafficking, protein-protein interactions, and enzymatic activity. Current research interests include imaging studies of synapse formation/development, and *in vitro* evolution of novel enzyme function.

Alice Ting has received the NIH Director's Pioneer Award, the Arthur C. Cope Scholar Award, the McKnight Technological Innovations in Neuroscience Award, the Technology Review TR35 Award, the Sloan

Foundation Research Fellowship, the Office of Naval Research Young Investigator Award, and the Camille Dreyfus Teacher-Scholar Award. She was appointed to the Ellen Swallow Richards Chair in 2011.

Flora Vaccarino

Dr. Flora Vaccarino is the Harris Professor, Child Study Center and Department of Neurobiology at Yale University. She directs a multidisciplinary research group with the goal of integrating knowledge from developmental neurobiology, genetics, human brain neuroanatomy and animal model systems to elucidate the pathophysiology of childhood neuropsychiatric disorders. She graduated from Padova University School of Medicine, Italy, and completed residencies in Neurology in Italy and in Psychiatry at Yale University. At Yale, she has been studying the development of rostral regions of the mammalian brain in animal models, with a focus on regulation of neural stem cells by both genetic and environmental factors. She contributed fundamental work on the regulation of cerebral cortical surface area expansion and neuronal specification by Fibroblast Growth Factor receptor signaling. With a key group of investigators, Dr. Vaccarino founded in 2009 the "Program in Neurodevelopment and Regeneration"(http://medicine.yale.edu/neurodevelopment/index.aspx), a collaborative interdepartmental program at Yale University that is leading interdisciplinary studies on human induced pluripotent stem cells (iPSCs), human neural stem cells and somatic mosaicism during brain development. We have led studies on copy number variation (CNV) and single nucleotide variation (SNV) of iPSC lines with respect to their somatic cells of origin, which have revealed extensive somatic mosaicism in normal human skin fibroblasts. Current work focuses on studying somatic genomic mosaicism in the brain and its implications for human development, evolution and disease. The long-term goal is to unravel how modifications in genomes and transcriptomes within cells of the brain contribute to the establishment of healthy or diseased neuronal networks during ontogenesis.

Viola Vogel

Professor Viola Vogel, Department Health Sciences and Technology at ETH Zurich, is heading the Laboratory of Applied Mechanobiology. With a PhD from the Max-Planck Institute of Biophysical Chemistry-Göttingen/Univ. Frankfurt, and after a Postdoc in the Department of Physics/UC Berkeley, she went through the ranks of Assistant/Associate/Full Professor in Bioengineering at the University of Washington, Seattle (1991-2004). She was the Founding Director, Center for Nanotechnology, University of Washington (1997-2003), and served on the PCAST subpanel finalizing the National Nanotech Initiative (1999-2000) and as US Representative on the Council of Scientists (HFSP, 2003-4) before moving to Switzerland (2004). Awards include the Otto-Hahn Medal, NIH FIRST Award, Julius Springer Prize 2006 for Applied Physics, ERC Advanced Grant (2008), Solvay Chair in Chemistry 2012 (Brussels), and receiving an Honorary Doctorate, University of Tampere Finland (2012). She represented the European Research Council in panel at the World Economic Forum in Davos 2013, and currently serves on several scientific advisory boards, including the Wyss Institute at Harvard (Boston), Institute of Bioengineering and Nanotechnology (A-Star, Singapore), Singapore-MIT Alliance for Research and Technology (SMART), Max-Planck Institute for Colloids and Interfaces (Golm), Nano-Initiative-Munich, Fondation Pierre-Gilles de Gennes Paris, and on the Board of Regents of the Ludwig-Maximilians-Universität, München.