



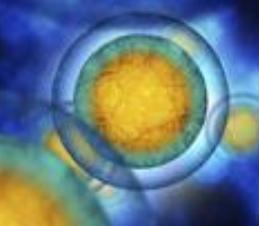
Poster Abstracts

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10	"Follow that Cell" Challenge Phase I Finalist	Nancy Xu	Photostable multiplexing nanoassays for real-time molecular imaging of single live cells
11	"Follow that Cell" Challenge Phase I Finalist	Eli Zunder	Tracking the phenotype of single cells using multicolor flow cytometry combined with cell-specific barcodes



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12	FY 2012 RFA-RM-11-013	Matthew Cai	Highly multiplexed in situ digital quantification of targeted RNA transcripts
13	FY 2012 RFA-RM-11-013	Ming-Yi Lin	Use of single-cell RNA-seq to molecularly define human Cajal-Retzius neurons
14	FY 2012 RFA-RM-11-014	Suman Ghorai	Near field laser ablation sample transfer mass spectrometry and application to single cell and tissue analysis
15	FY 2012 RFA-RM-11-014	Todd Gierahn	Correlating form and function: Integrating single cell transcriptome analysis into massively parallel single cell functional assays
16	FY 2012 RFA-RM-11-014	Deirdre Meldrum	Multi-parameter single-cell analysis for comprehensive biosignature discovery
17	FY 2012 RFA-RM-11-014	Qin Peng	Single cell imaging of epigenetic dynamics
18	FY 2012 RFA-RM-11-015	Yuri Bushkin	RNA temporal expression profiles in monitoring T cell activation using single-molecule fluorescence in situ hybridization and flow cytometry (FISH-Flow)
19	FY 2012 RFA-RM-11-015	Long Cai	Single cell in situ RNA profiling by sequential FISH (seqFISH)
20	FY 2012 RFA-RM-11-015	Daniel Colón Ramos	Towards the living connectome: Imaging of individual neurons in intact, developing embryos
21	FY 2012 RFA-RM-11-015	Craig Forest	High-throughput robotic analysis of integrated neuronal phenotypes
22	FY 2012 RFA-RM-11-015	Eliot McKinley & Michael Gerdes	Characterization of the intestinal stem cell landscape with MultiOmyx™ Single Cell Analysis
23	FY 2012 RFA-RM-11-015	George McNamara	Tattletales and T-Bow: Multiplex fluorescent protein biosensors to measure and make better serial killers
24	FY 2012 RFA-RM-11-015	Navin Varadarajan	Individual motile CD4+ T cells can participate in efficient multi-killing through conjugation to multiple tumor cells



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25	FY 2014 RFA-RM-13-020	Dipjyoti Das	Regulated transitions in levels of cell movement velocity and directional heterogeneity underlie vertebrate body elongation
26	FY 2014 RFA-RM-13-020	Bo Huang	Analyzing genomic elements in live animals by CRISPR imaging
27	FY 2014 RFA-RM-13-020	Tony Jun Huang	Validation of acoustic tweezers for single-cell analyses of purine metabolism
28	FY 2014 RFA-RM-13-020	Jeremy Norris	Single cell analysis using high spatial resolution and high sensitivity imaging mass spectrometry
29	FY 2014 RFA-RM-13-020	Arjun Raj	Validation and development of single nucleotide variant RNA FISH in single cells in culture and tissue
30	FY 2014 RFA-RM-13-020	Rui Sousa-Neves & Claudia Mizutani	Light-induced genetic alterations within single cell of a live vertebrate animal
31	FY 2014 RFA-RM-13-020	Jai-Yoon Sul	Nuclear pre-mRNA analysis of single cells in brain slice
32	FY 2014 RFA-RM-13-020	Tracy Young-Pearse	Single-cell detection of A β and sAPP α secreted from human iPSC-derived neurons and glia
33	FY 2014 RFA-RM-13-021	Ji-Xin Cheng	Microsecond Raman spectroscopy: Assessing single cell metabolism in a vital organism
34	FY 2014 RFA-RM-13-021	Zev Gartner	Identifying the intercellular networks regulating estrogen receptor expression with a high definition single cell printer
35	FY 2014 RFA-RM-13-021	Li He	Innovative reporters to characterize heterogeneous states among cells
70	FY 2014 RFA-RM-13-021	Jae Kyoo Lee	High spatiotemporal resolution mass spectrometry for single cell analysis
36	FY 2014 RFA-RM-13-021	Peter Nemes	Single-cell microsampling mass spectrometry for elucidating cell heterogeneity in the developing embryo
37	FY 2014 RFA-RM-13-021	John Parant	Light-induced genetic alterations within single cell of a live vertebrate animal



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38	FY 2014 RFA-RM-13-021	Guillem Pratx	Whole-body tracking of single cells by positron emission localization
39	FY 2014 RFA-RM-13-022	Olga Botvinnik	Novel computational metrics and approaches for evaluating population-wide differences in alternative splicing at the single-cell level
40	FY 2014 RFA-RM-13-022	Oleg Denisenko	Visualizing epigenetic mechanisms of megakaryocyte maturation at the single cell level
41	FY 2014 RFA-RM-13-022	Boyko Kakaradov & Janilyn Arsenio	Transcriptome-wide expression analysis of single cells during T-cell specification reveals distinct drivers of early differentiation states and final lineage fates
42	FY 2014 RFA-RM-13-022	Dinesh Rao	Characterizing tumor suppressive functions of microRNAs in B-cell neoplasia
43	FY 2014 RFA-RM-13-022	Ellen Rothenberg	Genomic site binding rules and regulatory factor function in developing T cells
44	FY 2014 RFA-RM-13-022	Assieh Saadatpour	Single-cell transcript profiles reveal multilineage priming in early progenitors derived from Lgr5+ intestinal stem cells
45	FY 2014 RFA-RM-13-022	Yan Song	Dynamic of splicing modality is uncovered from single cell transcriptomics during neuronal differentiation
46	FY 2014 RFA-RM-13-022	Daniel Tenen	Molecular biology of myeloid differentiation
47	FY 2014 RFA-RM-13-022	Guo-Cheng Yuan	Robust lineage reconstruction from high-dimensional single-cell data
48	FY 2014 RFA-RM-13-023	Jonathan Grinstein	The cellular landscape of cardiac development
49	Independent	Eric Batchelor	p53 pulses diversify and coordinate target gene expression
50	Independent	Omar De la Cruz Cabrera	Cycle-regulated genes via single cell RNA sequencing
51	Independent	Jean Fan	Bayesian hierarchical approach for CNV detection in single cells from RNA-seq data
52	Independent	Silvia Gravina	Aging, single-cell methylomes



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53	Independent	Kyungheon Lee	On chip analysis of CNS lymphoma in cerebrospinal fluid
54	Independent	Dylan Malayter	Correlation and kinetics of IFN γ and TNF α mRNA and protein in lymphocyte subsets at the single-cell level by flow cytometry
55	Independent	Rebecca McNaughton	High-throughput microdevice for temporal single-cell analysis
56	Independent	James Michaelson	The single cell basis for plasma protein synthesis by the liver
57	Independent	Gregor Neuert	Non-invasive perturbation and control of signaling activated gene regulation
58	Independent	Xinghua Pan	Single-cell genome-wide CpG methylation pattern analysis based on enzymatic discrimination and selective amplification promise a substantial coverage
59	Independent	Diego Presman & Ville Paakinaho	Analysis of glucocorticoid receptor dynamics by number and brightness and single-molecule tracking methods
60	Independent	Manisha Ray	The Polaris System: Integrating cell and molecular analysis at the single-cell level
61	Independent	Masahiko Sato	Characterization of cultured cells treated with low doses of formaldehyde by exhaustive single-cell lineage tracking analysis
62	Independent	Hirofumi Shintaku	On-chip preparation system for simultaneous cytoplasmic RNA and genomic DNA analyses of single cells
63	Independent	Vaishnavi Srinivasaraghavan & Jeannine Strobl	Content-rich measurements of single-cell biophysical properties via microfluidics
64	Independent	Jerilyn Timlin	TLR 4 distribution and dynamics in individual macrophage cells during immune response
65	Independent	Mohammed Uddin	Somatic variation in neurons in autism spectrum disorder



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66	Independent	Linwen Zhang	Metabolites and lipids in single human cells explored by capillary microsampling electrospray ionization mass spectrometry with ion mobility separation and stable isotope labeling
67	Independent	Shuqi Zhang	iTAST: Technology platform for high-throughput in situ TCR affinity and sequence test on human primary polyclonal CD8+ T cells
68	Independent	Weian Zhao	Rapid single bacterial detection from blood using Integrated Comprehensive Droplet Digital Detection



1

“Follow That Cell” Challenge Executive Summary

Self-destructing cellular barcode: A versatile tool for single cell analysis

James Ankrum

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The role of single cells in physiological and pathological environments has become critical to the study of stem cell niches, tumor biology, and regenerative medicine. While traditional biology tools provide a picture of biological processes, the picture is actually an average of the cell population and does not represent the diversity of phenotypes present in that population. Single cell analysis platforms, including single-cell PCR, flow cytometry, and single-cell microwells have begun to reveal the true diversity that is present in biology. However, the ability to fully characterize single cells across multiple platforms and in multicellular environments is limited by our inability to uniquely identify and track single cells. In this proposal, we provide a solution that is capable of uniquely labeling thousands of single cells across multiple cell generations, is stable for weeks, can be read repeatedly, and self-destructs when a cell dies. The proposed technology relies only on routine fluorescence microscopy and is applicable to a diverse array of cell types. This technology will enable full single cell characterization of cells alone or in multicellular environments.



2

“Follow That Cell” Challenge Executive Summary

Single-cell time-lapse gene expression profiling via an engineered self reporting pathway

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²*Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA*

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⁵*Ragon Institute of MGH, MIT, & Harvard, Cambridge, MA*

Single-cell gene expression (SCGE) profiling is an important analytical technique for studying mammalian cells. The ability to obtain high-resolution molecular phenotypes directly from individual cells is transforming the way in which we define cell states, understand cell circuitry, and study cellular responses to environmental cues. Nevertheless, there is tremendous interest in moving beyond static snapshots of SCGE in cell suspensions to understand how SCGE profiles change through time. Technologies that report the internal state and functional history of cells within tissues would enable novel insights into dynamic biological processes. Here, we outline a technology that allows cells to self-report their internal states in time-lapse measurements by barcoding and secreting a portion of their mRNA. We also highlight methods for integrating this approach with optical microscopy to collect complementary single cell information on protein levels, morphology, and spatial context. Our method is scalable and limited only by the throughput of next-generation sequencing (NGS). We propose to test our method by tracking the emergence of drug resistance in a BRAF mutant melanoma model.



3

“Follow That Cell” Challenge Executive Summary

BLINKER assessed live cell transcriptomics

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⁴*Department of Neurochemistry, Stockholm University, Stockholm, Sweden*

We propose to develop a live cell real-time RNA expression profiling methodology, called Blinker, to assess transcription in live mouse and human neurons. We will assess this in neurons that are dispersed in primary cell culture as well as the live slice preparation. This technology may also be transitioned into cortical cells of the intact mouse brain. We will integrate novel concepts in chemistry, enzymology, cell biology and imaging to exploit the live cell's innate transcriptional machinery to assess the identity of RNAs that this machinery transcribes from the cell's genomic DNA. In preliminary experiments, we have generated preliminary data showing the viability of this Blinker technology in vitro and after further in vitro optimization it will be transitioned into live cells. Blinker is enabled by engineering enzymes involved in transcription so that they contain a capture sequence near the site of RNA synthesis that will interact with a chemically-synthesized “transcriptional detector peptide” (TDP). This peptide is a chimera of: a peptide sequence that will bind to the capture sequence; a cell penetrating peptide (to transport it into the cell); a nuclear localization signal to move the peptide into the cell's nucleus; and a peptide nucleic acid (PNA) cassette that will permit interaction of the TDP peptide with RNA as it is synthesized. Upon binding of the PNA sequence to RNA (in a sequence specific manner), a fluorescence resonance energy transfer (FRET) signal is produced from the fluorophores that have been incorporated in the TDP flanking the PNA sequence. Each FRET signal will be detected as a signal blink in live cells using light sheet microscopy. Because the PNA will interact with a specific nucleotide motif on the synthesized RNA, the timing between blinks will correspond to the distance between the annealing sequences in the newly synthesized RNA. The distances between blinks are unique for any particular RNA, and using a set of algorithms that we have developed, we will identify the gene that is being transcribed. Preliminary data shows that this technology has worked in an in vitro model and in so accomplishing it shows promise to permit in vitro expression profiling of 100,000s of sequences from single cells in minutes decreasing the time and cost of RNA sequencing significantly. We have enabled parts of the Blinker technology and are refining it for expanded in vitro and in vivo use. In vivo use of the technology is being implemented and evaluated as a means to assess changes in gene expression that correlate with long-term potentiation, and pharmacological responsiveness of mouse and human neurons.



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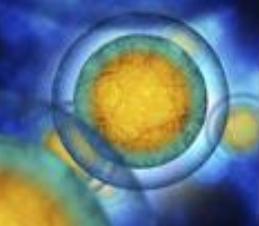
“Follow That Cell” Challenge Executive Summary

Microfluidic droplet based platform for dynamic single cell phenotype, secretion and interaction analysis

Tania Konry

Department of Pharmaceutical Sciences, Northeastern University, Boston, MA

Evaluation of the subtypes, numbers and functionality of tumor and immune cells as well as the activity of immune regulators on a single cell level is a complex scientific and technological challenge. The developed herein single cell micro-technology will allow researchers and physicians to identify and isolate specific cellular phenotype and rare cells in a mixed population based on multiplex analysis of both cell surface and secretion analysis as well as allow ultra fast screening of therapy protocols to identify cellular resistance to drugs over the time. The cellular phenotype is a conglomeration of multiple cellular processes, representing varying expression levels of genes and proteins that determine the cell's particular function in activities such as drug resistance, cellular communication and cell-cell interactions. While flow cytometry has traditionally been used to determine single fixed cell phenotypes, it cannot provide continuous measurements of proteins and secretions in the same individual live cells over time or measure the cellular phenotype during live cell-cell interaction. It is therefore different co-culture methods and immuno-assays are performed on groups of cells to study cell-cell interactions and secretions, under the assumption that all cells of a particular “type” are identical. However, recent evidence from studies of single cells reveals that this assumption is incorrect. Thus new approaches to single cell phenotype analyses and live cell-cell interaction on a single cell level are needed to uncover fundamental biological principles and ultimately improve the detection and treatment of disease. In this context, we have developed a sample-sparing assay that can conduct simultaneous multiparameter assessments of immune function that is critical for establishing a correlation between the immunological mechanism of the activation or inhibition of immune effectors and the targeted clinical response in cancer. In particular the proposed micro-technology allows to: 1) conducting sample sparing simultaneous multi-parameter analysis of both live and fixed cell surface and secretions, 2) controlled delivery of pico-liter volumes of immuno-regulatory agents and therapeutics to cell of interest to study their effect on functional phenotype of the cell, 3) monitoring of live cell-cell interactions on a single cell based level as well as secretion measurements during this interaction, and 4) fluorescenceactivated droplet sorting (FADS) for specific functional cell phenotype isolation.



5

“Follow That Cell” Challenge Executive Summary

Synthetic nano-antibodies for real-time monitoring of cellular biomarkers

Markita Landry

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The past decade has seen a rapid emergence in the use of nanomaterials for biomedical applications including the development of high-sensitivity and high-specificity optical sensors of biological activity. In particular, semiconducting single-wall carbon nanotubes (SWNT) exhibit three essential figures of merit required for their use as fluorescence-based optical sensors: 1) SWNT fluoresce with an extraordinary quantum yield, 2) are the only fluorophores to-date that have essentially infinite lifetimes, and 3) emit in an optical window where tissues, cells, blood, and other biological samples are transparent. Additionally, SWNT are small, measuring only 1 nm in diameter and are often under 100 nm in length. Because of their high aspect ratio, when bio-functionalized, SWNT can be passively uptaken into cells and tissues with more ease than conventional fluorophores and quantum dots. DNA-conjugated SWNT have even shown to passively enter the plant chloroplast, which currently have no other passive mode of entry for genetic information.

Very recently, my work has produced a platform for the production of synthetic optical sensors based on engineered functionalization of SWNT with synthetic polymers. This novel platform produces nanosensors based on the infrared fluorescence of SWNT-polymer conjugates, by producing a signature SWNT-mediated intensity and wavelength shift in the presence of an analyte molecule. While this platform has been very fruitful in the production of sensors, it relies on a screening-based approach. Such screens are labor intensive, time consuming, and limit target analytes to those that can be discovered serendipitously. If more was known about the fundamental interactions leading to selective molecular recognition, a design-based approach could be taken instead, and “synthetic sensors” could be produced to monitor any biomarker: small molecules, reactive species, and even proteins within single cells. Unfortunately, existing optical tools have insufficient overlap to study both polymers and nanomaterials simultaneously, which is becoming increasingly necessary to advance fields of research that hinge on polymer-bio interactions such as the design of SWNT-based sensors. This leaves us ill equipped to design novel high-specificity synthetic sensors for applications such as label-free detection of proteins within a single cell. The goal of this research proposal is three-fold: 1. To customize a microscopy platform that will enable the simultaneous study of polymers and multi-chirality nanotubes, to determine how to better engineer SWNT-based sensors (“synthetic sensors” and “synthetic antibodies”). 2. To apply our newly-developed design rules for the detection of biologically interesting molecules such as cell metabolites, and proteins. 3. To detect proteins in a label-free manner, and on a single-molecule scale, from live cells as they express protein targets.



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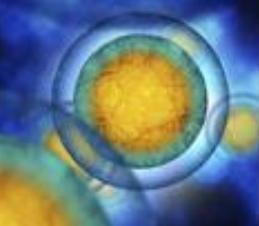
“Follow That Cell” Challenge Executive Summary

Single Cell Oncogenesis

Xin Lu

MD Anderson Cancer Center, Houston, Texas

It is believed that cancer originates from a single cell that has gone through generations of evolution of genetic and epigenetic changes that associate with the hallmarks of cancer. In some cancers such as various types of leukemia, cancer is clonal. Yet in other cancers like glioblastoma (GBM), there is tremendous tumor heterogeneity that is likely to be caused by simultaneous evolution of multiple subclones within the same tissue. It is obvious that understanding how a single cell develops into a clonal tumor upon genetic alterations, at molecular and cellular levels, holds the key to the real appreciation of tumor etiology and ultimate solution for therapeutics. However, surprisingly very little is known about the process of spontaneous tumorigenesis from single cells in human or vertebrate animal models. The main reason is the lack of technology to track the natural process of single cell changes from a homeostatic state to a progressively cancerous state. Recently, we developed a patented compound, photoactivatable ("caged") tamoxifen analogue 4-OHC and associated technique called optochemogenetic switch (OCG switch), which we believe opens the opportunity to address this urgent biological as well as clinical question about cancer. We propose to combine OCG switch with multiallelic genetically engineered mouse models (GEMM) of head and neck squamous cell carcinoma (HNSCC) and high grade astrocytoma (including GBM) to study how single cells, when transformed through acute loss of tumor suppressor genes PTEN and TP53 and gain of oncogenic KRAS (for modeling HNSCC), can develop into tumor colonies with cellular and molecular heterogeneity in these tissues. Specifically, we hypothesize that individual somatic cells of the same lineage and residing in the same tissue can display significant behavioral variations in terms of growth and invasiveness, after initial transformation by genetic manipulation, and furthermore this cellular behavioral heterogeneity can be correlated with accumulation of different additional genetic and epigenetic changes. To validate our hypothesis, we will perform doxycycline-induced lineage tracing and photoactivation-mediated single cell transformation of the tongue and brain tissue followed by longitudinal intravital imaging (IVM) with multi-photon microscopy (MPM). Next we will collect single cell-derived tumors as well as subsets within the same tumor colony using 3D intravital microdissection and perform genomic and transcriptomic profiling with next generation sequencing (NGS). We propose to apply mathematical modeling to the data on cellular behavioral variations to address theoretical questions associated with clonal evolution and cellular heterogeneity. More importantly, we aim to correlate the cellular heterogeneity with gene and pathway changes detected by NGS. Comparing conclusions drawn from two organ systems will generate interesting insights on tissue-specificity of oncogenic evolution. All together, we anticipate that our approaches with such novelty and comprehension will pave roads for paradigm-shifting new knowledge about single cell biology, oncogenesis, and clonal evolution of cancer, as well as generating potential new ideas on cancer therapy.



7

“Follow That Cell” Challenge Executive Summary

Study the longitudinal expression of the genome of a single cell

Nader Pourmand

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We have developed a novel technology to measure the longitudinal genomics of single cells with spatial and temporal resolution. To do this, we use a nanopipette with a tiny tip to sample miniscule samples of a cell in a manner that does not affect the function, genomic expression or viability of the cell. Using a novel targeting system, the nanopipette tip can sample the same cell repetitively over minutes, hours, days or weeks. We also developed a powerful single cell sequencing technology to conduct WTA analysis on the small cell samples to reveal changes in gene expression as the cell ages or undergoes other phenotypic alterations. This allows us to link genomic expression changes with phenotypic changes in any given cell. We have published results using this powerful single cell genomic technology to begin to study mechanisms of drug resistance in breast cancer cells. This is important because drug resistance is a major cause of relapse of chemotherapy and causes thousands of deaths a year because of the regrowth of tumors in cancer patients. We propose to use our nanopipette/nanoGenomics technology to monitor over time genomic changes in drug resistance in breast cancer cells from parent cells to daughter cells to study the molecular basis of how resistance is maintained in growing tumors and also how resistant cells revert to drug sensitive cells to identify genes needed to maintain drug sensitivity. We believe these studies will provide fundamental information on the basis for chemotherapy resistance and sensitivity which could not be obtained using any other technology. It will have implications in the development of adjuvant therapies to prolong and increase the efficacy of chemotherapeutics to treat breast cancer and allow patients to become cancer free.



8

“Follow That Cell” Challenge Executive Summary

Tracing cells by their mechanics

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Cells live by the flow of energy among three pools of free energy: chemical, electrical and mechanical. The chemical pool is studied with biochemical tools, the electrical pool by electrophysiology, and the mechanical pool is generally ignored. Attempting to understand cells without paying attention to mechanics is a bit like building a Ferrari given the periodic table; there are no missing parts, but...

We have learned about molecular mechanics from *in vitro* tools such as AFM and laser traps, but a dish with saline is not the same as the inside of a living cell. Reductionism can be misleading. We want to know what is going on inside *living* cells. To address these issues we designed genetically coded optical probes to measure the stress in specific structural proteins and we learned that:

- Structural proteins are generally under tension. This allows them to transmit signals across and between cells at speeds much higher than chemical messengers. Touching one part of a cell leads to changes of stress throughout the cell.
- There are gradients of stress in specific proteins within single cells, so that stress sensitive biochemistry will vary from place to place and over time, even in a single cell.
- Neighboring clonal cells can have very different distributions of stress.
- Osmotic stress is not confined to the cell membrane, but is distributed throughout the cytoplasm and will vary with local compliance.
- Stemness is associated with *high* stress in actin and α -actinin, and perhaps other proteins.
- Effector transducers of mechanical stress, such as mechanosensitive ion channels, are modulated by stress in the cytoskeleton.
- The force probes are nontoxic since we have made transgenic flies and worms and cell lines.
- We have MUCH MORE TO LEARN.



9

“Follow That Cell” Challenge Executive Summary

Development, optimization, and enhancement of fluorescent DNA-hairpin functionalized gold nanoparticles as imaging tools

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Convenient evaluation of gene expression in metastatic cells represents an important unmet need in translational cancer research. The pattern of gene or protein expression, the molecular signature of individual cells within a tumor, has clinical value. 1-3 Individual cells within a tumor mass exhibit unique transcriptional profiles that may influence the progression and outcome of disease.⁴ The diversity of cell types in the tumor microenvironment means that aggregate studies of a tumor may miss small, highly malignant cell populations that drive disease development.⁵ Therefore, inherent heterogeneity of gene expression among a population of cells necessitates single cell analysis in order to fully characterize a disease state. However, molecular analysis is cumbersome due to non-standard technical approaches, invasive collection of tissue, the multistep process for collection, processing, analysis of specimens, and the paucity of FDA-approved diagnostics. ^{3,6-9} Quantitative RT-PCR (qRT-PCR) is the gold standard for gene expression analysis for researchers, but this data does not reflect gene expression at a single cell level. Clinicians continue to rely on microscopy and histopathology for tumor diagnosis, prognosis, and prediction. These techniques cannot optically identify tumor tissue and report on functional or molecular status simultaneously. ⁹ Thus, readily available tools for evaluation of tumor cells or tissue do not capture the critical genotypic and phenotypic elements that define a tumor's metastatic potential. Tools to evaluate gene expression in real time within intact cells would provide accessible molecular insight to inform research questions and clinical decisions for treatment, surveillance, or assessment of therapy. We propose development, optimization, and enhancement of fluorescent DNA-hairpin functionalized gold nanoparticles as imaging tools that can directly identify tumor cells that have metastatic potential in a live animal model. Hairpin DNA-functionalized gold nanoparticle (hAuNP) probes pioneered by our group are an ideal bioimaging platform for molecular analysis of cells and tissues in situ (Gene-specific hAuNPs: 1) are readily synthesized, 2) offer high specificity, 3) retain high signal output and narrow spectral bandwidth, 4) are modular with interchangeable imaging and targeting moieties, and 5) remain small, deliverable, and biocompatible.^{10,11} The hAuNPs penetrate living cells without the need for transfection agents ¹¹ and emit quantifiable fluorescence upon intracellular hybridization with target mRNA.^{10,11} We have investigated several model systems, and now propose to translate these results into enhanced second generation tools for probing markers of metastasis in preclinical live animal models of tumor metastasis. We hypothesize that gene-specific hAuNPs can provide essential molecular information at a cellular level to enhance optical imaging and routine histopathology.



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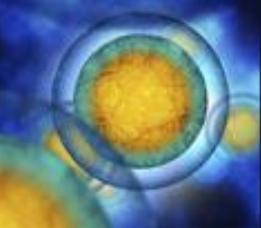
“Follow That Cell” Challenge Executive Summary

Photostable multiplexing nanoassays for real-time molecular imaging of single live cells

X. Nancy Xu

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Binding of just a few ligand molecules with its receptors on single live cells can initiate dynamic cascades of cellular signaling transduction pathways, alter cellular functions and cause diseases. Different ligand-receptor (L-R) interactions associated with the signaling pathways often work in concert to regulate and alter the cellular functions. Such signaling pathways can take minutes, hours or days. Furthermore, different types of cells express trace amounts of distinctive sets of receptors. Therefore, it is important to simultaneously and directly visualize and study different types of receptors on single live cells with single-molecule (SM) sensitivity in order to quantify various types of receptor molecules, and to directly capture how multiple types of L-R interactions work together to achieve given cellular functions. Currently, fluorescence microscopy using commercially available fluorescence imaging probes is the primary workhorse for live cell imaging. Unfortunately, fluorescence probes (fluorophor, fluorescence protein, QD) suffer intrinsic photobleaching effect, making them unable to continuously capture the dynamic events of the same single live cells over hours. Photobleaching also makes quantitative analysis over time difficult. Separation of different excitation and emission of various fluorophores leads to complex and expensive instrument and restricts their capacity of multiplexing study of multiple types of molecules simultaneously. All fluorescence imaging methods including conventional, confocal and super-resolution fluorescence microscopy suffer these same fundamental limitations. To overcome these fundamental and technological challenges, we aim to develop a novel molecular imaging platform including photostable multicolored and multifunctional single molecule nanoparticle optical biosensors (SMNOBS) and far-field photostable optical nanoscopy (PHOTON) for real-time study of functions of the same single live cells over a long desired period of time (hours-days) with high spatiotemporal resolution. To demonstrate the-proof-of-concept, we will use them to quantitatively image and molecularly characterize roles and functions of multiple types of the receptors on rare subsets of colon cancer stem cells (cCSCs) in highly heterogeneous tumor populations and to study their differentiation mechanisms over time. These powerful new tools are expected to address a wide range of pressing biochemical and biomedical questions about molecular and real-time characterization of functions of single live cells and their related signaling pathways in real time. They include molecular identification and characterization of functions of rare subsets of individual cells (e.g., cCSCs) in highly heterogeneous tumor cells in situ in real time for a wide variety of applications, ranging from addressing fundamental questions in cancer research to exploring new approaches for target therapy.



11

“Follow That Cell” Challenge Executive Summary

Tracking the phenotype of single cells using multicolor flow cytometry combined with cell-specific barcodes

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Monitoring the phenotype of individual cells over time in heterogeneous tissues is an important goal to facilitate improved understanding of normal development and disease pathologies such as the clonal evolution of cancer. Fluorescent flow cytometry has been widely applied to measure the phenotypes of single living cells by staining the surface markers with fluorescent antibodies. However, currently only snapshot measurements are possible. Even though the laser-based flow cytometry procedure is sufficiently non-invasive so that the cells can be cultured or reintroduced into the organism after measurement, there is no way to track the identity of individual cells between the measurements. We propose a novel genetic barcoding scheme that uses unique combinations of epitope tags that are expressed on the cell surface. This system can be used to track individual cells over time in several biological systems. Here we describe our plan to track the progression of leukemia between the flow cytometry measurements. At each time point, barcoded leukemic cells will be sampled out of the bloodstream, stained with barcode-specific as well as phenotype-specific antibodies, measured in the flow cytometer, collected and returned into the bloodstream of the animal. Since our barcoding scheme allows over 1,000,000 unique individual combinations, this setup will enable us to track the surface marker repertoire of a large number single cells over time *in vivo*. We will apply this strategy to track the development of clonal phenotypic heterogeneity in a mouse model of AML, identify and perform phenotypic characterization of leukemia stem cells (LSC) and assess the sensitivity of individual leukemia clones to the chemotherapy drugs *in vivo*.



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Highly multiplexed *in situ* digital quantification of targeted RNA transcripts

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Technology 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 and tumors. Advancements in single-cell sequencing techniques allow the full transcriptome of isolated cells to be profiled but it neglects the native spatial context of the cell. *In situ* methods address this by creating rolling-circle amplified colonies (rolonies) that provide high signal-to-noise targets for *in situ* sequencing or fluorescent *in situ* hybridization. However targeted *in situ* RNA detection methods are limited to a handful of RNA targets [1][2] while non-targeted *in situ* sequencing methods can waste limited cell volume with uninformative data such as ribosomal RNAs [3].

We are developing a highly multiplexed targeted method, DARTFISH (Decoding Analysis of Rolony Transcripts with Fluorescent *In Situ* Hybridization), that can selectively detect and localize hundreds of transcripts *in situ*. Similar to exome sequencing, the motivation is to focus the acquisition of information to a subset of most informative transcripts. We leverage the multiplex capability and high specificity of padlock probes [4] to detect targets of interest and include a hybridization-based barcode on each probe. The barcode scheme allows hundreds of probes to be identified with quick reaction kinetics and under isothermal conditions [5]. Preliminary results on DARTFISH in fixed fibroblasts and human brain tissue will be presented.

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13

Use of single-cell RNA-seq to molecularly define human Cajal-Retzius neurons

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The human nervous system is composed of diverse cell populations. Recently, next-generation single-cell RNA sequencing has enabled expression profiling of individual cells. Our group has been performing whole-cell patch clamp electrophysiology followed by cytoplasm extraction and RNA sequencing in neurons from human adult neocortex of temporal, parietal and frontal lobes and cerebellum. In addition, we have studied human fetal tissues, brain and spinal cord, from gestational weeks 10-20. We have sequenced over 197 cells using both the aRNA method (Van Gelder et al., PNAS 87:1663-7, 1990) and modified NuGen Ovation RNA-seq V2. These reads were then mapped and assigned to genes using GT-FAR (Genome- and Transcriptome-Free Analysis of RNA-Seq). On average, we detected 5,895 genes per cell and heterogeneity of gene expression was evident between cells from different types of tissue when the data were analyzed for Principle Components (PC). One cell type, Cajal-Retzius neurons, is of special interest, as they are morphologically distinct, large cells sparsely located in the marginal zone of the developing fetal cortex, and are thought to define the cortical structure of the human brain. Defects in these cells have been implicated in schizophrenia and autism. PC analysis of our data from the Cajal-Retzius neurons finds that they are molecularly distinct from fetal subplate neurons, and we have identified sets of genes that are significantly over- and under-expressed in these cells. Electrophysiological recording revealed for the first time that human Cajal-Retzius neurons have spontaneous synaptic activity and action potential firing. Pathway analysis of the gene expression data (using Ingenuity Pathway Analysis) suggests that this synaptic activity maybe mediated by GABA, glutamate, serotonin and dopamine. Our data illustrate the potential synergism of combining both functional and transcriptome analysis at single-cell level.



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Near field laser ablation sample transfer mass spectrometry and application to single cell and tissue analysis

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Sampling and analysis of large biological molecules with sub-micrometer spatial resolution is a challenge for mass spectrometric techniques. Secondary ion mass spectrometry provides high spatial resolution but it is limited to small molecules, MALDI imaging and ambient spray-based techniques can be used for high molecular weight with limited spatial resolution. Mass cytometry can be used for spatially resolved tissue analysis, but is limited to targets of available antibodies. We have developed a sub-micrometer spatial resolution sampling system for mass spectrometry based on near field laser ablation using an atomic force microscope probe. A pulsed nanosecond Nd:YAG laser is focused on to a gold coated AFM probe and the ablated material is captured for mass spectrometry analysis. The metal AFM probe 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. The system has been used to ablate and analyze molecules as large as small proteins without fragmentation. Small molecule components of cells have also been detected. The high resolution laser ablation sampling developed in this project has applications not only in mass spectrometry but also in conjunction with other analysis methods including targeted proteomics and genomics. Laser ablation capture also allows a separation step to be added to mass spectrometry sampling that will enable the detection of minor biomolecule components of tissue.



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Correlating form and function: Integrating single cell transcriptome analysis into massively parallel single cell functional assays

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Single cell transcript analysis has recently been demonstrated to be a powerful tool for interrogating the state of single cells. However, most methods are limited in the number of cells that can be profiled, preventing the characterization of all subtypes in heterogeneous populations. Several methods for massively parallel single cell transcript analysis have recently been demonstrated. Though vastly improving the scale of these assays, these methods are limited in their ability to link the transcript information to the functionality of the cell. To overcome this roadblock, we present our work towards integrating massively parallel single cell transcriptome analysis with functional assays in nanowell arrays. We demonstrate our ability to capture, reverse transcribe and uniquely amplify the transcriptome of each cell within a nanowell array. To enable matching each transcriptome to previously recorded functional data, we deliver a defined, unique barcode to each nanowell using an in situ-synthesized DNA microarray. All transcriptomes are then amplified in a single reaction and sequenced together. The nanowell barcode can then be used to trace each transcript back to a known nanowell. This method can be combined with many single cell functional assays that have been developed for nanowell arrays including cell cytotoxicity, protein secretion and 16-color cytometry. By combining massively parallel single cell RNA-Seq with massively parallel single cell functional assays, our method promises to decipher the functional consequences of observed transcript signatures.



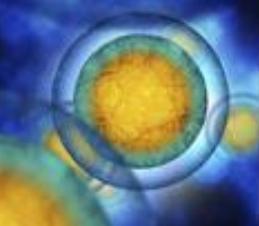
16

Multi-parameter single-cell analysis for comprehensive biosignature discovery

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The Center for Biosignatures Discovery Automation at Arizona State University has developed a suite of technologies for integrated multi-parameter analysis of molecular, metabolic, and morphological biosignatures at the single-cell level. These analytical platforms provide tools to understand the connection between cellular heterogeneity in tissues and disease onset and progression with the goal of identifying clinically actionable biosignatures and therapeutic targets. We employ optical computed tomography (CT), a true 3D imaging modality with isotropic sub-micron resolution, to perform quantitative morphometric studies of single live or fixed cells using absorption or fluorescence staining. The imaging process is compatible with commonly available fluorescence tags, reporters, and intracellular photochemical sensors to perform simultaneous, multi-parameter analysis. We developed custom intracellular pH and potassium sensors to accompany this imaging platform. Our second technology enables us to precisely lyse individual cells in an intact 3D tissue using two-photon laser excitation and to subsequently collect the single-cell lysate through microfluidic liquid manipulation for downstream DNA, RNA, and/or protein analyses (a SCAP funded project). This set-up can be coupled to the optical CT platform, permitting the creation of a combined 3D map of biological dynamics coupled with biomolecular profiles of the same individual cells. Finally, for dissociated single cells, we created the Cellarium, an automated platform that enables functional metabolic profiling of individual cells with high throughput. An array of microwells is loaded with live cells and combined with extracellular fluorescent sensors that respond to analytes such as O₂, pH and glucose. The array is imaged at predefined time intervals to determine oxygen consumption rate, change in extracellular pH, and glucose uptake. Integration of these technologies into one analytical platform will provide a unique tool for acquiring large sets of multi-parameter data with single cell resolution to generate multidimensional biosignatures for a broad spectrum of diseases.



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Single cell imaging of epigenetic dynamics

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Epigenetic modification including histone methylation at different residues can recruit differential sets of chromatin remodeling complexes to modulate chromatin structures and guide gene regulation networks/expressions. H3K9me3, which is necessary for transcriptional repression, plays a crucial role in heterochromatin remodeling. However, the dynamic epigenetic changes cannot be readily detected by static immunostaining using antibodies. Here, we developed a genetically-encoded H3K9me3 biosensor based on fluorescence resonance energy transfer (FRET) for the visualization and quantification of the dynamic H3K9me3 profile at single cell level during cell mitosis to understand the relationship between heterochromatin remodeling and H3K9me3. The dynamic tri-methylation of H3K9 was monitored by the biosensor in MEFs during cell division by real-time imaging. Our results showed that the biosensor can be introduced into live cells and localize to the nucleus, and more importantly, it can be methylated similarly in dynamics as endogenous histones. The biosensor FRET level was suppressed by H3K9 methyltransferase knockout and increased by the histone demethylase inhibitor TCP, indicating a high specificity of the biosensor in reporting H3K9 methylation. Furthermore, mutations of the FRET biosensor to change H3K9 residue or disrupt its functional domain suppressed the FRET signals, whereas mutations of H3K4 and H3K27 residues didn't affect the FRET signals. These results suggest that the biosensor functions as we designed. This biosensor further allowed the monitoring of the H3K9me3 dynamic change in MEFs during cell division. These results indicate that our H3K9me3 FRET biosensor can specifically monitor the H3K9 methylation with high sensitivity and precision. Hence, we have successfully developed a FRET biosensor for visualizing histone H3K9 tri-methylation in living cells, which provides a powerful tool for the spatial and temporal analysis of H3K9me3, as well as for our better understanding on how H3K9me3 modulates heterochromatin remodeling.

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RNA temporal expression profiles in monitoring T cell activation using single-molecule fluorescence in situ hybridization and flow cytometry (FISH-Flow)

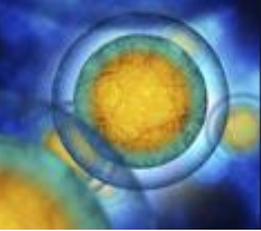
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Characterizing the “activated” status of stimulated T cells that fight infections, or provide a “memory” response to prior vaccinations and infections, typically relies on detection of induced proteins. Shifting the analysis to detection of induced mRNAs provides several significant advantages, which are illustrated by a new host immunity-based FISH-Flow platform for detection of infections that we have developed.

Production of canonical cytokines IL-2, IFN γ and TNF α is one of the major responses by stimulated T cells. The FISH-Flow platform yields improved sensitivity compared to detection of protein analytes and allows reproducible measurements of response kinetics. Moreover, multiplex detection of both RNA and protein analytes enables functional characterization of cells at the RNA level and their phenotypic characterization at the protein level. Memory of exposure to commonly encountered viruses such as CMV, EBV and influenza is reproducibly revealed by testing T cell responses in blood samples from healthy controls. FISH-Flow also detects the presence of latent infection with *M. tuberculosis* in an analysis of donors typed by skin tests or IFN γ release assay as infected or non-infected. Moreover, the presence of antigen-specific, cytokine-producing T cells in circulating blood from patients with active tuberculosis, but not in negative healthy controls, is readily demonstrated by FISH-Flow analysis. Further development and optimization may allow use of FISH-Flow to distinguish latent infection from active disease.

Additionally, clear differences in kinetics of cytokine production by T cells stimulated with agonists to PKC, TCR, and TLR are revealed by this method. Characteristic cytokine profiles induced by the same agonist, for example to TLR4, differ in T and non-T cells. Such kinetic differences in cytokine expression that are not apparent at the protein level should provide novel insights into gene expression programs expected to define different T cell subsets and non-T cell populations. Thus, the FISH-Flow method provides a new tool that can be extended to detection of many different RNAs induced in various types of immune cells as a means to identify a response to vaccination, the stage of an infection, or the effectiveness of a therapy for a wide range of infectious diseases and pathological conditions.



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Single cell in situ RNA profiling by sequential FISH (seqFISH)

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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. We will discuss application of this technology to brain sections, embryos, and human tissues.



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Towards the living connectome: Imaging of individual neurons in intact, developing embryos

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Long-term monitoring of single cells in the complex context of the tissue is crucial for the understanding of cell biological changes in physiology and disease. Two essential technological components are required: cutting-edge microscopy that enables prolonged imaging of the tissue at single cell resolution, and automated image analyses for systematic identification and tracking of individual cells. We have now succeeded in the implementation, validation and translation of a novel system that transform the highly desired technique of long-term monitoring of cells into an accessible practice by biologists. First, we created a robust dual-view selective plane illumination microscope (diSPIM) that enables sustained volumetric imaging at rates 10-1000X faster than other 4D microscopes, and at isotropic resolution (~350 nm in all three dimensions). To broaden the reach of this technology, we trained biologists on the assembly and use of diSPIM in multiple national and international imaging courses, we published papers with detailed protocols that enable a biologist to construct their own diSPIM from commercially available parts, and we collaborated with companies in the development of a fully turn-key instrument. We also collaborated with the u-manager open-source movement to develop freely available control and analysis software. As a results of these efforts, ~20 groups are currently using or are in the process of setting up diSPIMs.

Second, we developed methods for systematic measurements of single cell behaviors based on cell tracking in various model organisms. For example, these methods allow rule-based inference of in vivo developmental landscape from images, which includes computationally defined cellular states of differentiation, paths traversing the states, as well as predicted genetic pathways and cell-to-cell signaling events that regulate path choices. We demonstrate how these computational methods now give biologists unprecedented access to cell biological dynamics in the context of the developing tissue by examining neurodevelopmental dynamics in *C. elegans* during neurulation. We also discuss use of our enabling technologies towards the creation of a living connectome: a 4D atlas of the neurodevelopmental decisions made by single cells as they self-assemble into a functional nervous system in the embryo.



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High-throughput robotic analysis of integrated neuronal phenotypes

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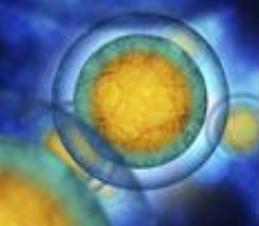
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For single cell characterization, we have established and implemented standardized protocols for transcriptional, electrophysiological and morphological profiling. For electrophysiology, we have used the single autopatching to record from >100 cells in the visual cortex of anesthetized mice and recorded intrinsic properties (n=21) and intrinsic and complete visually evoked responses from 8 cells showing distinct visual response dynamics. We also assessed our automated 4-neuron multipatcher in barrel cortex and visual cortex of anesthetized mice. In a total of 8 experiments run on 8 week old C57/BL6 mice, we performed a total of 41 multipatcher trials which yielded 52 stable whole cell recordings. Of the 41 trials, 2 trials resulted in no whole cell recordings in any of the 4 channels. Thus we were able to obtain whole cell recordings in 95.12% of multipatching trials (39/41 trials). We were able to obtain two or more whole cell recordings simultaneously in 29.26% of the trials (12/41 trials) with one of the trials resulting in a triple whole cell recording. We found that of the 164 pipettes that were inserted into the brain, 31 pipettes got blocked on descent to depth (18.9%) and were de-activated during the trials. Of the pipettes that continued on to find neurons, 77 pipettes formed successful gigaseal recordings (77/133 = 57.89%) of which 52 were successfully broken in to obtain whole cell recordings (52/77 = 67.53%). The whole cell patched neurons were recorded at depths of 350-700 micrometers below the pia. We have also performed multipatching experiments in awake headfixed animals. In 17 experiments on 13 mice, we had an overall success rate of 17.85% for whole cell recordings (65 whole cell recordings from 91*4 = 364 pipette insertions). We obtained 1 or more successful whole cell recordings in 47.25% of multipatching trials (n = 43/91 trials), and have obtained dual or triple whole cell recordings in 18.68% of trials (n = 17/91 trials). Average recording times were 30.5 minutes.

For transcriptional and morphological, we established two single-cell RNA-seq methods, Smarter and Cell-Seq, to routinely profile dissociated, FACS-isolated adult mouse neurons. We obtained high-quality single-cell Smarter RNA-seq datasets from >1000 neurons isolated from >17 Cre lines, and were able to use these benchmark datasets to classify, in an unbiased way, these neurons into at least 18 types. We also established standard procedures for staining, imaging and semi-automated reconstruction of biocytin labeled neurons. We developed a preliminary protocol for transcriptional profiling of cellular contents extracted from whole-cell patch-recorded cells using Fluidigm RT-qPCR with a set of genes selected from the above benchmark datasets that collectively allow us to distinguish all the identified cell types. Finally, we established protocols for in vivo blind or two-photon guided DNA electroporation, and used it to label the full morphology of single neurons in vivo. Combining large-scale, high-resolution confocal imaging with custom software packages for image stitching, stacking and program-assisted manual reconstruction, recently we completed our first reconstruction of the full 3D morphology of a mouse V1 layer 5 neuron that has extensive axonal projections within cortex, pons, multiple thalamic nuclei and superior colliculus.



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Characterization of the intestinal stem cell landscape with MultiOmyx™ Single Cell Analysis

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The intestinal crypt harbors a population of multipotent stem cells that are the progenitors for the differentiated cells of the rapidly renewing intestinal epithelium. A panoply of cellular markers have been identified to distinguish stem cell sub-populations that are quiescent, partially quiescent, or quickly cycling with competing models of intestinal stem cell dynamics. By crossing Lgr5-EGFP- to Lrig1-Apple-reporter mice, we generated a model marking both stem cell sub-populations. Tissues were collected at homeostasis, during fasting, and after different times of recovery to explore dynamic changes in the populations. Analysis of >30 protein targets that represent stem cell, differentiated progeny, associated signaling networks, and proliferation markers was enabled by MultiOmyx hyperplex immunofluorescence. Image analysis was applied for single cell segmentation and marker quantification with new algorithm approaches. Several approaches were used downstream to determine co-expression patterns at a cell level. The first approach was to manually assess co-expression by directed queries building, and the second was to use K-means clustering of the markers. These were compared to alternate methods for multi-dimensional single cell analysis including viSNE and SPADE. While all the approaches offer different end-point ways of visualizing the data, the co-localization patterns were found to be consistent across the platforms in defining sub-populations. These different methods uncovered diverse aspects of stem cell interrelationships, describing new subsets of cells with unique expression characteristics.

In separate studies, early events in tumorigenesis are being assessed in a mouse stem cell driven model of colorectal cancer and are being compared to results from human CRC cases. Mouse models of CRC and actual human CRC were evaluated for an overlapping set of stem cell markers. Based on stem cell marker co-expression patterns, initial results from the human cohort indicate there are several stem cell phenotypes associated with CRC and various degrees of heterogeneity of marker expression within the tumor mass. These different patterns are currently being examined in the context of patient survival and chemotherapeutic response.

1R01CA174377-03: Michael Gerdes



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Tattletales and T-Bow: Multiplex fluorescent protein biosensors to measure and make better serial killers

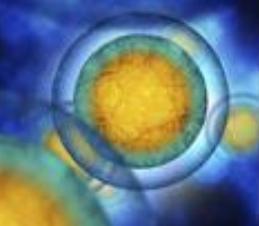
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We do single cell killing assays on nanowell chips imaging thousands of human cytolytic CD8+ T-cells, each with one or more target cells. We now identify kills by fluorescent markers and morphology changes. We want to go beyond killing to measure multiple parameters in our live cells, in real time. We aim to generate sets of sequence specific TALE-FPs (Transcription Activators-Like Effectors - fluorescent protein fusion proteins) 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 promoter response elements, and (ii) biosensors for five molecules.

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 our 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.

Please see <http://works.bepress.com/gmcnamara/65> for our 12/2014 NIH Single Cell Analysis Program “Follow That Cell” Innocentive Challenge submission for details and references.



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Individual motile CD4⁺ T cells can participate in efficient multi-killing through conjugation to multiple tumor cells

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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, and their ability to persist and participate in serial killing of tumor cells is a predictor of therapeutic success. We implemented Timelapse Imaging Microscopy In Nanowell Grids (TIMING) to provide direct evidence that CD4⁺CAR⁺ T cells (CAR4 cells) can engage in multi-killing via simultaneous conjugation to multiple tumor cells. Comparisons of the CAR4 cells and CD8⁺CAR⁺ T cells (CAR8 cells) demonstrate that while CAR4 cells can participate in killing and multi-killing, they do so at slower rates, likely due to the lower Granzyme B content. Significantly, in both sets of T cells, a minor sub-population of individual T cells identified by their high motility, demonstrated efficient killing of single tumor cells. By comparing both the multi-killer and single killer CAR⁺ T cells it appears that the propensity and kinetics of T-cell apoptosis was modulated by the number of functional conjugations. T cells underwent rapid apoptosis, and at higher frequencies, when conjugated to single tumor cells in isolation and this effect was more pronounced on CAR8 cells. Our results suggest that the ability of CAR⁺ T cells to participate in multi-killing should be evaluated in the context of their ability to resist AICD. We anticipate that TIMING may be utilized to rapidly determine the potency of T-cell populations and may facilitate the design and manufacture of next-generation CAR⁺ T cells with improved efficacy.



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Regulated transitions in levels of cell movement velocity and directional heterogeneity underlie vertebrate body elongation

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Body elongation during vertebrate embryogenesis is largely driven by collective and coordinated movement of cells, and to a lesser extent cell proliferation. The tailbud is the posteriorly advancing edge of a vertebrate embryo, and how it grows is not well understood. Elucidating this growth mechanism is important to understand the etiology of vertebral defects like scoliosis. We have used 3D confocal time-lapse imaging of the zebrafish embryo to systematically probe the cell dynamics of the tailbud. We found that a directed, ordered flow of cells symmetrically bifurcates at the tip of the tailbud. This bilateral and symmetric division of cells is crucial to maintain normal linear body elongation. We further found that inhibition of different signaling pathways (like Wnt or Fgf) can potentially break the bilateral symmetry, leading to curvature of the body axis. To mechanistically understand this symmetry-breaking phenomenon, we numerically investigated a simple theoretical model of cell migration, where cells interact via short-range repulsive and adhesive forces, and they are subjected to a random angular noise. Interestingly, we identify a continuous transition from a state of bilaterally symmetric cell flow to an asymmetric one when the noise strength is reduced below a critical value. Thus we argue that dynamical regulation of the noise (i.e. the heterogeneity of cell movement) is important. Moreover, we found that the symmetry breaking transition is not sensitive to the variation of adhesion strength between cells when the cells are densely packed. This parallels our observation that the wild type and Cadherin 2 null mutants both exhibit similar bilaterally symmetry in cell flow.



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Analyzing genomic elements in live animals by CRISPR imaging

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Distinct from the common impression, the genomes of individual cells in our body are not exactly homogenous or static. Heterogeneity and dynamic changes occur in many cases, such as aging, cancer and HIV infection. Detecting these single cell events in a live animal remains a major challenge. In the proposed project, we plan to validate and further develop our recently invented CRISPR imaging technique in a multicellular organism. This technique utilizes the CRISPR/Cas9 system to fluorescently label specific, endogenous genomic loci for microscopy detection. Here, we will use the nematode *C. elegans* as the model organism to validate and benchmark CRISPR imaging as a technology to measure the size of repetitive genomic elements such as telomeres as well as the copy number of genes. We will also improve the sensitivity of CRISPR imaging for the detection of non-repetitive genomic elements. Finally, we will apply CRISPR imaging to the study of telomere length change at the single cell level during *C. elegans* development and aging.



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Validation of acoustic tweezers for single-cell analyses of purine metabolism

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The lack of a single-cell manipulation technique that can simultaneously achieve high throughput, high precision, and high cell integrity is a major roadblock for studies of intercellular communication. Recently, our interdisciplinary team has developed a surface acoustic wave (SAW)-based microfluidic platform called “acoustic tweezers” that possesses significant advantages over existing cell-manipulation techniques for single-cell analysis. Our acoustic tweezers platform is able to modulate the distances between individual cells with sub-micron precision. In addition, it is highly scalable and capable of creating a large array of cellular arrangements for high-throughput studies. Cells do not need to be labelled and can be cultured in their native media. Furthermore, the acoustic power and frequency used to manipulate cells are in the same range as those used in ultrasonic imaging, which has proven to be highly biocompatible. Finally, the components required for SAW generation are small and inexpensive, and the device itself is easy to operate. With these advantages, the acoustic tweezers are groundbreaking in their ability to provide precise spatiotemporal control of intracellular communication at the single-cell level in a high-throughput manner while preserving cell integrity. The transformative potential of acoustic tweezers has already been demonstrated in studies on gap junction-mediated functional intercellular communication in several homotypic and heterotypic cell populations by visualizing the transfer of fluorescent dyes between cells. Our objective in this project is to conduct advanced development of acoustic tweezers and validate them in studies on the effects of intercellular communication on metabolic pathways within the cell. We are pursuing the following specific aims: (1) advanced development of acoustic tweezers for high-yield, high-throughput characterization of intercellular communication and purinosome assembly at the single-cell level; (2) multi-parametric investigation of purinosome assembly in a primary cell model using acoustic tweezers; and (3) single-cell analyses of purinosome assembly and purine metabolism in a neuronal model using acoustic tweezers. At the completion of the proposed project, we hope to uncover the mechanism for how a genotype affects complex phenotype using Lesch-Nyhan disease (LND) as the disease model and purinosome as an indicator of metabolic state. Due to its unique ability to create multicellular assemblies with prescribed architectures in high throughput, we expect that the acoustic tweezers will become an invaluable tool for single-cell analysis and will fulfill many unmet needs in the bioengineering, biomedical, and pharmaceutical research communities.



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Single cell analysis using high spatial resolution and high sensitivity imaging mass spectrometry

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Mass spectrometry is one of the primary analysis techniques for cell biochemistry. However, due to the small size of cells and low concentrations of lipids and metabolites, instrument spatial resolution and sensitivity have been major challenges for single cell mass spectrometry. To overcome these barriers, we are developing high spatial resolution and high sensitivity imaging mass spectrometry using customized laser optical system coupled to a MALDI Fourier transform ion cyclotron resonance mass spectrometer. Sample preparation procedures including conditions for cellular deposition on the MALDI targets and application of matrix are optimized to maximize signal. We demonstrate that this technology has sufficient sensitivity and specificity for single cell analysis of lipids and metabolites and provides novel insights into the heterogeneity of molecular expression in various mammalian cells including pancreatic islet beta cells. We will apply this technology to obtain molecular profiles from single cells to determine the influence of different treatments on the molecular expressions of the cells.



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Validation and development of single nucleotide variant RNA FISH in single cells in culture and tissue

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As methods for gene expression measurement have improved, there has been a growing appreciation that biological behavior in single cells can vary dramatically in ways not captured by conventional bulk assays. Single molecule RNA FISH has developed into a powerful and generally applicable method for single cell gene expression analysis, having been adopted by dozens of labs worldwide. However, while it is able to precisely locate individual RNA molecules in single cells, it has not been able to discriminate single base differences on those RNA, thus precluding many potential biological applications. Such applications include differences in expression between the maternal and paternal alleles of a gene, which typically only differ by single nucleotides. These expression differences are relevant in many areas of genetics, including gene imprinting, in which only the maternal or paternal copy of the gene expresses. Many of these issues arise in biological contexts (e.g., complex and heterogeneous tissues such as brain and placenta) that make even single cell biochemical assays problematic because they do not preserve the spatial context of the cells in question. Thus, these biological areas of research have been unable to harness many of the advances that single cell biology has conferred. Recently, we have developed a method that enables us to distinguish individual RNAs based on single base differences. The goal of this proposal is to quantitatively validate this method, first in cell lines and then in mouse and human tissues and then applying to specific biological questions that underscore the utility of the approach. Methodologically, we perform a series of experiments aimed at understanding the parameters relevant to our method with the goal of making the method robust, reliable and easily accessible to other researchers. In aim 1, we will perform these experiments in cell lines to characterize the probes themselves and develop the capability to measure multiple single nucleotide variants simultaneously within single cells. In aim 2, we will apply our method to tissue sections from mouse and human with the goal of solving common problems specific to tissue, such as RNA degradation and high background. As an application demonstrating the utility of our method, we will study Beckwith-Wiedemann Syndrome, a genetic disorder in which loss of appropriate imprinting leads to a highly varied phenotype. We will study the manifestations of this disorder by examining allele-specific expression in developing mouse tissues and then in de-identified human patient samples, examining how cell-to-cell variability in imprinting leads to the disease phenotype.



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Light-induced genetic alterations within single cell of a live vertebrate animal

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Stem cells have the remarkable ability to self-renew and differentiate during development. In addition, adult self-renewal and differentiation also play an essential role in injury response and overall tissue maintenance. In the past two decades, a considerable effort was made to develop and apply methods of gene expression profiling which are now used to generate atlases of gene expression in different tissues throughout development and in adults. In contrast, considerably less progress has been achieved to develop tools that simultaneously allow multi-gene profiling, manipulation of single cells in situ with minimal perturbation in developing or fully differentiated and aging tissues. Such tools are indispensable to determine how tissues are maintained during the adult life and understand how cells can sometimes enter in an abnormal path that leads to disease. The main challenges posed to capture, interfere and analyze the effects of gene expression in situ in adult and aging tissues with single cell resolution stem from: (1) technical limitations to label and visualize specific cell lineages in intact tissues, (2) the need of non-invasive tools to interfere with specific cell populations and (3) the availability of a reliable system to determine the onset of gene expression in adult and aging tissues. To begin addressing these issues, we have been using a combination of three technologies that allow us to report the temporal birth order of cell lineages by a color-code in intact complex tissues; visualize the expression of several genes with single cell resolution using a combinatorial spectral barcoding in situ hybridization; and finally remove genes within specific cell lineages using RNA interference. In this proposal, we will optimize and expand the range of applications of these technologies to other cell types, which include adult and senescing cells, and make it more accessible to the end-user.



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Nuclear pre-mRNA analysis of single cells in brain slice

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This project focuses on the gene expression profile of a single nucleus to identify pre-mRNA transcript signatures in vivo. Pre-mRNA is a precursor of cytosolic translation-ready mRNA in the cytosol, which is translated into protein, a fundamental biological element. The existence of pre-mRNA in the nucleus has long been known and recent progress in single cell biology raises the importance of profiling it for comprehensive research in genetic information flow from DNA to protein. However, due to the lack of proper technology, investigation of nucleic pre-mRNA profile in vivo has not been possible. Although much different pathology have been linked to genetic information flow, the correlation between nucleic and cytosolic mRNA has never been studied. One of the main reasons is a lack of methods to capture the total pre-mRNA in a single nucleus in vivo. We suggest that pre-mRNA content reflects a cell's natural microenvironment in tissue. In this proposal, we will first perform pre-mRNA profile analysis of single nuclei of different cell types from multiple brain locations in order to build a database to correlate pre-mRNA signatures with their resultant cellular phenotype in vivo. A cell-type specific genetically modified mouse model will be used to identify specific cell types and used to snap capture the pre-mRNA within a single nuclei of each cell type. High resolution microscopy and optical manipulation of a novel mRNA capturing compound (TIVA-Nuc) will be employed to capture the pre-mRNA transcript profile with and without physiological stimulation. The main goal of this project is to establish the heterogeneous nature of pre-mRNA profiles based on their cell types and anatomical location in brain as well as their functional connections, such as interacting with other neurons in tissue. These results will generate a novel database for assessing the role of pre-mRNA, which will greatly facilitate understanding their specific roles in regulating and maintaining brain cellular function.



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Single-cell detection of A β and sAPP α secreted from human iPSC-derived neurons and glia

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Secreted factors play a central role in normal and pathological processes in every tissue in the body. The brain is composed of a highly complex milieu of different cell types, and few methods exist that can identify which individual cells in a complex mixture are secreting specific analytes. By identifying which cells are responsible, we can better understand neural physiology and pathophysiology, more readily identify the underlying pathways responsible for analyte production, and develop and test novel therapeutic strategies that target the cell types of relevance. We present here a method for detecting analytes secreted from single hiPSC-derived neural cells, and have applied the method to measure A β and sAPP α , analytes central to Alzheimer's disease pathogenesis. Through these studies, we have uncovered the dynamic range of secretion profiles of these analytes from single iPSC-derived neuronal and glial cells, and have molecularly characterized subpopulations of these cells through immunostaining and gene expression analyses. By examining cells at the single cell level, we have uncovered novel biological insights into A β and sAPP α secretion. We have found: 1) unexpectedly heterogeneous responses to secretase inhibitors in putatively "homogeneous" stable clonal cell lines; 2) a previously unappreciated subpopulation of cells that secrete high levels of A β in the absence of detectable sAPP α ; 3) that cell state can affect the relationship between α - and β -secretase cleavage of APP; 4) that during the time of neural differentiation, the number of cells secreting detectable levels of A β and sAPP α increases; and intriguingly 5) that cells expressing forebrain GABAergic neuronal markers are overrepresented in subpopulations of cells that secrete high levels of A β and sAPP α , although a variety of cell types can secrete high levels of each. Finally, while there is a widespread belief that neurons are the major source of A β in the CNS (1-3), the results presented here show that astrocytes are competent to secrete high levels of A β and may therefore be a significant contributor to pathology in Alzheimer's disease. Taken together, these results using APP as a model protein describe a novel methodology to examine and quantify analyte secretion from hiPSC-derived neural cells at a single cell level, and they demonstrate the utility of such a system with studies directly relevant to unsettled questions regarding AD pathobiology.



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Microsecond Raman spectroscopy: Assessing single cell metabolism in a vital organism

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Quantitation of metabolic conversions in live cells and in real time is essential to determining how a cell responds to an intervention such as drug treatment or exposure to a risk factor. Nevertheless, most of our knowledge about cellular content is derived from in vitro analysis of isolated cells or measurement of tissue homogenates, either by biochemical assays, omics or sequencing technologies. This gap highlights a need of developing new techniques that are able to repetitively assess the same single cell in a vital organism. We propose to develop a new Raman imaging platform to enable repetitive assessment of single cell metabolism in a vital organism, using *C.elegans* as a test bed. Our innovation is spectral scanning of a femtosecond pulse at the Fourier plane of an angle-to wavelength pulse shaper, through which a SRS spectrum can be acquired on the scale of 20 ms per pixel. The long-term goal of our research is developing next generation technology to enable quantitative analysis of single live cell response to a stimuli or a treatment in 3D cultures or live animals. The specific objectives of this R21 application are constructing a ms time scale spectroscopic imaging system and longitudinally assessing the fat metabolism in vital *C.elegans*. An interdisciplinary team has been formed. Dr. Ji-Xin Cheng (PI) is an expert in label-free spectroscopic imaging. Dr. Heidi Tissenbaum (co-PI) is an expert in dissecting molecular mechanisms of the aging process using *C.elegans* as a model organism. The two investigators have an established collaboration in developing coherent Raman scattering microscopy to study lipid metabolism in live *C.elegans*. In feasibility studies, the team has demonstrated hyperspectral SRS imaging of lipid oxidation, lipid desaturation, and cholesterol storage in adult worms using fingerprint Raman bands. Moreover, the Cheng lab recently demonstrated spectral modulation SRS imaging with an angle-to- wavelength pulse shaper. Such development paves the foundation for acquisition of a SRS spectrum on the ms time scale. Our hypothesis that ms Raman spectroscopy is able to longitudinally assess lipid metabolism in vital *C.elegans* during aging, diet restriction or overfeeding. To test this hypothesis, we will design and construct, and test a microsecond Raman spectral imaging with an angle to wavelength pulse shaper. We will then use the system to longitudinally assess energy metabolism of single cells in wild-type and mutant *C. elegans*. Though *C.elegans* is used as a test bed, our platform heralds a broader impact on biomedical research via assessing single live cell response to an intervention, including monitoring live cell response to a risk factor or tissue regeneration in response to a stimulus.



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Identifying the intercellular networks regulating estrogen receptor expression with a high definition single cell printer

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The long-term objective of this proposal is to reinvent live-cell printing by combining advances in droplet microfluidics, robotic automation, and microscopy techniques. **The immediate scientific goal** of this proposal is to recreate the *in situ* microenvironment by building combinatorial cellular interaction arrays using primary or limited life-span human mammary epithelial cells. Specifically, we will apply the multicellular interaction arrays to dissect the cellular network that contributes to heterogeneous patterns of estrogen receptor expression. Estrogen receptor (ER) is the key regulator of human mammary gland growth and is necessary for the most prevalent forms of human breast cancer. Remarkably, however, the estrogen receptor is only expressed in one cell type in the human mammary gland – luminal epithelial cells. More remarkably still, the receptor is expressed in a binary fashion, and only in 5-20% of luminal epithelial cells in a normal tissue. Little is known about the mechanisms through which ER expression is regulated within the luminal population, but available evidence suggests that multiple other cell types in the microenvironment are critical. We will use the proposed high definition single cell printer to explore a large combination of primary human cell types, soluble factors, and ECM components to identify the minimal intercellular circuit necessary to sustain ER expression in the mammary gland. We hypothesize that ER expression in the luminal population is regulated by a combination of cell-cell contact with myoepithelial cells and a paracrine circuit involving fibroblasts and at least one other stromal component responsible for stabilizing estrogen receptor expression.



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Innovative reporters to characterize heterogeneous states among cells

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Our proposal addresses the goals stated in the Research Objectives: "to develop next-generation tools that distinguish heterogeneous states among cells in situ", in particular "Tools that provide significant advances in sensitivity, selectivity or spatiotemporal resolution of molecules/structures/activities within single cells and between ostensibly similar cells in situ". Gene activation patterns vary widely in complexity. Some are straightforward and static, such as the pattern of many housekeeping genes, that are expressed in all cells at all times in life. Others are extraordinarily intricate with expression fluctuating from minute to minute or from cell to cell. Changes in gene expression reflect changes in the transcriptional landscape of the cell, which in many cases occur in response to extracellular signals. Characterizing the spatiotemporal changes in gene expression is therefore a critical question in cell and developmental biology. In addition, besides temporal dynamics within particular cells, the spatial architecture of cell-cell connection and communication are also critical. In multicellular organisms, direct intercellular contacts play vital roles in almost every biological process, from basic functions like cell proliferation, differentiation, and migration; to higher functions like immune system activation, body movement, and memory formation. To address some of the limitations with current technologies, we propose to develop a series of tools that will provide significant advances in the analysis of cellular heterogeneity and cell contact in vivo. Specifically, we propose to develop: Aim 1. Sensitive sensors of gene expression to visualize heterogeneity among groups of cells within tissues. Transcriptional reporters that drive the expression of fluorescent proteins (FPs) are commonly visualized to analyze the activation of pathways; however, the stability of these reporters makes it impossible to achieve the temporal resolution needed to dissect dynamic gene expression changes. We have begun to develop a series of new fluorescence reporters (Dynamic Fluorescence Reporters) that improve dramatically the temporal visualization of gene activities to address these fundamental questions. We propose to further optimize these tools and demonstrate their in vivo applications to: 1. study heterogeneity among apparently homogeneous cell populations; and 2. examine the transcriptional response of individual cells to a morphogen molecule. Aim 2. Transcriptional sensors to detect contacts between cells. We have developed robust in vitro synthetic ligand-receptor systems that activate a transcriptional reporter or effector following direct cell-cell contact. We propose to establish these systems in vivo and evaluate their use for: 1. the study of cytonemes, cellular projections making long-range contact with other cells, in imaginal disc epithelia; and 2. the study of neuronal connectivity. The tools developed in Aims 1 and 2 will have many additional applications and enable studies of cross-talk between signaling pathways; blood cell homing; the binding of secreted ligands to their cellular targets by generating secreted ligand-GFP molecules; as well as isolation of specific cell types at different developmental/functional states for RNAseq transcriptome analyses. Finally, although the experiments described in this R21 will be performed in *Drosophila*, the tools once established will be easily transferrable to other systems, especially vertebrates.



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Single-cell microsampling mass spectrometry for elucidating cell heterogeneity in the developing embryo

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Establishment of a heterogeneous population of cells is critical to embryogenesis and normal development. A substantial portion of bioanalytical methodologies that are used in cell and developmental studies, however, work *ex vivo* or rely on long-term cell cultures, which conditions potentially change the molecular composition of cells, complicating result interpretation. We and others have found that the metabolome is particularly sensitive to environmental influences due to its highly dynamic and complex nature. Uncovering cell heterogeneity during embryogenesis requires new, innovative single-cell technologies that can detect multiple types of biomolecules, operate rapidly, preferably *in situ*, and are compatible with the three-dimensional structure of the embryo's body plan without chemical fixation or sectioning. Furthermore, measurement of the metabolome necessitates high information-content detection, preferably by mass spectrometry, the modern analytical technique for the measurement of these types of small molecules.

Our goal is to develop a new type of single-cell mass spectrometry platform to determine metabolomic cell heterogeneity in the actual developing embryo. To this end, we have developed an *ex vivo* single-cell mass spectrometry platform that allows for measuring small molecules at 10 nM (or 60 amol) lower limit of detection, sufficient to detect endogenous metabolite concentrations. Using this platform, we have uncovered small-molecular heterogeneity between embryonic cells that give rise to different tissue types from the 16-cell embryo of the South African clawed frog (*Xenopus laevis*), and have validated these findings in functional experiments that change the fate of the cells. At present, our goal is to transform this single-cell mass spectrometry platform to *in situ* and scalable operation as this will be discussed during our talk. We envision that the single-cell mass spectrometer will be adoptable to other cell types including embryonic cells from different models as well as neurons to facilitate the qualitative and quantitative assessment of small-molecular cell heterogeneity.

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Light-induced genetic alterations within single cell of a live vertebrate animal

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Animal modeling of disease states and bioreporters for in situ analysis of cellular dynamics are an essential aspect of biomedical research; however, most studies focus on genetic manipulation within the whole organism or tissue of interest, which does not accurately reflect disease states that are often associated with defects in individual cells or groups of cells. This discrepancy is largely due to the technical challenges of single cell manipulation and analysis within an intact organism. While ongoing SCAP studies are characterizing the transcriptome, proteome, and chromatin alterations within single cells, a biological system to test the impact of these single cell alterations is necessary. Therefore, consistent with this FOA, we believe that a system for controlled genetic manipulation (resulting in loss of function alleles or expression of exogenous cDNAs/bioreporters) within a single cell of a viable organism is required. This system should not alter or perturb the surrounding environment and should allow for potential systems based analysis and biomarker incorporation. Therefore we hypothesize that: 1) through the use of optogenetic (light inducible) Cre transgenic animals, single cell Cre recombination can be achieved allowing for single cell inducible gene alterations (i.e. conditional knockout or bioreporter/gene of interest expression); and 2) through the use of a Cre inducible Cas9 system, single and multiple gene ablations can be rapidly achieved within an individual cell of an intact organism. These two systems will be established within the zebrafish embryo but can be applied to all model organisms. Upon completion of this proposal we will have established versatile and efficient single cell tools for selective genetic alterations that will facilitate single cell analysis in a multitude of research fields. We anticipate that this research will have a broad positive impact on a number of other human diseases including (but not limited to) neurobiology, immunology, cancer biology, and developmental biology. This proposal is in alignment with the SCAP and this FOA objectives: 1) to develop tools that "minimize cell perturbation and permit viability of cells for repeated measures over time"; 2) "Systems-level single cell dataset analysis or modeling... in the context of tissues or whole organisms; and 3) "the discovery of new, innovative tools for spati-otemporal imaging, manipulation, analysis and modeling of a biologically relevant population of cells with minimal perturbation".



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Whole-body tracking of single cells by positron emission localization

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Current applications of positron emission tomography (PET) involve reconstructing one or more images representing the spatial distribution of a radiotracer. However, PET is increasingly used in cell-tracking applications, for which the “imaging” paradigm may not be optimal. We are investigating an alternative approach, which consists in reconstructing the time-varying position of individual cells directly from raw list-mode PET, thereby bypassing conventional image reconstruction entirely. The hypothesis of this work is that this method would enable the spatiotemporal trajectory of single cells to be resolved for physiologically relevant activities and velocities. As an initial step, we have formulated an algorithm to reconstruct the trajectory of a single cell from list-mode PET measurements. The trajectory is modeled as a 3D B-spline function of the temporal variable. We used non-linear optimization to estimate the spline coefficients that yield the trajectory closest to the list-mode coincidence lines in the mean-square sense. To validate this new algorithm, we simulated the acquisition of list-mode microPET data using the Monte Carlo method. A single cell was moved inside a static water phantom meant to approximate the dimensions of a mouse. We also experimentally acquired data from a moving point source placed inside an Inveon PET scanner. Overall, we found that single-cell tracking is possible for a wide range of activities and velocities. We determined that a source could be tracked if its activity [Bq] was greater than four times its velocity [mm/s]. This relationship is however dependent on the sensitivity of the PET system used and on the desired tracking accuracy. Experimental results obtained on an Inveon microPET system have validated our simulation studies but highlighted an issue specific to that particular system: the radiation detectors it uses contain the element Lutetium, which is naturally present as two different isotopes, one of which is slightly radioactive (half-life of 40 billion years) and interferes with the weak signals emitted by single cells. Thus we are now investigating a different PET system with no such background radioactivity. In conclusion, single-cell tracking using PET presents the advantage that a single cell can be tracked over the entire body of an animal, in real time, with 1 mm spatial resolution and 1 s temporal resolution. This new capability will be useful for a number of biomedical investigations.



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Novel computational metrics and approaches for evaluating population-wide differences in alternative splicing at the single-cell level

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The recent availability of technologies that enable the large-scale isolation of single cells and generation of whole transcriptome sequencing data has led to the development of computational methods to evaluate gene expression at the single cell level. This has enabled exciting studies of cellular heterogeneity and gene regulatory networks. However, there is a dearth of computational approaches to analyze alternative splicing at the single cell level. We have recently generated deep (>30 million reads per cell) RNA-seq data for hundreds of single cells during stem cell differentiation towards mature motor neurons. Here we present a computational, open-source, framework that enables the analysis of alternative splicing at the single-cell level, using Bayesian methods to assign splicing “modalities” and non-negative matrix factorization (NMF) to reveal how “modes” change across our profiled single cells. We identify hundreds of “bimodal” alternative splicing events that have, surprisingly, distinct sequence and transcript properties that distinguish them from single “modal” events, revealing the power of these technologies and analytical tools in revealing the biological importance of alternative splicing at the single cell level.



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Visualizing epigenetic mechanisms of megakaryocyte maturation at the single cell level

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Epigenetic changes, including transitions in chromatin structure and histone/DNA modifications, determine the path that an individual cell takes during differentiation. To facilitate epigenetic studies of cell differentiation, we are developing a novel method for chromatin analysis at individual genes with single cell resolution.

These studies are focused on basic molecular mechanisms underlying maturation and polyploidization of megakaryocytes (Mk), which are essential for effective platelet formation. In a tightly controlled process, megakaryocyte-erythroid progenitors (MEP) differentiate into diploid megakaryoblasts; undergo a progressive increase in ploidy, resulting in large polyploid nuclei; and undergo extensive cytoplasmic maturation in preparation for proplatelet release. Previously, we have identified a link between the MKL1/SRF signal transduction pathway and polyploidization. SRF (Serum Response Factor) is a ubiquitous transcription factor that regulates its target genes by integrating with highly regulated cell-type specific transcriptional cofactors (e.g. MKL1), to activate gene transcription. We have shown that MKL1 expression is upregulated during Mk maturation, and that MKL1 is essential for normal Mk polyploidization by regulation of target genes including EGR1, and the guanine exchange factor GEF-H1 (ARH-GEF2). Our goal is to probe changes in the epigenetic state at these genes as primary human MEP cells undergo megakaryocyte lineage commitment and differentiation. Specifically we are interested in determining whether MEP cells are epigenetically homogeneous or if there is a distinct subpopulation pre-determined to become Mk. Analysis of primary human MEP cells and megakaryocytes at different stages of maturation will allow comparison of the kinetics of epigenetic changes at the EGR1 and ARH-GEF2 genes in individual cells with changes in gene expression levels, as well as expression of other genes that are critical for Mk differentiation.

Our epigenetic assay is based on a proximity *in situ* biochemical reaction, where the fluorogenic substrate fluorescein diacetate (FDA) is tethered to the gene as a streptavidin conjugate via a biotinylated DNA probe, while the cognate enzyme (esterase) is bound to the protein of interest as an antibody conjugate. Given close proximity, the *in situ* reaction produces fluorescent signal visualized and quantified by microscopy. We have previously demonstrated the feasibility of this idea in a budding yeast model system. Although these observations served as a proof of principle, attempts to use these reagents in mammalian cells failed due to instability of FDA in mammalian cells. Therefore we have focused on an alternative approach using a more stable substrate, FDP. New reagents were developed and tested in human Jurkat cells, including FDP-streptavidin, a biotinylated probe to one of the loci of interest (EGR1), and control probes to centromeres and COT1 (repeats). With these reagents, we have demonstrated that the assay allows for detection of DNA methylation present at a locus of interest in a single cell. For method validation, we used a conventional ChIP assay. Method challenges, limitations and alternative routes are discussed.



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Transcriptome-wide expression analysis of single cells during T-cell specification reveals distinct drivers of early differentiation states and final lineage fates

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In response to microbial infection, naive T cells undergo asymmetric division into short-lived effector cells, which mount an acute response, and long-lived memory cells, which provide durable immune defense against the pathogen. However, the molecular determinants for their lineage specification are not well understood. To address this, we gathered transcriptome-wide expression measurements from hundreds of individual CD8⁺ T cells during lineage progression during the course of microbial infection. Unsupervised clustering demonstrated separation between effector- and memory-fated cells but also revealed two subgroups of cells within the first division of activated T cells, which was consistent with their eventual fates. To investigate these novel findings, we constructed precise and accurate gene expression classifiers that distinguish between effector-like and memory-like cells in both early and late stages of specification. Largely non-overlapping sets of genes were identified as predictive of effector-like or memory-like states, suggesting that different regulatory programs are dominant within the same intermediate cell-types, depending on their stage of differentiation. To investigate this hypothesis, we applied both classifiers to cells isolated 4 days after microbial infection. Thus, the early classifier was deployed forwards in time and the late classifier was deployed backwards to an intermediate timepoint. Relying on the dissociative power of single-cell measurements, this approach found that the two classifiers agreed on the unsorted day 4 cells ($p < 5e-05$) despite the differences in their dominant genes.

Unlike existing methods for analyzing single-cell expression data from time-course RNA-seq experiments [1], which assume a single lineage for computational efficiency, our forward-backward approach not only handles the bifurcating lineage of T cell specification, but also generalizes to more complex lineages with multiple bifurcation points, such as stem cell differentiation. We believe our results suggest novel molecular drivers for lineage specification.

[1] Trapnell, C. *et al.* The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat Biotech* **32**, 381–386 (2014).



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Characterizing tumor suppressive functions of microRNAs in B-Cell neoplasia

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B-cell lymphoma is a significant clinical problem that is rising in incidence and prevalence as the American population ages. The most common subtype of B-cell lymphoma, diffuse large B-cell lymphoma, is a disease which arises from mature B-cells at various stages of antigenic activation and differentiation into plasma cells. Recently, it has become apparent that microRNAs (miRNAs), small RNA molecules that regulate gene expression, are intimately involved in oncogenesis and developmental regulation. In the parent R01, we had proposed to examine how miRNAs act to modulate the activation and oncogenic transformation of B-cells. The three aims were to (i) Identify and characterize microRNA-dependent regulation of B-cell activation and terminal differentiation; (ii) Characterize B-lymphomagenesis as a function of tumor-suppressor microRNAs; and (iii) Characterize the molecular mechanisms of miRNA-mediated regulation in B-cell development and B-cell lymphoma. Since the original grant submission, we have found that the microRNA miR-146a, a feedback regulator of the NFkB pathway, shows a highly important role in B-cell responses to antigenic stimulation. Although the overall role for miR-146a is as a factor that restrains activation of B-cells, individual B-cells show heterogeneity in the developmental decision following activation: proliferation/differentiation, or cell death. Here, we hypothesize that miR-146a regulates heterogeneity in the expression of critical targets that guide the cell fate decisions of individual B-cells. Recently, the laboratory of Dr. Alexander Hoffmann, an eminent computational and experimental biologist with expertise in the NFkB pathway, has utilized a sophisticated microscopy and modeling system to follow how these developmental decisions are made at the level of individual B-cells. In collaboration with their laboratory, we plan to further analyze the role of miR-146a in B-cell activation by answering the following questions: (i) do single B-cells from miRNA deficient mice show different developmental trajectories during activation? (ii) How does miR-146a regulate the transcriptome of a single B-cell during activation? And (iii) what is the relationship between miRNA activity and the decision of a B-cell to grow and divide or to undergo apoptosis? The answers to these questions will allow us to understand whether miRNAs make a unique and biologically non-redundant contribution to developmental regulation. Furthermore, we will model NFkB activity during B-cell development and how it is regulated by miR-146a. These studies will help us develop predictive models of cell-fate and developmental decisions, which will inform both basic research and therapeutic applications for small RNA techniques. All of the necessary methodology, training, resources, mentorship and personnel are in place for the successful completion of these goals. The completion of these goals promises to significantly increase our understanding of critical biological and pathological processes, in turn leading to improvements in cancer diagnosis and treatment.



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Genomic site binding rules and regulatory factor function in developing T cells

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We propose to use an innovative multiplex fluorescent in situ method for determining transcript numbers from multiple genes in the same cells, seqFISH, in a model system to address the general problem of how transcription factor effects are exerted on different classes of target genes in a dynamic developmental system. The proposal is a Revision for the existing, funded project, "Genomic site binding rules and regulatory factor function in developing T cells" (R01HD076915), and it adds a crucial single-cell analysis component to clarify the interpretation and extend the insights from the project. In parallel, the biological system offers many advantages for enhancing the power and demonstrating the utility of the seqFISH technique. We exploit a well-characterized framework of developmental events through which multipotent hematopoietic progenitors undergo commitment to become T-cell precursors, a system that can be studied in parallel in vivo and in vitro and which is highly defined at the cellular level and in terms of patterns of gene expression. The project focuses on the crucial but enigmatic role of the transcription factor PU.1, which is needed to support the early stages in T-cell development but does so apparently at the price of maintaining a regulatory bridge to an alternative set of developmental fates, i.e. macrophage, granulocyte, and dendritic cell fates. PU.1 has lineage-specific differences in its patterns of genomic occupancy in different hematopoietic precursors, but many of its binding sites do not appear to be linked with function. The parent proposal combines acute perturbation assays, genome-wide RNA-seq, and diagnostic CHIP-seq approaches to determine the rules that relate PU.1 occupancy to PU.1 regulatory functions in this early T-cell context. However, it is important to determine how homogeneous each baseline state is, and how uniform PU.1 actions are on all the cells at a given early T-cell stage, to resolve which PU.1 target genes are actually responding to PU.1 in the same regulatory-state context and which are responding in a different one. To answer this question and reveal in detail how different target genes "process" changes in activity of the same regulator, we propose a new collaboration between the Ellen Rothenberg and Long Cai groups. Specific aim 1: Use seqFISH to characterize the variation in PU.1 expression in T-cell precursors and variation in levels of known and suspected PU.1 target genes, relative to expression of genes that can modulate PU.1 effects. Specific aim 2: Optimize seqFISH technology for detection of transcripts from >20 different genes per cell in individual cells. Specific aim 3: Analyze the effects of PU.1 deletion and PU.1 antagonism on expression of different classes of candidate target genes in single cells, and determine their correlation with levels of different modulating factors in those cells.



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Single-cell transcript profiles reveal multilineage priming in early progenitors derived from Lgr5⁺ intestinal stem cells

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Cycling Lgr5-expressing intestinal stem cells (ISC) drive gut epithelial self-renewal. Lgr5⁺ cell populations differ in stem-cell activity, but the nature and extent of this heterogeneity is unclear, and ISC produce cells of distinctive absorptive and secretory lineages by passing through uncharacterized bipotential progenitors. Our profiling of 192 single Lgr5⁺ intestinal crypt cells for known stem cell and lineage-restricted mRNAs identified two distinct, equally abundant subpopulations. One population carries high levels of known ISC markers, whereas single cells of the second pool express genes that mark differentiated enterocytes and secretory cells. Distinct transcriptional networks indicate that the first population gives rise to the second, which shows features of bipotential progenitors. Immunofluorescence analysis verified expression of a lineage-restricted gene in a subset of Lgr5^{hi} cells in the crypt base. These findings define the earliest steps in ISC differentiation and reveal key properties of bipotential intestinal progenitors.



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Dynamic of splicing modality is uncovered from single cell transcriptomics during neuronal differentiation

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The human brain is comprised of billions of neurons that can be categorized into hundreds of different cell types based on their morphological, molecular and electrophysiological properties. One crucial mechanism to establish neuron diversity is alternative pre-mRNA splicing. Though the transcriptomics diversity created by mRNA splicing has been appreciated, the variation and complexity of splicing demands investigation at higher resolution, such as at the single cell level. Here we investigated the prevalence and characteristics of alternative splicing in single human neural progenitors (NPCs) and motor neurons (MNs). We deeply sequenced single NPCs and MNs derived from the induced pluripotent stem cells. Gene expression analysis uncovered individual cells failed to commit to neuronal fate *in vitro* differentiation. Alternative splicing analysis in single cells revealed multiple modalities of splicing. Interestingly, some of the alternative splicing events undergo significant modality change through differentiation, highlighting the dynamic and complexity of splicing during neuronal differentiation. This dynamic can be visualized in non-negative matrix factorization space. Taking advantage of the variation of splicing measurements and RBP expression at single cell level, we model splicing regulation and predict RBP networks, which converge to a similar model extracted from mouse spinal cord motor neurons. Taken together, our study underscores the strength of single cell sequencing as a powerful stagey to understand splicing regulation in heterogeneous populations.



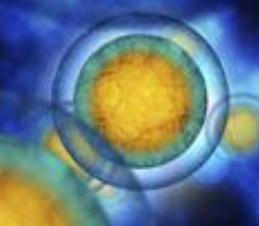
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Molecular biology of myeloid differentiation

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DNA methylation is linked to a growing number of human diseases, such as myelodysplastic syndrome, acute myeloid leukemia, almost all solid tumor cancers, and a number of genetic syndromes such as Prader Willi Syndrome (PWS). There has been moderate success of treatments of myelodysplastic syndromes with the FDA approved demethylating nucleoside analogs, including azacitidine and decitabine. Unfortunately, these agents are also highly toxic by virtue of acting indiscriminately on the entire genome, thus bringing about the numerous side effects. Here we propose to develop novel tools and protocols for targeted gene-specific alteration of genomic methylation. These tools and protocols will not only be applicative in research, but, most importantly, could eventually ground the bases for novel therapeutic agents in cancer and other diseases. This proposal builds on our recent discoveries: (1) RNA deep sequencing ("RNA-seq") analysis on RNAs immunoprecipitated with DNMT1 antibody, revealed ~6,000 transcripts interacting with DNMT1, suggestive of global involvement of transcription in the establishment and maintenance of cell type-specific DNA methylation patterns; (2) among these transcripts, the CEBPA gene locus noncoding RNA, chosen as a model for this study, was shown to be directly involved in inhibition of DNA methylation by forming complexes with DNA methyltransferase (DNMT1); and (3) expression of this RNA in cells in which CEBPA was not expressed resulted in promoter demethylation and activation of gene expression in a gene selective manner. These three major findings prompted us to propose the development of targeted gene-specific demethylation agent(s). Introduction of this novel gene-specific demethylating approach will lead to new treatments with great advantages over existing 5-aza-cytidine-based protocols. These advantages will include: a) high gene specificity; b) lower cytotoxicity; and c) absence of drug based side effects. These technologies will add to our ability to understand the basis of cancer development and progression, and form the basis of potentially novel therapeutic approaches.



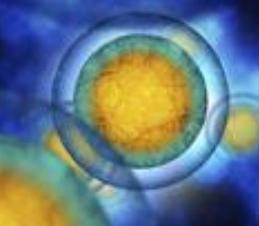
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Robust lineage reconstruction from high-dimensional single-cell data

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Single-cell gene expression profiling technologies provide an excellent opportunity to uncover rare cell types and cell-fate transition events during development. However, it remains a major challenge to systematically identify rare cell types and the lineage relationships among different cell-types, mainly due to the technical difficulty of precise gene expression profiling in single cells. While several methods have been developed to reconstruct cell lineages from data, one common limitation is that their results are sensitive to measurement uncertainty and sample sizes. It is desirable to account for such uncertainty in subsequent functional validation and mechanistic investigations. Toward this end, we have developed a novel computational method using an ensemble-based technique. The results from different subsamples are assembled together to infer probabilistic information regarding the robustness of cell-type classification and lineage relationships. The method is fast and applicable for analysis of large datasets. We have applied this technique to several datasets, obtained from various developmental processes. Compared to existing methods, our method not only significantly improved the robustness of the lineage reconstruction, but correctly inferred the uncertainty of lineage relationships. Our method provides a powerful tool for systematic exploration of single-cell data.



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The cellular landscape of cardiac development

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CHD is the most common type of birth defect with an estimated incidence of ~2-3%. Although recent advances in human genomic sequencing have revealed that many mutations disrupt gene regulatory events, the mechanisms by which these cardiovascular (CV) gene regulators control the progression of cardiovascular progenitor cells (CVPCs) along distinct lineages into mature specialized CV cell types and heart structures remains incompletely understood. In large part, much of our knowledge of how the heart develops has been based on large-scale bulk cell population analysis, including ensemble averaging of transcriptional profiling. As a result, elucidating how key CV developmental regulators create the complex regulatory networks to specify CV progenitors into each specialized CV lineage remains challenging due to the inability to study individual CV cell types as they transition developmentally, particularly at the earliest developmental time points during cardiogenesis where many of these key decisions are likely determined.

To overcome these roadblocks, we have utilized single-cell analyses to begin to discover the diverse array of CV cell-types that arise during cardiogenesis. Because many key CV fate decisions occur early, we have focused on analyzing the earlier events of cardiogenesis when mesodermal cells are becoming CVPCs and CVPCs are determining their lineage fate. To this end, we have employed a hPSC-CV system, which greatly aids in the ability to reproducibly obtain large amounts of various human CV cell types at the earliest CV developmental stages. As a result, we have begun to use single cell sequencing to not only identify new CV cell types not previously observed but also define the relationship of these CV cell types to each other. Completion of these studies to define the distinct CV cell types and their transcriptional profiles will position us to further computationally define the gene regulatory networks that guide the progression of CV cell types as they mature. Overall, these studies will inform us better in the future on how affected CV regulators in CHD patients may influence the CV regulatory network and heart development, and more importantly, how we might be able to alter their disrupted CV regulatory networks to improve patient outcome.



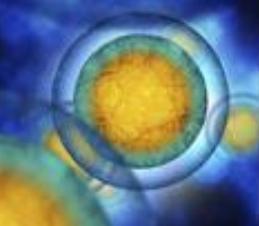
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p53 pulses diversify and coordinate target gene expression

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Cellular signaling pathways can transmit information about environmental stimuli and cellular state by controlling the expression dynamics of signaling molecules. The p53 tumor suppressor protein responds to DNA double-strand breaks with a series of pulses of fixed amplitude, duration, and period, regulating transcription of genes in the DNA damage response. While p53 pulses control cell fate, the mechanisms by which this regulation occurs are poorly understood. Here we show that p53 pulses generate different temporal patterns of target gene expression, which can be predicted by the mRNA decay rates of the genes and are not specific to gene function. Moreover, using transcriptional profiling of 1680 individual cells, we show that the expression of a subset of pulsatile p53 target genes is coordinated in a time-varying manner. These two functions of p53 pulsing enable it to orchestrate the complex response to DNA damage. Our results give new insight into the function of a growing number of signaling pathways that use pulsatile dynamics and may inform chemotherapeutic strategies based on selective manipulation of p53 dynamics.



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Cycle-regulated genes via single cell RNA sequencing

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We introduce a method for using single-cell RNA-seq to characterize cycle-regulated genes in a population of actively dividing cells: CRESCA (Cycle-regulated Expression via Single Cell Analysis) is a combination of advanced statistical methods with cutting edge single cell high throughput technology.

CRESCA starts with single-cell gene expression measurements. Then, regarding each cell as a point in expression space, manifold regression techniques are used to fit a closed loop curve through the points, and assign to each cell an artificial time stamp (ATS), measuring its progress through the cell division cycle. These ATS's are then used to scan for cycle-regulated genes on a transcriptome-wide basis.

For proof of concept, we obtained full transcriptome data for single cells from a colon cancer cell line by using using microfluidic capture (Fluidigm C1) and multiplexed cDNA paired-end sequencing (Illumina Nextera XT and HiSeq 2500).

This powerful methodology enables in-depth analysis of cell cycle gene expression in populations of actively dividing cells. It holds great promise in the genetic dissection of proliferation in cancer, of embryonic tissue development, of regeneration and wound healing, and other processes relevant to human health.



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Bayesian hierarchical approach for CNV detection in single cells from RNA-seq data

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Uncovering the impact of genetic heterogeneity on gene expression requires linking genetic and transcriptomic information at the single cell level. However, DNA and RNA cannot currently be reliably isolated jointly from the same cell. Here, we demonstrate the feasibility for linking somatic copy number variations (CNV) with transcriptional heterogeneity on the single cell level using data from single cell RNA-seq and prior knowledge from bulk whole exome-seq.

Previous efforts to infer CNVs on the single cell level from RNA-seq data have been limited to whole chromosome and chromosome arm-level changes. Inferring smaller CNVs from RNA-seq data remains difficult due to uneven coverage across CNV sites. Mono-allelic and highly variable gene expression introduces additional sparsity and noise. We developed BADGER, a Bayesian hierarchical approach for CNV detection using single cell RNA-seq data. BADGER leverages information from across multiple heterozygous SNP loci within a candidate CNV to make probabilistic inferences on the CNV status in single cells. BADGER also takes into consideration biases introduced by mono-allelic expression and other technical artifacts such as sequencing errors.

We apply BADGER to assess the genetic heterogeneity in one case of multiple myeloma. We identify two distinct subclonal populations marked by different deletions. A second sample derived from the same patient reveals clonal expansion of one subpopulation. We further apply differential expression analysis to identify gene expression differences between the two subclonal populations to provide potential explanations for the clonal expansion.



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Aging, single-cell methylomes

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Recent data suggest that the epigenome is highly dynamic and serves as an interface between the environment and the inherited static genome. The large volume of epigenomic events and its continuous need of maintenance, i.e., after DNA repair or replication, suggest a high chance of errors. The question we wish to address is how unstable the epigenome really is. Do epimutations accumulate with age and do they occur in a random fashion, i.e., as 'epigenomic drift'? Do they ever reach levels that are high enough to have functional consequences?

To experimentally determine epigenetic drift we focused on DNA methylation, a major layer of epigenomic control. To study intra-organ variation in DNA methylation during aging, it is necessary to have access to procedures that allow assessing DNA methylation patterns at the level of single-cells or single-molecules. Such methodology is currently entirely lacking: to fill this void we have optimized bisulfite sequencing for single cell analysis.

The procedure developed allows us to analyze DNA methylation patterns in single cells, within promoter regions of genes or genome-wide. Data on mouse fibroblasts, neuronal nuclei and hepatocytes will be presented and discussed. We are currently applying the method to test the hypothesis that random DNA methylation changes accumulate in the mouse liver during aging, contributing to functional decline of somatic cells that gives rise to chronic pathology and aging.



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On chip analysis of CNS lymphoma in cerebrospinal fluid

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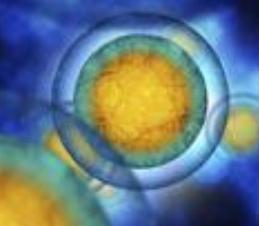
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Diagnosis and monitor molecular profiling of central nervous system lymphomas in cerebrospinal fluid (CSF) samples can be challenging due to the paucicellular nature of the limited samples. We herein present a microfluidic platform able to process the entire CSF sample volume, capture single lymphoid cells in sub-nanoliter volume trapping site, and then profile the cells on-chip by fluorescence image analysis or by response to chemotherapeutic agents. We used three-step strategy to profile lymphocyte cell populations, 1) the use of CD19 and/or CD20 to determine B cells; 2) the use of kappa or lambda light chains to identify clonal populations and 3) additional phenotypic markers for subtyping and prognostic tasks. In the present study, we show that the system can detect scant lymphoma cells and quantitate their kappa/lambda immunoglobulin light chain restriction patterns. The approach provides a flexible platform to profile lymphoma cells from paucicellular samples, thus enhancing the accuracy and ease of CNS lymphoma diagnosis, the potential for biomarker-based treatments, and the ability to track the efficacy of those treatments over time.



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Correlation and kinetics of IFN γ and TNF α mRNA and protein in lymphocyte subsets at the single-cell level by flow cytometry

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Flow cytometry with its ability to look at millions of cells, target multiplexing capabilities and straightforward workflow to detect both cell surface and intracellular proteins with single-cell resolution has been the standard for the study of heterogeneous cell populations. However flow cytometry has historically been constrained by the availability and adequacy of antibodies to measure analytes. Non-coding RNA, messenger RNA, viral transcripts, unique model organisms and targets or targets for which antibody development is troublesome have not been able to utilize the power of flow cytometry and have required numerous disconnected experiments to analyze their impact on cell subsets.

Intracellular flow cytometry is commonly used to assess cytokine production at the single-cell level in heterogeneous samples. Here, we describe a novel *in situ* hybridization (ISH) based flow cytometry assay (PrimeFlow™ RNA Assay) used in combination with intracellular antibody staining to study the kinetics of transcription and translation of IFN γ and TNF α in lymphocytes at the single-cell level. Normal human PBMCs were stimulated with PMA and Ionomycin for 0-5 hours. Cells were fixed, permeabilized, and intracellularly stained with antibodies for CD8, IFN γ , and TNF α . Next, cells underwent a series of hybridization steps to label mRNA for IFN γ and TNF α . IFN γ mRNA was upregulated in CD8+ and CD8- lymphocytes within 1 hour after stimulation, while protein levels were detected at 2 hours, after which both mRNA and protein were maintained for the next 3-4 hours. In contrast, TNF α mRNA and protein were both upregulated within 1 hour after stimulation; expression was maintained in CD8+ cells while expression in CD8- cells peaked between 1-2 hours and then decreased over the next 4 hours, with mRNA decrease preceding protein decrease. Using the PrimeFlow™ RNA Assay, we found that induction of IFN γ and TNF α mRNA and protein exhibit unique kinetics and that TNF α protein and mRNA are differentially regulated in CD8+ and CD8- lymphocytes.



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High-throughput microdevice for temporal single-cell analysis

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One critical need for advancing the field of single-cell analysis is the ability to nondestructively sample individual cells over time with experimental throughput that is statistically relevant. This would be a major advance beyond the currently available lysate-based single-cell analysis techniques. The rich information obtained from single-cell, temporal analyses would lead to fundamental understanding of the molecular machinery of a cell and its response in a complex environment, enabling novel temporal studies of disease mechanisms, biological variability, or molecular input-output relationships that control mechanistic pathways.

To this end, we are developing a microfluidic device that will provide on-chip cell culture, transfection, nondestructive cytosol sampling, and eventually, subsequent on-chip biomarker detection. The device is comprised of a cell culture chamber with a porous membrane substrate, microfluidic channels, and integrated electrodes for electroporation and enhanced molecular detection. As an initial step, we prepared a localized electroporation device for on-chip cell culture and transfection and tested it by first performing electroporation of HeLa and HT1080 cells. We then demonstrated on-chip differentiation of neural stem cells and transfection of postmitotic neurons with a green fluorescent protein plasmid.



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The single cell basis for plasma protein synthesis by the liver

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In a conventional histological preparation, the predominant cells of the liver, the hepatocytes, appear to be homogeneous. However, these cells possess a cryptic heterogeneity, which can be visualized by immunofluorescence with antisera to a number of liver-produced plasma proteins. By this method, each plasma protein appears to be present in a small, separate, subpopulation of hepatocytes. This cellular heterogeneity appears to be generated by a process of plasma protein gene activation that is stochastic. Albumin is present in somewhat less than 1% of hepatocytes in adult liver, while cells containing each of the other proteins are present in lower numbers, roughly in parallel with the relative rates of synthesis of the corresponding plasma proteins, a relationship seen in a wide range of contexts: in neonatal and adult life, during aging, liver regeneration, experimental hormone administration, and inflammation. The specific mix of these specialized cells appears to be molded by a process of cellular selection that is reminiscent of clonal selection in the immune system.



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Non-invasive perturbation and control of signaling activated gene regulation

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Signal transduction and gene regulatory pathways exhibit dynamic profiles that cause distinct cellular phenotypes. Manipulating these profiles required genetic and drug perturbations of known proteins. We propose an orthogonal approach to perturb and control dynamic signaling and gene regulatory pathways, without genetic or drug perturbations, using extracellular temporal concentration profiles. To demonstrate the feasibility of this approach we interrogate and control the osmotic stress response in yeast, enabling the manipulation of signaling intensity, duration, and shape. Combining quantitative single cell and single molecule experiments with predictive modeling, enables the quantification of thresholds for signal transduction activation, signal transduction saturation and gene expression activation. This approach is independent of the biological pathway or organism and presents a general methodology to interrogate and control signal transduction and gene expression pathways non-invasively.



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Single-cell genome-wide CpG methylation pattern analysis based on enzymatic discrimination and selective amplification promise a substantial coverage

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We report a method combining methylation-sensitive restriction enzyme digestion (MSRE) and multiple displacement amplification (MDA), followed by reduced representative sequencing for determining the CpG methylation patterning (MM-CpGmp) of single cells. We first characterized this method using K562 cell line, each sample containing approximately 10 to 100 cells, and we found a good correlation between the global methylation pattern obtained with our method and the Infinium 450K methylation beadChips data (m450K) from the ENCODE database. We then analyzed 30 samples in total from 4 different cell types (K562, GM12878, iPSC and fibroblasts) and found that the data was clustered accordingly. When 8 K562 and 8 GM12878 single cells were analyzed, we detected from a single cell up to 86.8% of the CpG islands (CGIs) that the conventional reduced bisulfite sequencing (RRBS) with bulk DNA detected (22,037 CGIs), and the total and the average CGIs covered for the 16 single cells were 93.5% and 63.4% respectively. Importantly, we detected 28.1% CGIs across all 16 single cells; this intersection coverage was 20x higher than those obtained with the recently reported scRRBS across 16 samples (1.46%). In addition, we confirmed the reliability of the single cell data by comparing it with the ENCODE bulk data for a series of 40 epigenomic events available for K562 cells and 15 epigenomic events available for GM12878 cells, and both cell types demonstrated significant correlations between the ENCODE data (m450K and RRBS) and single cell analyses for these events. Finally, we identified heterogeneity in both K562 and GM12878, which show distinct and specific CpG methylation patterns, correlated with ENCODE data. Our method faithfully captures the methylation status of a single cell at a genome scale. It provides a new and robust tool for elucidating DNA methylation heterogeneity at the single cell level.



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Analysis of glucocorticoid receptor dynamics by number and brightness and single-molecule tracking methods

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The Glucocorticoid receptor (GR) is a ligand-regulated transcription factor and one of the most targeted proteins in the pharmaceutical industry due to its powerful anti-inflammatory and immunosuppressive activities. GR transcriptional activity and clinical outcome have been linked to its dimeric/monomeric state. Here we have analyzed GR's oligomerization state in vivo using the Number and Brightness assay, a fluorescent fluctuation technique implemented in single cells. Our results suggest a complete, reversible, and DNA-independent ligand-induced model for GR dimerization. Contrary to dogma, no correlation between the GR monomeric/dimeric state and transcriptional activity was observed. These results have major implications on future searches for therapeutic glucocorticoids with reduced side effects.

GR dynamically associates with a subset of glucocorticoid response elements (GREs) present in chromatin. Dynamic binding of the receptor depends on the context-specific chromatin landscape. Although GR binding to DNA has been extensively studied, mechanisms involved in GR dynamic exchange with chromatin in living cells remain elusive. We are developing a two-color single-molecule tracking approach to characterize the dynamic interaction between GR and its cofactors (including chromatin remodelers) within a tandem array of GREs inside living cells. Here we present preliminary results of GR's residence time on chromatin and the general strategy for the two-color experiments.



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The Polaris System: Integrating cell and molecular analysis at the single-cell level

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Single-cell biology has opened up vast new frontiers of discovery, however, until now an entire dimension of space has been missing. While probing the genome and protein expression of the fundamental unit of life has revealed profound scientific insights, by and large we've been unable to study molecular and cell biology that reflects the actual conditions in which cells live and change. This is because we primarily conduct single-cell research outside of a precisely controlled environment. With the Polaris System, which consists of an integrated fluidic circuit (IFC) and instrument, researchers will be able to actively select individual cells based on phenotype, precisely control the environment to which the cells are subjected and analyze the mRNA from each individual cell. Here we show proof of concept experiments on the Polaris System isolating and processing multiple types of cells through mRNA Seq. We also demonstrate the ability to perform and monitor transfections on the Polaris 48 IFC.



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Characterization of cultured cells treated with low doses of formaldehyde by exhaustive single-cell lineage tracking analysis

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A chronological analysis of events occurred in individual cells provides a powerful means of understanding the heterogeneity of cultured cells. Thus, we have been developing an exhaustive single-cell lineage tracking analysis. We built a made-to-order microscope that is optimized for long-term live cell imaging (up to 4 weeks) and developed a series of software that can automatically create up to 16 live cell imaging movies simultaneously, perform single-cell tracking, create a cell lineage database, determine cell lineage maps and perform data analysis. We used the analysis to characterize cells exposed to a low dose of a carcinogen.

In various laboratory studies using a carcinogen, high dose has been routinely used for the induction of cellular responses, e.g. cell death, simply to have the responses detectable by conventional methods. However, human cancers are likely caused by exposure to environmental carcinogens at extremely low dose. Because cellular responses induced by such a dose of carcinogens cannot be detected by existing methods, we employed exhaustive single-cell lineage tracking analysis to ask whether low doses of carcinogens induce any cellular responses. We found that a minor group of cells underwent abnormal cell division or cell fusion after exposure of cells to the dose of a carcinogen. While it still requires more extensive studies to relate the observed abnormalities with the risk of developing cancers in humans, our analysis approach would provide a new option to investigate the process of cancer development.



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On-chip preparation system for simultaneous cytoplasmic RNA and genomic DNA analyses of single cells

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Single cell analyses of RNA and DNA are crucial to understanding the heterogeneity of cell populations. The number of tools and approaches to analyze single cells are expanding, but sequence specific measurements of nucleic acids have typically been limited to studies of either DNA or RNA, and not both. This analysis still remains a challenge as RNA and DNA have very similar physical and biochemical properties, and cross-contamination with each other may introduce false positive results.

We here present an on-chip system that leverages selective electrical lysis and isotachopheresis to deliver cytoplasmic RNA and genomic DNA (gDNA) from the same single cell to independent downstream analyses. Our system selectively lyses the cytoplasmic membrane while leaving the nucleus intact. We then extract, focus, and separate (away from the gDNA-containing nucleus) the released cytoplasmic RNA and absolutely quantify it. Absolute RNA mass quantification is performed using fluorescence measurements without enzymatic amplification, all in less than 5 min. The cell nucleus is left intact and the relative gDNA amount in the nucleus can be measured. We fractionate the nucleus and focused RNA to two separate downstream wells for further analysis. We demonstrate the technique using single mouse B lymphocyte cells, for which we extracted an average of 14.1 pg cytoplasmic RNA per cell and observed heterogeneity associated with cell cycle in RNA and DNA masses. We also demonstrate our system with simultaneous, sequence specific quantitation of cytoplasmic RNA and gDNA analyses using off-chip RT-qPCR and qPCR, respectively.



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Content-rich measurements of single-cell biophysical properties via microfluidics

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CellMECh (Cell Mechano-Electrical Characterization) technology is a high-throughput, label-free assay with single-cell resolution for screening cell biophysical (mechanical and electrical) properties. The microfluidics-enabled technology offers a high throughput of cells (≥ 3000 s cells per channel per minute) for biophysical profiling of individual cell properties in heterogeneous cell populations. The micro-chip is fabricated by a simple, low-cost molding technique and is equipped with embedded electrodes for fully automated measurements of single-cell electrical (multi-frequency impedance) and mechanical (transit time) properties. On this microchip, impedance is continuously monitored in real-time as cells transition between a mechanically non-disruptive channel into a narrow deformation region producing mechanical stress by deforming the cell membrane, cytoplasm, and nuclear structures, and as cells relax upon exiting the deformation region. The technology provides rapid data acquisition and yields high-content and high resolution data of single-cell biophysical properties. The link between cell biophysics and disease has been documented in cancer, malaria, sepsis, and diabetes. Disease pathology directly impacts and dysregulates cell biophysical behaviors through alterations in cell membrane, cytoskeleton, and cytosol composition and biochemical organization. We used this assay to analyze mechanical and electrical properties of breast cells representing transition states between normal and metastatic cancer. The experimental data from single-cell populations showed the assay capability to distinguish between benign, low metastatic potential, and highly metastatic tumorigenic breast cell lines. The CellMECh technology is highly sensitive to alterations in cell biophysical properties and has applications in the detection of diseased cells and cellular responses to toxicants, pharmaceuticals, and other biologically active agents.



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TLR 4 distribution and dynamics in individual macrophage cells during immune response

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Macrophage cells have a repertoire of mechanisms for sensing and responding to invading pathogens. Among these, Toll like receptors (TLRs) stimulate immune response to a variety of pathogen associated molecular patterns (PAMPs). Bacteria have adapted to avoid immune recognition by altering the structure of lipopolysaccharide (LPS), a molecule present on their surface which specifically binds Toll-like receptor 4 (TLR4) and initiates a signaling cascade for immune response. For example, *Yersinia pestis* makes a hexaacylated form of LPS that is stimulatory to macrophages resulting in secretion of cytokines and subsequent immune response when grown in the flea gut at 21°C. However, when *Y. pestis* infects humans and shifts to 37°C, the LPS becomes tetraacylated and non-stimulatory.

In this study, we compare and contrast the TLR4 distribution and dynamics in RAW 264.7 macrophage cells upon stimulation with five different LPS chemotypes. Multifocal plane microscopy (MFM) was employed to determine the diffusion behavior of individual TLR4 molecules in the plasma membrane, prior to and immediately following stimulation by LPS. Using interferometric photoactivation and localization microscopy (iPALM), we visualize the three-dimensional, nanoscale distribution of single TLR4 molecules over the surface of individual cells at 15 minutes post-stimulation. We discover multiscale reorganization of TLR4 into various structures including dimers, clusters, and flat patches. Our work provides the basis to elucidate fundamental principles that govern differential TLR4 receptor reorganization at the plasma membrane during immune response.

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Somatic variation in neurons in autism spectrum disorder

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Autism spectrum disorder (ASD) is a common complex neuropsychiatric condition with strong genetic background affects 1 in every 68 children. Rare pathogenic *de novo* events (single nucleotide variation (SNV), and copy number variation (CNV)) are enriched with ASDs cases. Such germline *de novo* mutations only present in ~10-15% of ASD cases, implying that much of the genetic risk remains unexplained. Regarding phenotype, there exist notable discordance within monozygotic twin pairs for diagnosed ASD, and often considerable symptom severity differences within ASD-concordant MZ twins. Due to the inaccessibility of brain tissue and technological limitations, most analyses were conducted in pooled DNA derived from peripheral blood and the brain somatic variation is an unexplored opportunity to detect robust genotype-phenotype correlation. Somatic *de novo* mutations and expression regulatory variations are extremely heterogeneous within tissues, such as in the brain. Conventional mRNA analysis derived from a pool of cells suggests that 86% of the genome is expressed in the brain, with over 90% of these genes showing different patterns of expression prenatally. Until recently, neuronal somatic variations (DNA/RNA) were largely overlooked. Although most neurons do not divide when an individual reaches adulthood, during the first half of human gestation the proliferation of these cells may be higher than in any organ at any period of development. Recent analysis on single neurons captured from human frontal cortex revealed that 13 to 41% of neurons have at least one megabase-scale somatic *de novo* CNV. From our analysis, we have identified 2-7% mosaic variations analyzing multiple datasets of autism spectrum disorder (ASD) families. Our study emphasizes the genome-wide rate of mosaic variants in ASD. In addition, we are also quantifying the rate of mosaic variants within clones of neurons obtained from postmortem ASD and control individuals. The detection of somatic variation in neurons in ASD subjects and controls promises to uncover more of the factors that lead to ASD. This, in turn, will support the development of strategies to treat individuals with, or at risk for ASD, through targeted and personalized interventions.



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Metabolites and lipids in single human cells explored by capillary microsampling electrospray ionization mass spectrometry with ion mobility separation and stable isotope labeling

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Advances in mass spectrometry (MS) have enabled simultaneous detection and identification of a wide range of metabolites and lipids in single cells. Metabolic analysis of single human cells, however, still presents significant challenges due to the small cell volume, low amounts of metabolites, fast turnover rates, and complex molecular species. Here, we apply capillary microsampling and electrospray ionization (ESI) MS with ion mobility separation (IMS) for the metabolic analysis of single hepatocytes. To determine lipid turnover rates, this technique is combined with stable isotope labeling pulse-chase analysis. Cellular heterogeneity of the adenylate energy charge (AEC) and its response to rotenone treatment are investigated.

Individual cells were sampled by a pulled glass capillary using a micromanipulator and an inverted microscope. The capillary was backfilled with electrospray solution and a platinum wire electrode was inserted. Applying a high voltage to the wire produced an electrospray, the generated ions were separated by a traveling wave IMS system according to their different collision cross sections (CCS) and then analyzed by a mass spectrometer. Based on both CCS and mass-to-charge ratio measurements, 21 metabolite and 46 lipid ions were identified. The results showed that the range of the AEC levels in single cells changed from 0.49-0.90 to 0.02-0.54 after rotenone treatment, corresponding to a significant shift in the mean value from 0.70 ± 0.10 to 0.14 ± 0.13 . Phosphatidylcholine lipid turnover rates, e.g., $t_{1/2} = 46.8 \pm 3.0$ h for PC(16:0,16:1), were determined in single cells by stable isotope labeling pulse-chase analysis.



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iTAST: Technology platform for high-throughput in situ TCR Affinity and Sequence Test on human primary polyclonal CD8⁺ T cells

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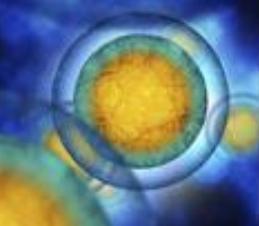
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Through thymic selection, the immune system generates a diverse repertoire of CD8⁺ T cells each bearing a unique T cell receptor (TCR), which recognizes specific peptide antigens bound to major histocompatibility complex (pMHC). While it is known that the physical interaction of the TCR with pMHC can dictate the quality of the T cell response, such as proliferation rate and differentiation status, this parameter has been difficult to measure owing to the need to produce soluble TCRs. To remedy this problem, we introduce the in-situ TCR Affinity and Sequence Test, named iTAST, that can process 50 single T cell affinities and sequences in one day directly from human blood, while requiring as few as 200 starting T cells.

Using single-cell in-vitro expansion techniques, we prove that the affinities measured by iTAST is predictive of in-vitro function and more so than tetramer staining intensity. We used iTAST to measure the affinity distribution of the inexperienced HCV-specific CD8⁺ T cell repertoires within healthy human donors and found a surprisingly large dynamic range of over 100-fold. Paired TCR $\alpha\beta$ sequences were successfully obtained from 70% of the primary T cells that had an affinity test making it possible to track both TCR sequences and affinity simultaneously. In addition, by comparing the CMV-specific T cell repertoires between CMV seropositive and seronegative individuals, we also observed a significant affinity increase in the former compared to the latter, indicating that the underlying mechanism of T cell functional avidity maturation is based on physical kinetics of the TCR-pMHC interaction. These results demonstrate the wide applicability of iTAST, from basic research into TCR-pMHC affinity to a diagnostic tool for measuring the quality of in-vivo T cell responses.



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Rapid single bacterial detection from blood using Integrated Comprehensive Droplet Digital Detection

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The high mortality of blood stream infections is associated with the ineffectiveness and time-consuming process of bacterial detection and treatment. Unfortunately, blood culture, the gold standard for the detection of bacteremia, takes several days to obtain results. New molecular diagnosis methods, such as polymerase chain reaction (PCR), are often not sensitive enough to detect bacteria that occur at low concentrations in blood (1-100 colony-forming unit (CFU)/mL). Moreover, all these techniques are sophisticated and expensive, and therefore not well-suited for routine testing. Therefore, simple methods are urgently needed for rapid and sensitive identification of bacteria in blood, which has the potential to significantly reduce the mortality rate and the cost of medical care associated with blood stream infections.

In this study, we present a new technology termed 'Integrated Comprehensive Droplet Digital Detection' (IC 3D) that can selectively detect bacteria directly from milliliters of diluted blood at single-cell sensitivity in a one-step, culture- and amplification-free process within 1.5–4 h. The IC 3D integrates real-time, DNAzyme-based sensors, droplet microencapsulation and a high-throughput 3D particle counter system. Using *Escherichia coli* as a target, we demonstrate that the IC 3D can provide absolute quantification of both stock and clinical isolates of *E. coli* in spiked blood within a broad range of extremely low concentration from 1 to 10,000 bacteria per ml with exceptional robustness and limit of detection in the single digit regime. This IC 3D technology can serve as a platform for other single cell analyses, such as circulate tumor cells detection in the blood stream.



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“Follow That Cell” Challenge Executive Summary

Next generation automated cell tracking software to Follow That Cell and its progeny accurately in complex multicellular environments

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Time-lapse microscopy is a commonly used technique to study dynamic cell behaviors, such as proliferation and differentiation, on a single cell level. Rapid developments of fluorescent markers, microscopes, cameras, and computers have made it possible to generate extremely large time-lapse datasets in both 2D and 3D. Currently, the main bottleneck preventing a widespread use of technologies that rely on time-lapse microscopy is the lack of automated methods to outline and track cells in time-lapse sequences.

In this project we will develop an open source software package for cell tracking, which is user-friendly for biologists, achieves high tracking performance, and is applicable to many different cell types and imaging conditions. We currently have prototype software that we have used to monitor muscle stem cell fate. Performing cell tracking using this software, we have revealed the profound impact of substrate rigidity on maintaining the stem cell fate [1]. This platform has in turn enabled us to screen for and identify compounds that enhance stem cell function and rejuvenate the aged muscle stem cell population [2]. In this project we propose to extend our existing software to incorporate novel fluorescent markers of stemness and differentiation, for example dynamic readouts of transcription factors, such as the stem cell marker Pax7-eGFP. We will continue to improve the performance and the broad utility of our software. For example, we will develop methods to select and optimize algorithm parameters in an automated manner. This will entail either manually labeled training data or unsupervised learning. This will allow individuals with no background in computer science to achieve tracking results which only researchers in computer science can produce today.

Our software can already be used to track almost any cell type imaged using different kinds of transmission microscopy, as well as nuclear and cytoplasmic fluorescence microscopy in both 2D and 3D. This general applicability is ensured by the modularity of our solution, where the tracking algorithm is designed to work jointly with any segmentation algorithm tailored to a specific cell type and imaging modality. We have also empirically demonstrated the strength of this approach through our participation in the ISBI Cell Tracking Challenges, held at the International Symposium on Biomedical Imaging 2013, 2014, and 2015. The challenges evaluated the performances of different cell tracking systems on diverse datasets with cells in culture and in whole embryos, imaged using different imaging techniques in both 2D and 3D. The tracking challenges were global and open to both academia and industry. In 2014 we achieved the best performance on all 14 datasets of the challenge, showing that we are leading the world in terms of both tracking performance and applicability to different cell types and imaging conditions. The results for 2015 will be announced on April 16.

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High spatiotemporal resolution mass spectrometry for single cell analysis

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We propose to develop a new ambient ionization mass spectrometry for single cell analysis. We call this new method desorption/ionization droplet delivery mass spectrometry (LDIDD MS). It enables MS imaging with high resolution and sensitivity such that a single cell or even subcellular components can be analyzed for its chemical content. The LDIDD MS utilizes a pulsed laser for desorption and ionization of molecules on a substrate; liquid droplets directly sprayed onto the focused laser irradiation spot delivers the desorbed ions to a mass spectrometer. The spatial resolution we have currently achieved is 2~3 microns, and limit of detection is 50 femtomoles. LDIDD MS will be optimized for better spatial resolution and sensitivity. In the proposed research, an array of printed single cells will be analyzed and imaged with LDIDD MS for high-throughput single cell analysis. A database of single cell metabolomic changes and secreted molecules under apoptosis will be constructed to address the mechanism of onset and progression of cell death as a model system. LDIDD MS is also capable of direct real-time analysis of samples in the liquid phase. Peptides and protein dissolved in water were successfully analyzed using LDIDD MS. Secreted peptides from cultured live PC12 cells were also successfully detected. The performance of the live cell analysis with LDIDD MS will be further optimized to enable the measurement of secretions from single live neurons. We propose to construct single- neuron secretomics and to learn about the interactions between single neurons by analyzing molecular species from single cells. These combined features of LDIDD MS would enable the collection of unprecedented information on spatially resolved metabolomic profiles as well as the spatiotemporally resolved secretomic profiles at the single cell level. The collected metabolomic and secretomic data will be statistically analyzed to extract the most significant and relevant species to a cellular response model. We have chosen cell apoptosis as the first model for study. By analyzing cell-to-cell variation in metabolomic and secretomic changes upon the same amount of apoptotic stimulus at different time scales of apoptosis, we hope to learn about those factors causing cell death. This scored molecular database will be used for constructing a stochastic model of cell apoptosis. At present, to our knowledge this type of information does not exist.