Cutting-edge protein analysis technologies

Advancing quantitative proteomic research, biomarker assessment and molecular diagnostics

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Collaborative Protein Technology Resource (CPTR)

The Nanoscale Protein Analysis Section
We offer expertise and provide state of the art immunoassays to support CCR investigators on rapid, precise and cost-effective functional proteomic studies for:

- **Comprehensive and quantitative cell signaling profiling**
- Cytokine, chemokines, growth factors and immune response measurement
- **Single-cell protein analysis**
- Biomarker & therapeutic target identification and validation
- **Preclinical and clinical applicable assay development and implementation**
- On- and off-target drug activity assessment, pharmacodynamics evaluation

-- A CCR resource specializes in evaluating, developing and implementing cutting-edge proteomic analysis technologies to facilitate discovery and translational research in CCR/NCI/NIH
Cutting edge protein analysis technologies

**Capillary immunoassays**
- Simple Western system

**High multiplex immunofluorescence imaging**
- CODEX technology

**In-solution multiplex sandwich ELISA**
- Luminex xMAP technology

**Single-cell western system**
Pressure to publish, selective reporting, poor use of statistics and finicky protocols can all contribute to wobbly work. 

"Researchers can also be hampered from building on basically solid work by difficult techniques, poorly described methods and incompletely reported data."

http://www.nature.com/news/reproducibility-1.17552

**Western blot**,  
-- 30 year old technology, gold standard for cell signaling pathway study

- poor reproducibility
- lack of accurate quantitation
- extensive time to result
- reliability issues
Automated Capillary Immunoassay System

– Simple Western™

- Employs high-resolution MW (size-based) or isoelectric-focusing (IEF, charge-based) separation, followed by target-specific immunoprobing to profile proteins and respective post-translational modification isoforms
- Integrates and automates all manual operations associated with Western blotting
- Provides bioanalytical labs with reproducibility and sensitivity in western blotting methods
- Have been applied for quantitative proteomic analysis in both discovery research and clinical practice
The Simple Western Technology

**Step 1: Load Matrix** (size based assay only)
Stacking and separation matrices are loaded into capillaries.

**Step 2: Load Sample**
~10-40 ng protein samples, prepared with SDS-containing buffers (for MW separation) or solution-phase carrier ampholytes (for IEF separation), were loaded into the capillaries.

**Step 3: Separate**
Proteins and fluorescent standards are separated by MW or isoelectric point.

**Step 4: Immobilize**
UV light is used to immobilize proteins to the capillary wall using a proprietary linking chemistry.

**Step 5: Immunoprobe**
The capillary is immunoprobed for specific proteins. Luminol and peroxide are added to generate chemiluminescent light, which is captured by a CCD camera.

**Step 6: Quantitate**
The digital image is analyzed and quantitative results are presented in the software.
Assay performance

- **Fully automated and robust**, all steps computer programmed, including sample loading, protein separation, immunoprobing, washing, detection and data analysis.

- **Precise and accurate measurement**, digital data quantitation, good assay sensitivity, reproducibility and dynamic range.

![Graph showing assay performance](image)

1.5 log dynamic range improvement
Assay performance

- Fully automated and robust, all steps computer programmed, including sample loading, protein separation, immunoprobing, washing, detection and data analysis.

- Precise and accurate measurement, digital data quantitation, good assay sensitivity, reproducibility and dynamic range.

- Nanogram (ng) level protein analysis, capillary platform allows protein analysis in extremely small and precious samples, such as stem cells, primary cells, fine needle aspirates, other patient specimens etc.

- Multiplex analysis with fast assay turn-around time, allows simultaneous measurement of multiple protein targets, analyzes up to 96 sample / analyte combinations per run in ~16 hours.

- One analysis run, ~16-hour;
- 5µL of sample, 8 different markers
Assay performance

- **Fully automated and robust**, all steps computer programmed, including sample loading, protein separation, immunoprobing, washing, detection and data analysis.

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- **Multiplex analysis with fast assay turnaround time**, allows simultaneous measurement of multiple protein targets, analyzes up to 96 sample / analyte combinations per run in ~16 hours.

- **Increased sensitivity and specificity**, multi-analyte analysis using a parallel single-analyte format
  
  - One analysis run, ~16-hour;
  - 5μL of sample, 8 different markers
Sample stability through analysis

AKT pathway: MCF7+/−Insulin

Up to 16 data points per 5 µL sample

Samples are kept on 4 °C during analysis run

Insulin - + - + - +

Anti-total antibodies

Day 1 Day 2

PRAS40
p70S6
S6 protein
AMPKα
AMPKβ
AKT
cRaf
Tubulin

Anti-phospho antibodies

Day 1 Day 2

- + - +
Consistent with Western data

**Quantitation of PKCβII and PKCδ down-regulation in U937 cells treated with phorbol esters and bryostatins**

![Conventional Western Data](image1)

![Simple Western Data](image2)
Consistent with Western data & precise quantitation

Quantitation of PKCβII and PKCδ down-regulation in U937 cells treated with phorbol esters and bryostatins

Quantitative proteomic assessment of differential ligand responses downstream of protein kinase C activation

**Targets:**
- 40 signaling molecules, PKC isoforms, NFkB pathways, MAP kinases, AP1 transcription factors etc.

**Lysate amount:**
- ~30µg (5-10ug with newly improved protocol)

In collaboration with Drs. Noemi Kedei & Peter Blumberg
Quantitative proteomic assessment of differential ligand responses downstream of protein kinase C activation

In collaboration with Drs. Noemi Kedei & Peter Blumberg
Quantitative proteomic assessment of differential ligand responses downstream of protein kinase C activation

**Total lysates**

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**Nuclear extracts**

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In collaboration with Drs. Noemi Kedei & Peter Blumberg
Established Simple Western assays

Over **250** targets established at CPTR:
https://cptr.cancer.gov/technologies/simple_western/assays

**Key Pathways:**
Apoptosis/Cell Death, Cell cycle and checkpoint control, Cellular metabolism, Chromatin Regulation/Epigenetics, DNA damage and repair, Gene regulation and DNA repair, JAK/STAT signaling, MAP Kinase signaling, NFkB signaling, PI3K/AKT/mTOR signaling, Protein Kinase C signaling, Receptor tyrosine Kinase signaling, Rho signaling, RNA regulation, TGF-β/SMAD signaling, Transcription regulation, Ubiquitin-proteasome, Wnt Signaling etc.

We **continuously develop new assays** based on the demand from the CCR/NCI researchers; **Antibody transfer rate** from conventional western: > **80%**

- **Flexible assays** to provide **custom pathway network profiling** based on the disease, drug target(s) etc.
- The ability to analyze a number of key pathways is enabling investigators to **identify critical pathways** involved in the behavior of newly developed cell lines, PDXs, patient tumor samples, as well as molecular functioning mechanisms.
Clinical application I: Phase II Study of **SMAC-Mimetic** Birinapant

**Birinapant (TL32711):**

A first in class bivalent peptidomimetic of SMAC, which mimics SMAC’s modulation of inhibitor of apoptosis proteins (IAPs)

**Treatments** – Relapsed platinum resistant epithelial ovarian cancer (EOC), primary peritoneal cancer (PPC) or fallopian tube cancer (FTC) patients received Birinapant 47mg/m² IV on days 1, 8 and 15 of a 28-day cycle.

<table>
<thead>
<tr>
<th>Sample type</th>
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<th>Planned analysis</th>
<th>Markers</th>
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<tr>
<td>Frozen tumor, fine needle aspirate</td>
<td>11 pre, 7 cycle 2 (day 15)</td>
<td>Size-Simple Western</td>
<td>IAP1, IAP2, caspase 3, caspase 8, PARP, NFκB-p65, IkBa, NFκB-p52/100, cFLIP, RIP</td>
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<tr>
<td>Fixed tumor</td>
<td>11 pre, 7 post B</td>
<td>IHC</td>
<td>TNF, TRAIL, CD3, CD19, CD56, CD68</td>
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<tr>
<td>Plasma</td>
<td>11 (x6) cycle 1 PK</td>
<td>Drug levels</td>
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<tr>
<td>Plasma</td>
<td>11 (x2) pre/post B</td>
<td>Cytokines</td>
<td>TNF, TRAIL, IL-6, IL-8</td>
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<td>PBMC</td>
<td>11 pre (0, 4hr, 24hr), 10 cycle 2 (0, 4hr)</td>
<td>Size-Simple Western</td>
<td>IAP1, IAP2, caspase 3, NFκB-p65, IkBa, NFκB-52/p100,</td>
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<tr>
<td>Whole blood</td>
<td>11 pre, 9 post B</td>
<td>T, B, NK cell counts</td>
<td>CD3, CD4, CD8, CD19, CD56, CD16</td>
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In collaboration with Dr. Christina Annunziata
Clinical application I: Phase II Study of SMAC-Mimetic Birinapant

PBMC sample analysis

- Clinical benefit was not observed in participate patients, but the drug is well tolerated and shown favorable pharmacokinetic (PK) properties.

- Simple Western assay clearly showed that the drug consistently suppressed targeted signaling pathway. This helped the drug developer to re-formulate treatment strategy.

Tumor core biopsy analysis

Noonan et al, Cancer 2016, 122:588-597
Simple Western™ assays in PBMC samples (~120 targets)

-- monitoring drug responses with specimens collected in a non-invasive manner, thus enables samples from more treatment time points to be analyzed

**Adhesion and cell-matrix:** Fibronectin, Integrin β1

**Apoptosis/Cell Death:** Bad (pS112), Bax, Bcl-xL, BIM, Caspase 3, Caspase 7, Caspase 8, cleaved cIAP1, cleaved cIAP2, FADD, PARP, XIAP, SMAC/Diablo

**Autophagy:** LC3A/B

**Cell Cycle and checkpoint control:** Bmi1, CyclinE1, CyclinD3, EZH2, MCM5,

**Cellular metabolism:** AMPKα (pS485, pT172), ATGL

**Chromatin Regulation/Epigenetics:** DNMT1

**DNA damage/repair:** XPC, PTIP

**JAK/STAT signaling:** STAT3 (pY705), STAT5 (pY694), JAK2

**Loading Controls:** α-Tubulin, β-Actin, Thioredoxin 1, ALAS1, HSP70, Vinculin, Glucose-6-phosphate dehydrogenase (G6PD), Rho-GDI, GAPDH

**MAP Kinase signaling:** ERK1/2 (pT202/pY204), MEK1/2 (pS218/222, pT292, pT386, pT394), p90-RSK (pT359), p38 alpha MAP Kinase (pT180/182), JNK (pT183/185), JNK2, A-Raf, B-Raf, c-Raf

**NFκB signaling:** IkBa, IkxB, NFKB p65 (pS536, pS529), NFKB1 p105/p50, NFKB2 p100/p52, c-Rel, RelB, RIP

**PI3K/AKT/mTOR signaling:** AKT1/2/3 (pS473), GSK3α/β (pS9, pS21), PI3 Kinase p110 α/β, PTEN (pS380), 4E-BP1 (pT37/46, pT45), p70 S6 kinase (pT389), p90 RSK (pT359), PTEN (pS380)

**Protein Kinase C signaling:** PKCδ, PKCa, and PKCβII

**Receptor tyrosine Kinase signaling:** Shc (pY239/240), Src (pY527, pY416), VEGFR

**Rho signaling:** Cofilin (pS3), Rho-GDI, ROCK-1, ROCK-2

**RNA regulation:** S6 Ribosomal protein (pS235/236)

**TGF-β/SMAD pathway:** SMAD1, SMAD2 (pS465/467), SMAD3 (pS423/425), SMAD4, SMAD5

**Transcription regulation:** c-Myc (pS62, pT58), FosB, FoxO3A (pS318/321), JunB, JunD

**Ubiquitin-proteasome pathway:** Ubiquitin

**Wnt Signaling:** β-Catenin
Clinical application II: **Resistance to trametinib** in pediatric melanoma patient

**Patient NCI0155**—a 16 year-old female with metastatic cutaneous melanoma, somatic GNAQ Q209R mutation (exome and transcriptome sequencing), treated with trametinib (MEK inhibitor), and had an initial response to therapy, but ultimately progressed on treatment.
Clinical application II: **Resistance to trametinib** in pediatric melanoma patient

**45 signaling molecules**
- ERK1/2 (pT202/204), MEK1/2 (MEK1pS298, MEK pS218/222, MEKpT292, MEK1pT386, MEK2 pT394)
- AKT (pS473, pT308), 4E-BP1 (pThr37/46)
- PKCδ (pS299, pY311), PKCθ, PKCα, PKCε, PKCβII, PKD1 (pS744/748), RasGRP3 (pT133)
- NFkB p52/100, NkB p65 (pS536)
- STAT3 (pY705), c-Raf, SRC pY527, SRC, JNK (pT183/185), c-Jun (pS63), c-Jun, Cyclin D1
- S6 Ribosomal Protein (pS235/236), P70 S6 Kinase (pT389)
- ALAS1, GAPDH, Vinculin

*In collaboration with Dr. Mari Yohe*

**Biopsies** – before treatment and after drug resistance developed
Clinical application II: **Resistance to trametinib** in pediatric melanoma patient

- **Potential treatment targets**
  - Trametinib resistance mediated by reactivation of MAP kinase pathway and activation of AKT
  - Pak and PKC pathways as potential therapeutic targets in melanoma with GNAQ mutation
Use isoelectric-focusing to separate proteins by charge

- Distinguish and detect different post-translationally modified states of a protein without using modification-specific antibodies

The IEF immuno-assay
Divergent PKC activation patterns by different ligand stimulation

Distinguishes Erk phosphorylation in human lung cancer from mouse stromal in xenograft samples
The IEF immuno-assay

- Reveals additional level of signaling molecule activation status that are not accessible by conventional western blots
- Provides a novel platform for biomarkers and therapeutic target identification
Data normalization, new publication requirements

Scientific publishers look more closely at experimental methods and data analysis

JBC requirement

1) **Normalization of signal intensity to total protein loading** (assessed by staining membranes using Coomassie blue, Ponceau S or other protein stains) is preferred.

2) “House-keeping” proteins should not be used for normalization without evidence that experimental manipulations do not affect their expression.

3) Signals obtained using antibodies specific for phosphorylated epitopes should be normalized to the total protein level of the target protein”

http://www.jbc.org/site/misc/ifora.xhtml
House keeping protein analysis

Simple Western house keeping protein assays:
α-Tubulin, GAPDH, β-Actin, Thioredoxin 1, ALAS1, HSP70, Vinculin, Glucose-6-phosphate dehydrogenase (G6PD), Rho-GDI
Impact of protein abundance and signal saturation

Band intensity and signal saturation affects analysis accuracy

- Many housekeeping proteins and structural proteins used as internal loading controls are highly abundant, but target proteins are often expressed at much lower levels.
- The impact of protein abundance and saturation on Western blot normalization is often overlooked.
House keeping vs total protein analysis

Simple Western assay for total protein analysis:
- Total protein assay is performed by labeling lysate proteins with biotin followed by HRP conjugated streptavidin detection
- The process is automated, and signals are captured by CCD camera and quantified with Compass software as Simple Western immunoassays
Normalize phospho-signal with pan-target-protein signal
The Simple Western™ technology applications

- **Samples**: cultured cells, mouse tissues, PDX, patient specimens (e.g. PBMC, OCT tumor tissues, bone marrow or tumor aspirates, etc.)

- **Applications**: Characterize cellular signaling networks; Determine drug selectivity and identify therapeutic targets; Define regulatory mechanisms; Drug treatment and pharmacodynamic evaluations

**Collaborative projects with shared cost on capillary usage**

$5 / per data point, eligible for a 50% subsidy from OSTR

$2.5 / per data point final cost
In-solution multiplex sandwich ELISA

-- Luminex xMAP technology

➢ Combines advanced fluidics, optics, and digital signal processing with proprietary microsphere technology to deliver multiplexed assay capabilities with small sample consumption

➢ Analysis of cytokines, chemokines, growth factors, hormones, metabolite, immune response, cell signaling, inflammation and cancer markers etc. in cell supernatant and plasma/serum samples

➢ Most widely cited multiplex immunoassay platform in life science research. Application areas include cancer, immunology, cardiovascular disease, metabolic disease, inflammation, neurological disorders, drug discovery, and vaccine development etc.

➢ Clinical applicable assay performance
xMAP assays

Over 800 research xMAP assays have been developed and provided by different vendors providing a broad selection of preconfigured and custom assay panels


xMAP kit finder: [https://kitfinder.luminexcorp.com/](https://kitfinder.luminexcorp.com/)

The MAGPIX platform at CPTR supports xMAP assays configured on magnetic beads with up to 50-Plex analysis capability

Collaborative or “self-service”
Researcher purchased assay kits, maybe eligible for OSTR subsidy
Operations

- Concepts of good laboratory practice (GLP concepts) are integral to the core operation, to ensure data accuracy & reliability, and assay readiness for bench to transferring from bench to bed-side

- A web-based interface (https://cptr.cancer.gov) is employed for ease of accessibility to our technologies and protocols, as well as more efficient project review, communication and management.

- Offer expertise throughout all project stages, including project feasibility, experiment design, method/analysis strategy development, sample preparation/analysis, data evaluation/summary, further project advancement, and assistance with manuscript preparation.

- General consultation on your questions of proteomic analysis
Our team

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xiaoling.luo@nih.gov
Tel: 240.760.6932

Building 37, Room 1044
CODEX 2
A highly multiplex immunofluorescence imaging platform

- it provides multidimensional quantitative information about target expression at single cell level, similarly to FACS analysis, but preserving spatial information

- the technology is a modified version of the recently published method developed in Gary Nolan’s laboratory (Stanford), commercialized by Akoya Biosciences (Goltsev et al: Deep Profiling of Mouse Splenic Architecture with CODEX Multiplexed Imaging. Cell. 2018 Aug 9;174(4):968-981)

- CPTR is currently evaluating the CODEX2 technology, running an early access instrument; the commercial version of the CODEX instrument is expected to be released in November

- CODEX: CO-Detection by indEXing

- Uniquely, tissue samples are stained with the antibody cocktail (~ 24-30 antibodies) at once and the signal is visualized through cycles using target-specific fluorescent probes (max. 3 targets per cycle); the visualization is automated, relatively fast and non-degradative to the tissue

- Currently is available for fresh frozen tissues only; protocols and antibodies for FFPE tissues are being developed

- Antibodies need to be customized for the technology: need to be conjugated with unique oligonucleotide tags
20X objective  
Later versions could work with 40X

4 detection channels:  
DAPI, fam, CY3, CY5

Automated image acquisition on 4 channels with multiple Z-stacks, generating ~6000 images per hour

3 buffers with different amount of DMSO  
Dyes for each cycle are prepared in 96 well plate

Tissue chamber  
Sample on the coverslip  
The coverslip is currently mounted using double side tape
## CODEX-ready ANTIBODIES

### Current human antibody panel for fresh frozen tissues

<table>
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<tr>
<th>Target</th>
<th>Clone</th>
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<td>CD11c</td>
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<td>Collagen IV</td>
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**B cell markers:**
- CD19, CD21, CD22

**T cell markers:**
- CD3, CD7

**Myeloid markers:**
- CD15, CD11c

**Vascular markers:**
- CD31, CD34, podoplanin

**ECM markers:**
- Collagen IV

**Epithelial markers:**
- Pan-cytokeratin

### Current mouse antibody panel for fresh frozen tissues

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<td>1A8</td>
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<tr>
<td>IgD</td>
<td>11-26c.2a</td>
</tr>
</tbody>
</table>

**B cell markers:**
- B220, IgD, IgM, CD79

**T cell markers:**
- TCRb, CD4, CD8A, CD5

**Myeloid markers:**

**Vascular markers:**
- CD106

**ECM markers:**
- none

*Conjugation kit with additional 8 oligonucleotide tags is available for addition of new custom targets to the panel; antibody requirement: to be additive and preservative free (50-100 ug)*
Mouse 4T1 tumor
Selected region
(3 x 3 tiles show after deconvolution)

CD11b CD8A Ki67

Collaboration with David Wink (Cancer and Inflammation Program), Stephen Lockett, David Scheiblin (OMAL)
4T1 tumor region stained with CD11b CD45 CD8a Ki67
Staining pattern in the tumor bearing mouse lung suggests specificity of the antibodies

Collaboration with Alex Wu, Patricia Steeg (Women’s Malignancies Branch) and Lalage Wakefield (LCBG)
CD44 staining in immune cells present between heart muscle fibers (mouse)

Collaboration with Meera Murgai, Sabina Kaczanowska and Rosandra Kaplan (Pediatric Oncology Branch)
Adenocarcinoma of the lung
Human
(3 x 3 tiles show after deconvolution)

Staining:
- CD3 (T cells)
- CD19 (B cells)
- Pancytokeratin

Also detected:
- Autofluorescent connective tissue
- Non-specific CD19 binding or precipitation to cytokeratin

Collaboration with Bríd Ryan
(LHC)
Adenocarcinoma of the lung (zoomed in image)

Collaboration with Bríd Ryan (LHC)
CD45+ immune infiltrate in human mesothelioma

Collaboration with Qun (Queena) Jiang (TGMB)
Majority T cells, relatively separate B cells (human mesothelioma)

With deconvolution

Without deconvolution

Collaboration with Qun (Queena) Jiang (TGMB)
CODEX workflow

TISSUE PREPARATION and SECTIONING onto coverslips
- OCT fresh frozen tissues
- Poly-L-Lysine coated coverslips
- 10 um sections

ANTIBODY STAINING including pre- and post-fixation
- 5 hrs
- Multiple samples can be prepared in parallel

IMAGING = cyclic signal reveal
- 24-48 hours depending on the size of regions of interest and number of antibodies
- Only 1 sample at a time

Image STITCHING for macroscopic evaluation
- Several hrs — typical ~ 4 hrs

TRANSFER of files to the analysis computer
- Several hrs — typical overnight

Data SHARING and STORAGE
- Several hrs, depending on the project

Data VISUALIZATION (graphs, powerpoint)

SEGMENTATION for quantitative analysis
- Several hrs — typical overnight

Image OVERLAY for detailed microscopic analysis
- Several hrs, depending on the project

PROCESSING for microscopic analysis
- Several hrs — typical overnight

Color key: automated, manual, mixed, implementation/improvement needed
Procedure for getting access to the CODEX technology and details about the upcoming service

1. Setup a meeting with Noemi and Jessie at CTPR to discuss about a potential project, to get detailed information about the technology, available targets, analysis software, etc.

2. Submit a new project request through https://CPTR.cancer.gov

   - **Advantage:** - keeps communication and data in a single platform, easy to track changes
     - easy access to the oversight committee for approval

   - **Needed information**
     - Project background: information about the project; paragraph stating why using CODEX
     - Supporting data: it helps us understand the project better
     - Proposed experiments: think about experiments in phases
       - phase 1: feasibility assay: how the tissue behaves during sectioning, fixation, staining, how much autofluorescence
       - phase 2: testing staining (multiplex and/or subsets of targets) on smaller sample set to establish experimental conditions
       - phase 3: experiments answering scientific problems/questions

3. Experiment:

   **Tissue preparation and staining:**
   - coverslips provided by CPTR,
   - tissue sectioning by investigators (recommended to use Histoserve or PHIL, especially for difficult tissues)
   - staining the tissue and running CODEX by CPTR

   **Data analysis:**
   - Primary data analysis by CPTR: image processing, annotation using Akoya pipeline, basic segmentation with Akoya software
   - Secondary and tertiary analysis by investigator in collaboration with CPTR and the bioinformatics support

   **Data sharing and storage:** server space for temporary data storage and analysis in progress, long term storage with Cleversafe in progress, developing data analysis platform in progress
4. New target development:
   - conjugation kit (up to 4-5 antibodies) and protocol provided by CPTR
   - antibody clone selection (by IHC staining), purchase, conjugation and post-conjugation testing by IHC to be done by investigators
   - post-conjugation testing by CODEX as single stain and in combination by CPTR

5. Cost/Resources:
   - based on current calculations staining a tissue section with the full antibody panel comes to ~ $600
   - staining a tissue section with selected targets: ~ $260 + $15/antibody
   - eligible for OSTR subsidy of 30-50%
   - feasibility test covered by CPTR
MHCII

White pulp: B + T cells

Red pulp

White pulp

Red pulp

TCRB

CD79b

Spleen 56/8
The pattern of CODEX ab staining matches the one described in the literature.

**Literature:** Staining of T and B cells in the spleen of C57BL/6 mice (OCT fresh frozen)

Markers identifying B cells (selected region)

- B220 (fam #5)
- CD79b (CY5 #9)
- CD21/35 (fam #8)
- Blank (fam)
- IgD (CY3 #9)
- IgM (fam #10)
Markers identifying T cells (selected region)

- TCRB (CY3 #10)
- CD90 (fam #11)
- CD4 (CY3 #4)
- Blank (CY3)
- CD8A (CY3 #5)
- CD8A (CY3 #8)

Literature: staining of mouse spleen (BC: B cell region)

CD8 B220

J Immunol. 195:5227
Markers staining cells in the red pulp (selected region)

- CD11b (CY3 #6)
- CD11b (CY3 #11)
- F4/80 (CY5 #6)
- LY6C (CY5 #4)
- LY6G (CY5 #5)
- CD71 (fam #3)
The good image segmentation on spleen using the current Akoya software enables quantitative analysis at single cell level
The specific signal is washed off with DMSO

Separation of specific signal from background

- Fam channel
  - MHCII cycle 6 (Reg1X9Y9 08.02.2018)
  - blank cycle 7 (Reg1X9Y9 08.02.2018)
  - CD90 cycle 11 (Reg1X9Y9 08.02.2018)
  - blank cycle 12 (Reg1X9Y9 08.02.2018)
The specific signal is washed off with DMSO

Separation of specific signal from background

CD11b cycle 6 (Reg1X8Y9 08.02.2018)  
CD11b cycle 11 (Reg1X8Y9 08.02.2018)  
blank cycle 7 (Reg1X8Y9 08.02.2018)  
blank cycle 12 (Reg1X8Y9 08.02.2018)

CY3 channel

CD11b - average blank (signal intensity per cell)
The specific signal is washed off with DMSO

Separation of specific signal from background

F4/80 cycle 6 (Reg1X8Y9 08.02.2018)
blank cycle 7 (Reg1X8Y9 08.02.2018)

CD11b cycle 11 (Reg1X8Y9 08.02.2018)
blank cycle 12 (Reg1X8Y9 08.02.2018)

Ki67 cycle 11 (08.02.2018 reg1X8Y9 uncompensated)

- CY5 blank 1
- CY5 blank 2
- CY5 blank 7
- CY5 blank 12
- F480 6

Ki67 - average blank (signal intensity per cell)
Reproducibility of staining between different spleen samples: CD8A
Reproducibility of staining between different spleen samples: MHCII
Similar CD4 staining pattern for CODEX and immunofluorescent staining (macroscopic comparison)

<table>
<thead>
<tr>
<th>CODEX</th>
<th>Immunofluorescent staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>Using CD4 RM4-ROU AF488 BD Bioscience antibody (same clone as CODEX)</td>
</tr>
<tr>
<td>DAPI</td>
<td></td>
</tr>
</tbody>
</table>

Spleen 56 slide 8

Spleen 56 slide 9 or 10

Staining performed by David Scheiblin (OMAL) and imaged with widefield microscope
Comparable CD45 staining pattern for CODEX and immunofluorescent staining (macroscopic comparison)

CODEX

CD45  DAPI

Spleen 56 slide 8

Immunofluorescent staining

Using CD45 30-F11 AF647 Biolegend 1:400 (same clone as CODEX)

CD45  DAPI

Spleen 56 slide 9 or 10

Staining performed by David Scheiblin (OMAL) and imaged with widefield microscope
Single cell western system using Milo

- It performs **Western analysis on 1000-2000 single cells** in parallel.
- Quantitatively detects multiple proteins in a single cell including hard to detect targets by FACS and other single cell analysis technologies, such as **isoforms, post-translational modifications, intracellular proteins, transcription factors**, etc.
- Simple workflow, **quantitative data analysis** with Scout Software, **multiplexing ability** depending on the targets (up to 12-15 targets), option to **re-probe archived samples** months later.

**Offers a tool for studying cell signaling in single cells and for dissecting population heterogeneity,**

**proved applications including:**

1. Target expression heterogeneity of tumors,
2. Identify differentiated stem cell subtypes,
3. Measure activation of intracellular signaling pathway — including phosphorylated targets or transcription factors;
4. Complement single-cell RNA results with the protein expression information;
5. Identify the efficiency of genetically engineered CRISPR, transduction, or transfection;
6. Detect rare events.
Single cell Western workflow

**Preparation of single cell solution**
- Cultured cells
- Dissected tumor tissues
- PBMCs
- CTCs

**Loading cells into the wells**
- Cells enter the wells by gravity
- Controlled by cell number, well size and loading time

**Visualization of cell loading (optional)**
- Scanning the loaded slides using brightfield, phase contrast or epifluorescence (Keyence; 10X objective)

**Lysis, electrophoresis and immobilization of separated proteins using Milo**
- Short lysis (10-15 sec) by denaturing but non-reducing conditions in running buffer
- Short electrophoresis based on the MW of the target (45-90 sec)
- UV immobilization of separated proteins to the poly-acrylamide gel (480 sec)

**Quantitative analysis of scanned slides**
- Scout software enables quantitative analysis of the signal for each individual cell, as well as calculation of stripping efficiency

**Stripping**
- 90 min at 65°C in SDS and b-MEA containing buffer

**Detection of targets by immunostaining**
- Incubation with primary ab (generally 2 hrs at RT)
- incubation with fluorescently labeled secondary ab (1 hr RT)
- Detection of the fluorescent signal using microarray scanner with 4 color filters: (488, 550, 594, 635 nm)
Some project examples

1. Downstream signaling at single cell level when using EC$_{50}$ doses of PKC activation (3 nM PMA in LNCaP cells)
2. Correlation between the level of PKC delta and the induced downstream signaling events
   - expression of cFos, EGR1, pPKCdeltaSer299
3. Efficiency of NOX1 reduction in genetically engineered CRISPR clones of colon cells
4. LRRK2 expression in purified microglia of the brain
Successful separation and detection of 12 targets after multiple stripping steps using 4 color detection

<table>
<thead>
<tr>
<th>#</th>
<th>Target</th>
<th>Characteristics</th>
<th>Antibody species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cFos</td>
<td>Newly synthesized protein</td>
<td>mouse</td>
</tr>
<tr>
<td>2</td>
<td>EGR1</td>
<td>Newly synthesized protein</td>
<td>Rabbit</td>
</tr>
<tr>
<td>3</td>
<td>GAPDH</td>
<td>Housekeeping/loading control</td>
<td>Goat</td>
</tr>
<tr>
<td>4</td>
<td>Beta Tubulin</td>
<td>Loading control</td>
<td>Mouse</td>
</tr>
<tr>
<td>5</td>
<td>pPKCdeltaSer299</td>
<td>Phosphorylation/activation</td>
<td>Rabbit</td>
</tr>
<tr>
<td>6</td>
<td>ERK total</td>
<td>Loading control</td>
<td>Mouse</td>
</tr>
<tr>
<td>7</td>
<td>pPKD1</td>
<td>Phosphorylation/activation</td>
<td>Rabbit</td>
</tr>
<tr>
<td>8</td>
<td>PKC alpha</td>
<td>Target protein</td>
<td>Rabbit</td>
</tr>
<tr>
<td>9</td>
<td>PKC delta</td>
<td>Target protein</td>
<td>Rabbit</td>
</tr>
<tr>
<td>10</td>
<td>cFos (repeat)</td>
<td>Newly synthesized protein</td>
<td>Rabbit</td>
</tr>
<tr>
<td>11</td>
<td>P65 NFKB</td>
<td>Transcription factor</td>
<td>Mouse</td>
</tr>
<tr>
<td>12</td>
<td>PKC delta</td>
<td>Target protein (repeat)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>13</td>
<td>Vinculin</td>
<td>Loading control</td>
<td>Mouse</td>
</tr>
</tbody>
</table>
Good separation of 4 targets with 40-130 kDa molecular weight range

Representative composite images

Treated LNCaP cells (PMA 1000 nM 60 min)
Stripping efficiency for different signals

<table>
<thead>
<tr>
<th>Target</th>
<th>Wavelength (nm)</th>
<th>Median stripping efficiency (%)</th>
<th>95% of signal is between %-% efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>594</td>
<td>93.1</td>
<td>77.3 - 103.3</td>
</tr>
<tr>
<td>EGR1</td>
<td>635</td>
<td>88.9</td>
<td>80 - 95.3</td>
</tr>
<tr>
<td>Tubulin</td>
<td>488</td>
<td>100</td>
<td>77.1 - 128.3</td>
</tr>
<tr>
<td>pPKCdelta Ser299</td>
<td>635</td>
<td>89.8</td>
<td>74.9 - 111</td>
</tr>
<tr>
<td>ERK</td>
<td>635</td>
<td>96.4</td>
<td>80.9 - 109.4</td>
</tr>
<tr>
<td>pPKD1</td>
<td>532</td>
<td>100.6</td>
<td>76.5 - 110.9</td>
</tr>
<tr>
<td>PKC alpha</td>
<td>488</td>
<td>99.8</td>
<td>-11.2 - 129.1</td>
</tr>
<tr>
<td>PKC delta</td>
<td>532</td>
<td>96.7</td>
<td>6.7 - 110.0</td>
</tr>
<tr>
<td>P65 (mouse)</td>
<td>635</td>
<td>94.2</td>
<td>24.0 - 102.5</td>
</tr>
<tr>
<td>Vinculin (mouse)</td>
<td>532</td>
<td>101.8</td>
<td>65.3 - 108.5</td>
</tr>
<tr>
<td>PKC delta b</td>
<td>635</td>
<td>93</td>
<td>72.4 - 109.1</td>
</tr>
<tr>
<td>Cyclin A (rabbit)</td>
<td>635</td>
<td>90.4</td>
<td>68.1 - 117.7</td>
</tr>
</tbody>
</table>
The effect of PKCdelta siRNA on downstream signaling events (EGR1 expression) after PMA and bryo treatment of LNCaP cells

Larger decrease in EGR1 expression in bryo treated sample suggests preference of bryostatin 1 toward PKC delta; PMA is more promiscuous towards different PKC isoforms
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     - phase 1: feasibility assay: work out the cell loading and target detection conditions
     - phase 2: test the established staining and analysis parameters on smaller sample sets
     - phase 3: experiments answering scientific problems/questions

3. Experiment:
   - Preparation of single cell solution by the investigator
   - Loading the cells onto the chip, electrophoresis and UV cross-linking using the Milo: CTPR with or without the investigator
   - Immunostaining, scanning the signal, stripping, re-staining: CTPR and/or the investigator depending on manpower and the project
   - Data analysis using Scout 2.0: CTPR and the investigator

4. Cost:
   - the cost of 8 chips is ~$1300; $160-170/chip
   - no additional cost if the antibodies are provided by the investigator; (relatively large amount of antibody is used)
   - eligible for OSTR subsidy of 30-50%
   - feasibility test covered by CTPR
ACKNOWLEDGEMENTS

OSTR
David Goldstein
Mariam Malik

OMAL
Stephen Lockett
David Scheiblin

PHL
Lawrence Sternberg
Elijah Edmondson
Donna Butcher

NCI Investigators
Amber Giles
Alex Wu
Qun Jiang (Queena)
Meera Murgai
Sabina Kaczanowska
Rosandra Kaplan
Lalage Wakefield
David Wink
Brid Ryan
Agnes Juhasz
Peter M. Blumberg
Alice Kaganovich

Akoya Biosciences
Ash Wilson
Chris Streck
Meenu Perera
Michael Hansen

CBIIT/CIB
Addepalli Kanakadurga
Sunita Menon

NCI/CCR/OD
Maggie Cam
Janelle Cortner

NCI/CGB
Liz Conner