Cutting-edge protein analysis technologies

Advancing quantitative proteomic research, biomarker assessment and molecular diagnostics

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Collaborative Protein Technology Resource (CPTR)

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

The Nanoscale Protein Analysis Section

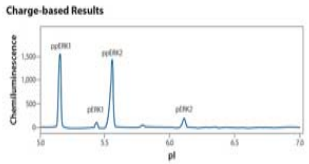
We offer expertise and provide state of the art immunoassays to support CCR investigators on rapid, precise and cost-effective functional proteomic studies for:

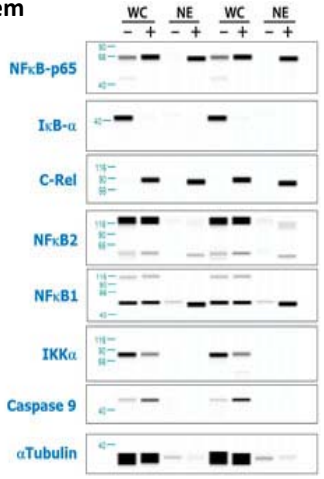
- **Comprehensive and quantitative cell signaling profiling**
- Cytokine, chemokines, growth factors and immune response measurement
- **Single-cell protein analysis**
- Biomarker & therapeutic target identification and validation
- **Preclinical and clinical applicable assay development and implementation**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

Cutting edge protein analysis technologies

Capillary immunoassays

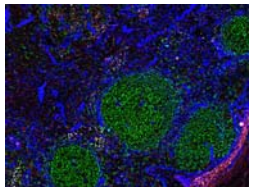
- Simple Western system

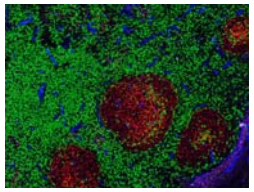


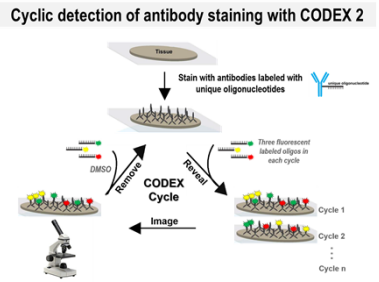


High multiplex immunofluorescence imaging

- CODEX technology

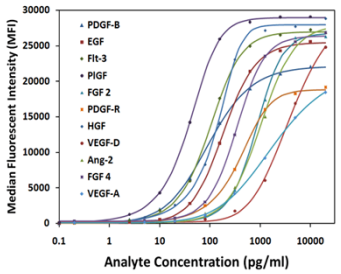




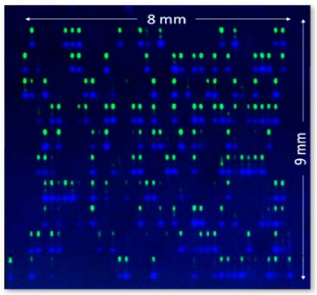


In-solution multiplex sandwich ELISA

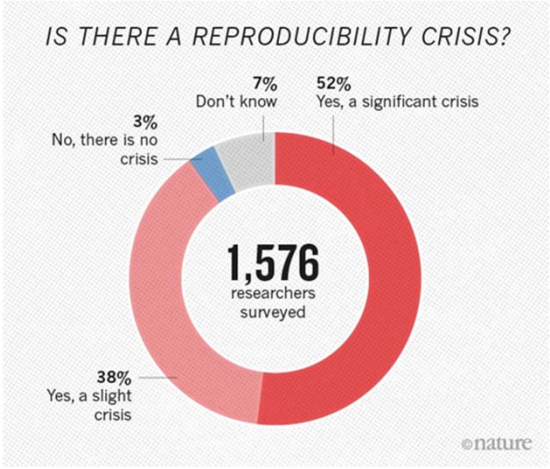
- Luminex xMAP technology



Single-cell western system



Data quality issues:



"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

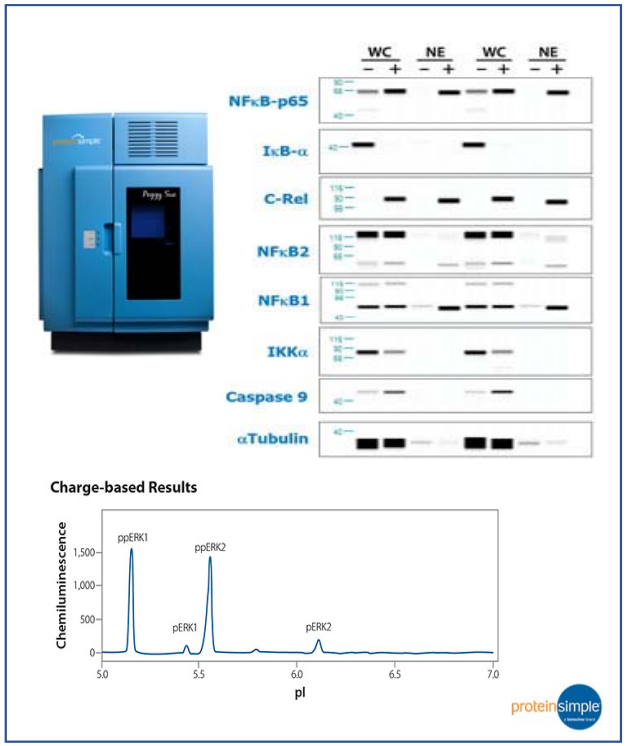
Western blot,

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

- poor reproducibility
- lack of accurate quantitation
- extensive time to result
- reliability issues

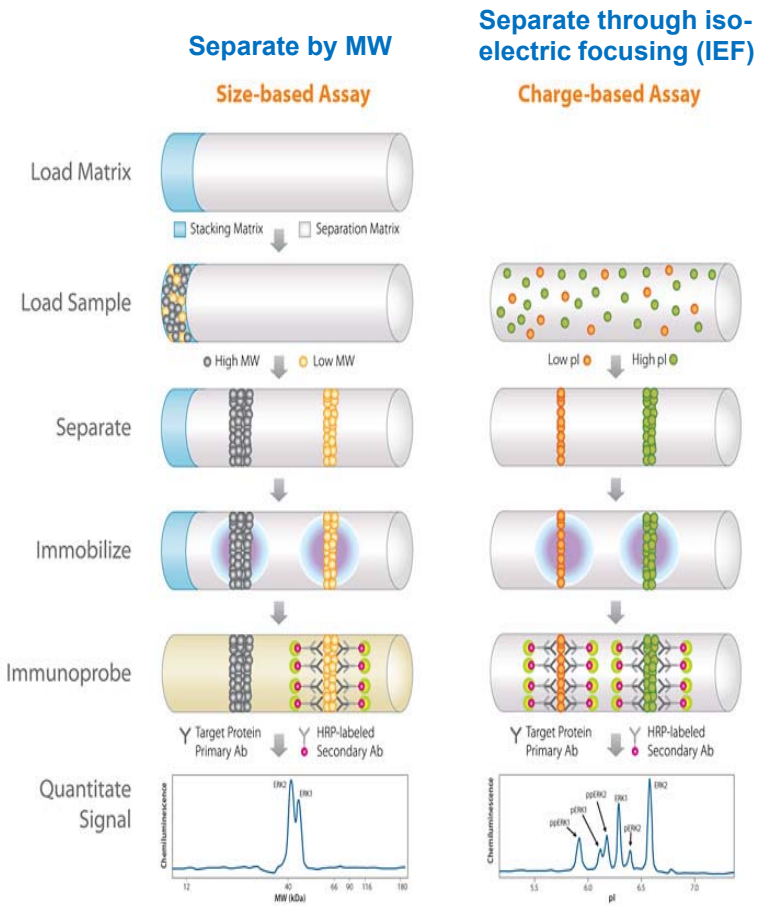
Automated Capillary Immunoassay System

– 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

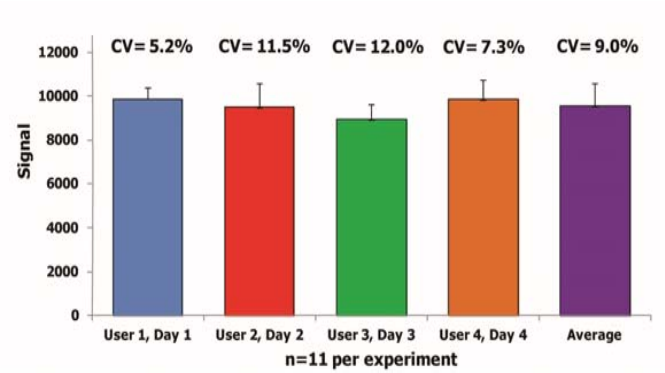
The Simple Western Technology

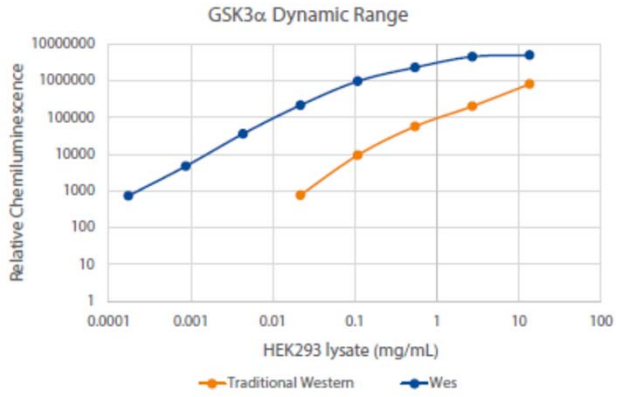


- **Step 1: Load Matrix** (size based assay only)
  Stacking and separation matrices are loaded in to capillaries.
- **Step 2: Load Sample**
  ~10-40 ng protein samples, prepared with SDS-containing buffers (for MW separation) or solution-phase carrier ampholytes (for IEF separation), were loaded into the capillaries.
- **Step 3: Separate**
  Proteins and florescent standards are separated by MW or iso-electric 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.

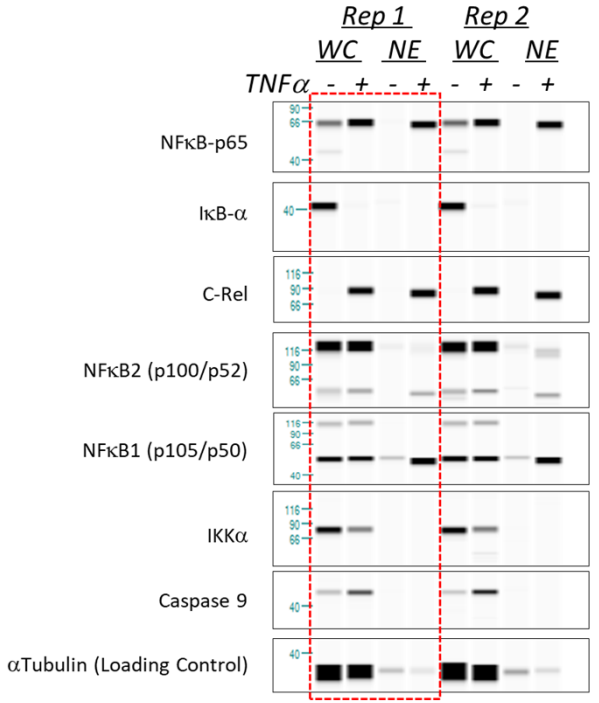




1.5 log dynamic range improvement

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

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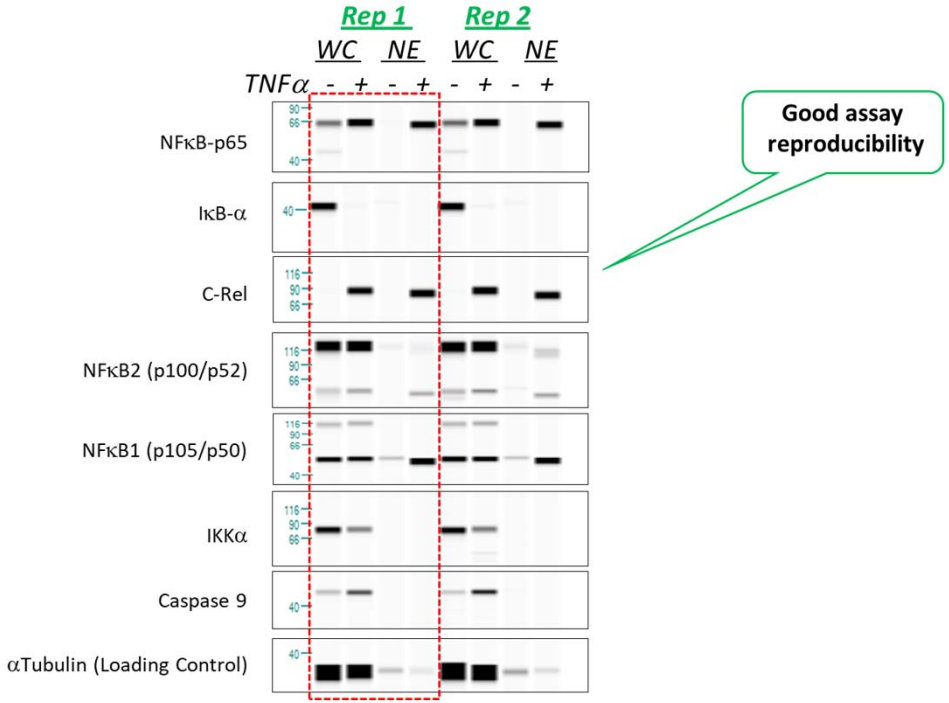
- **Multiplex analysis with fast assay turn-around time,** allows simultaneous measurement of multiple protein targets, analyzes up to 96 sample / analyte combinations per run in ~16 hours.
- **Increased sensitivity and specificity,** multi-analyte analysis using a parallel single-analyte format



- **One analysis run, ~16-hour;**
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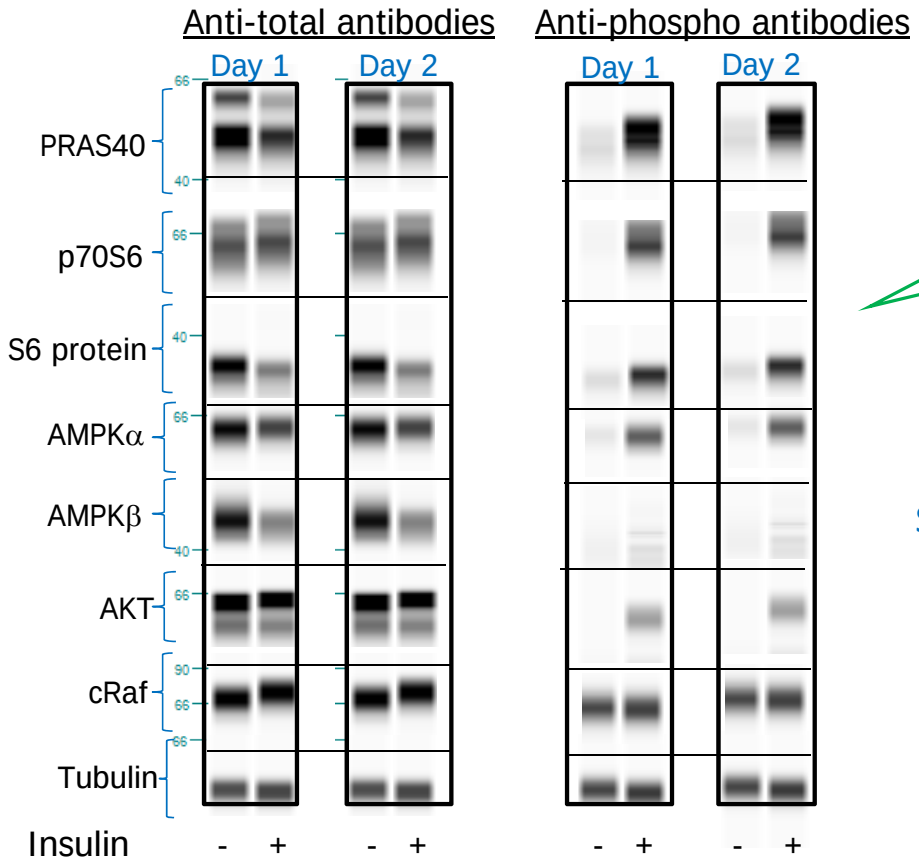
Sample stability through analysis



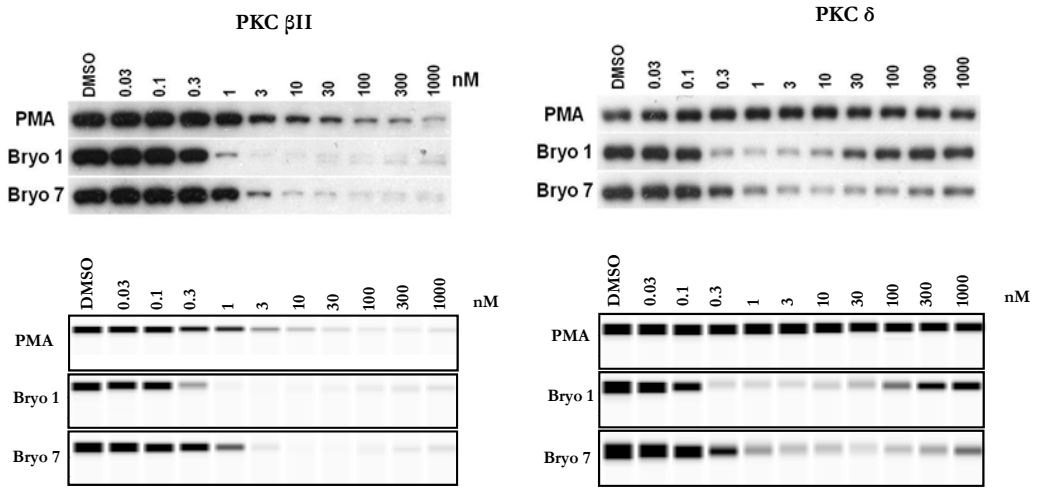
Up to 16 data points per 5 µL sample

Samples are kept on 4 °C during analysis run

AKT pathway: MCF7+/-Insulin

Consistent with Western data

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

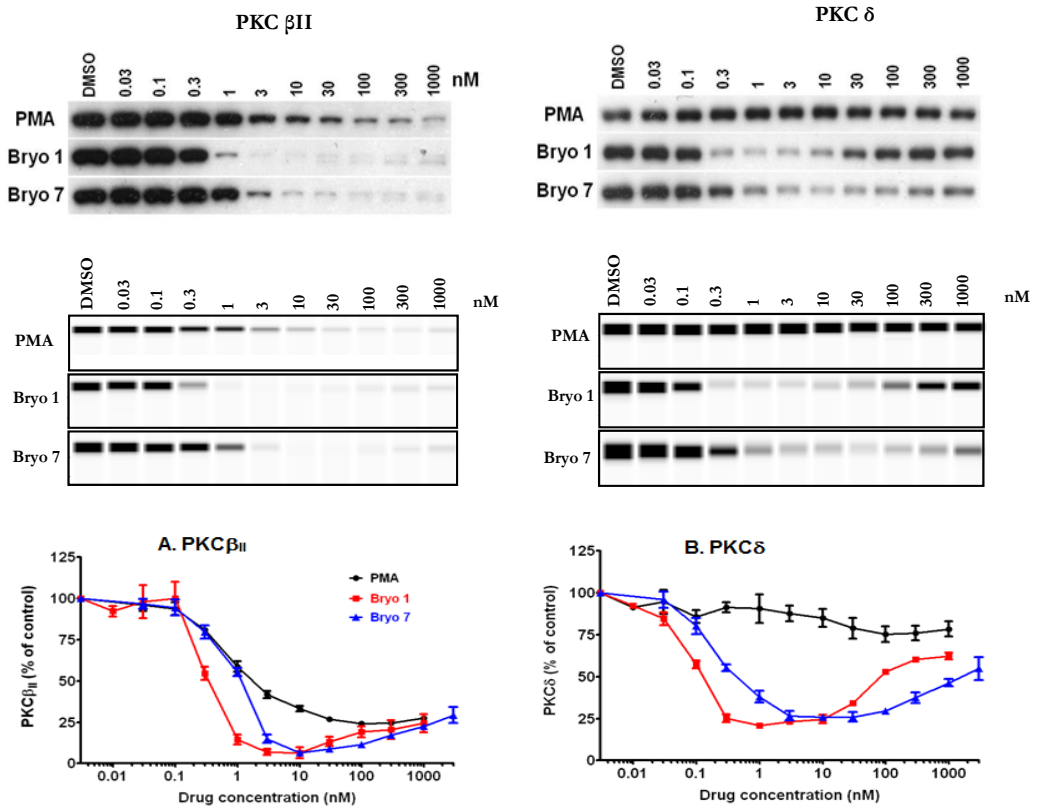


Conventional Western Data

Simple Western Data

Consistent with Western data & precise quantitation

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



Conventional Western Data

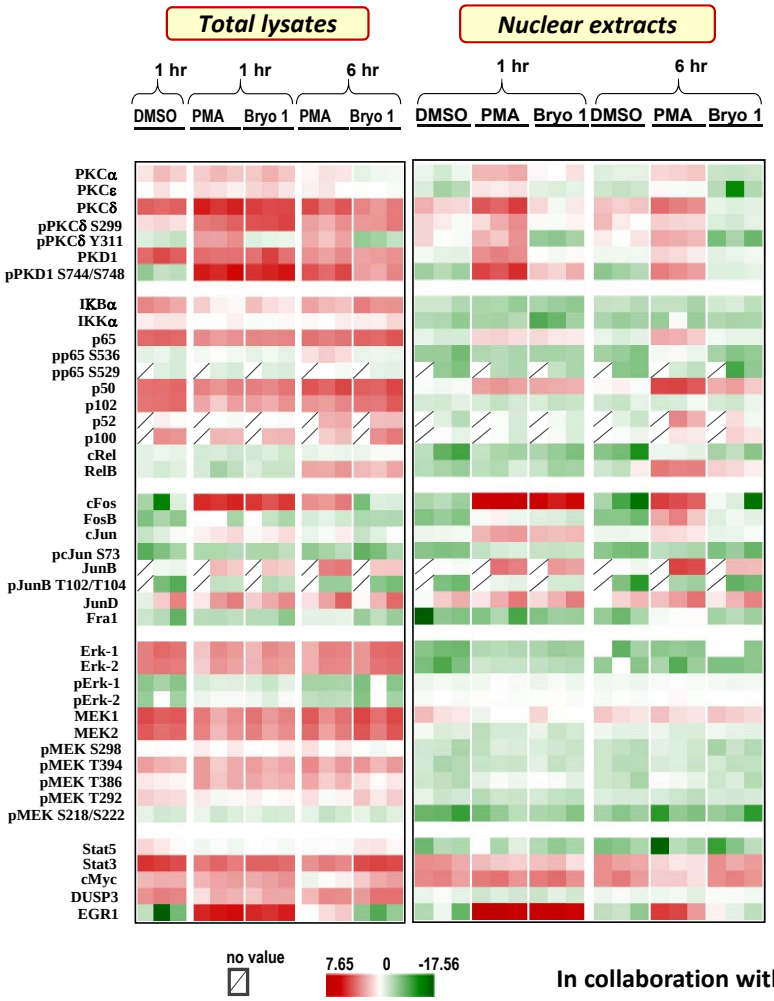
Simple Western Data

Simple Western Data Quantitation*

* mean ± SEM of three independent experiments

Kedei et al, ACS Chem Biol, 2013, 8(4): 767-777

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



Targets:

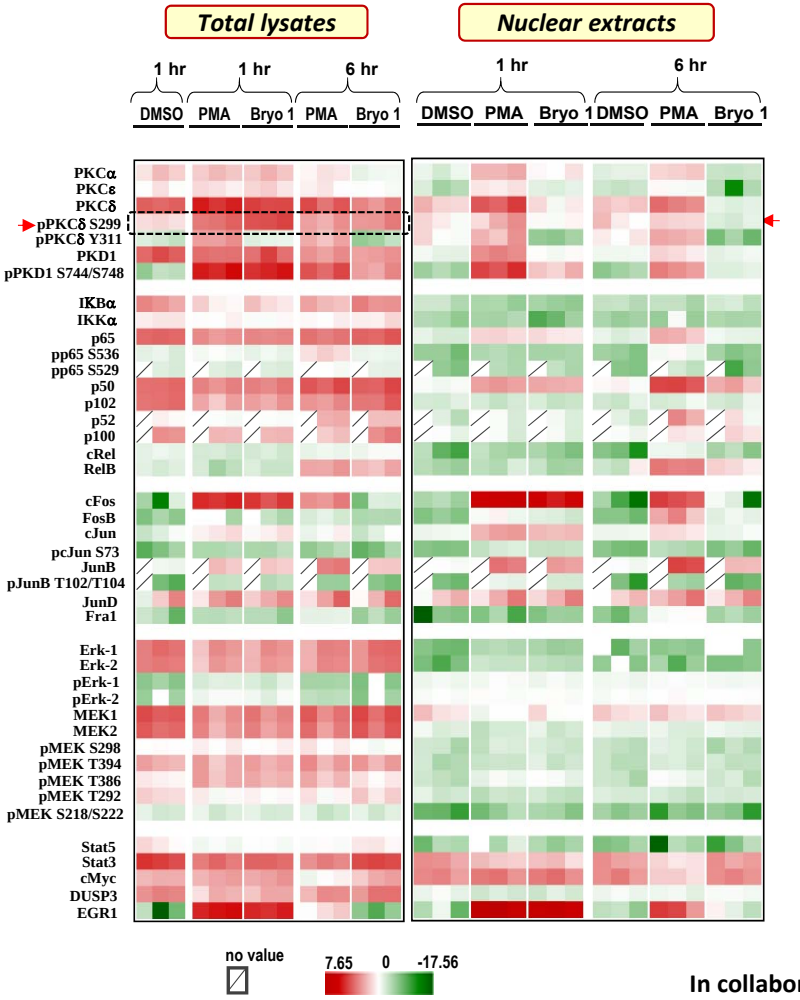
- 40 signaling molecules, PKC isoforms, NFkB pathways, MAP kinases, AP1 transcription factors etc.

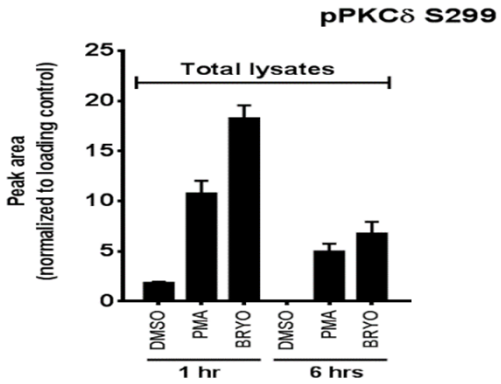
Lysate amount:

- ~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





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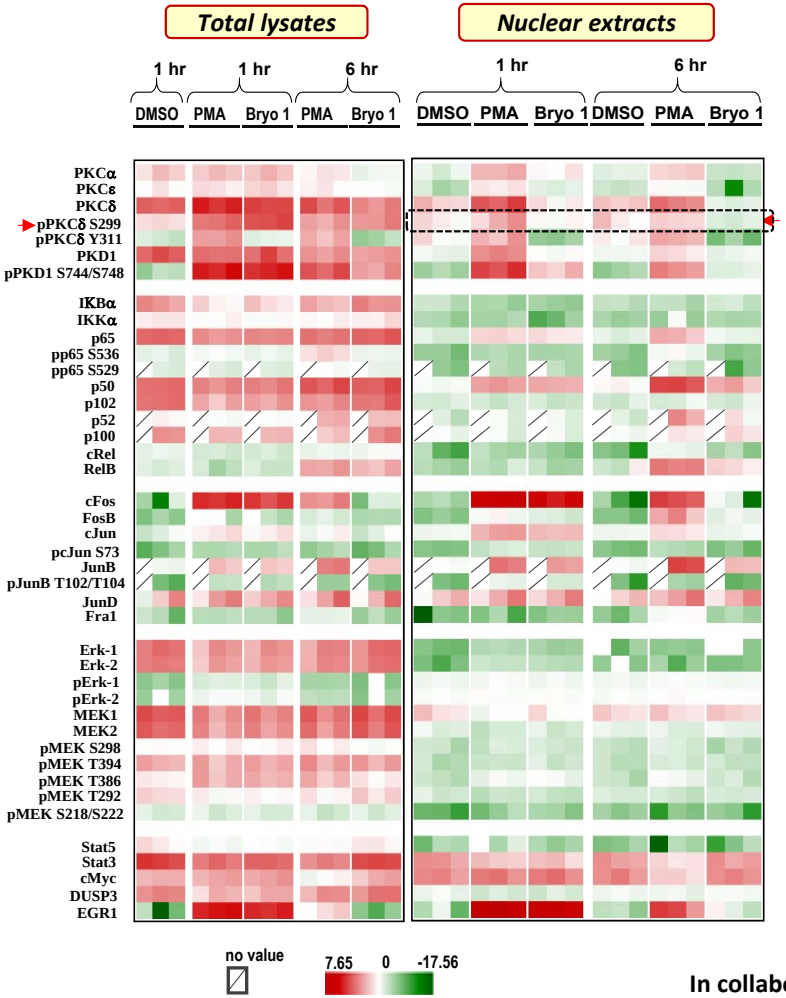
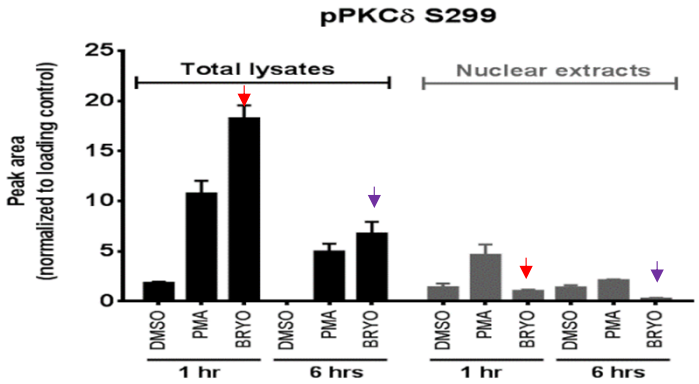




In collaboration with Drs. Noemi Kedei & Peter Blumberg

Established Simple Western assays

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

Key Pathways:

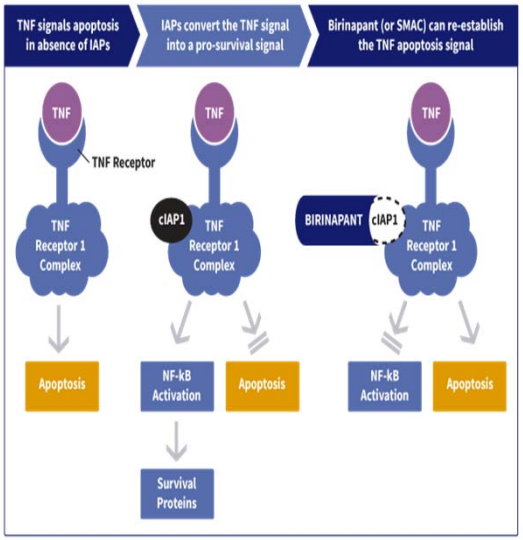
Apoptosis/Cell Death, Cell cycle and checkpoint control, Cellular metabolism, Chromatin Regulation/Epigenetics, DNA damage and repair, Gene regulation and DNA repair, JAK/STAT signaling, MAP Kinase signaling, NFκB signaling, PI3K/AKT/mTOR signaling, Protein Kinase C signaling, Receptor tyrosine Kinase 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)

TetraLogic Pharmaceuticals

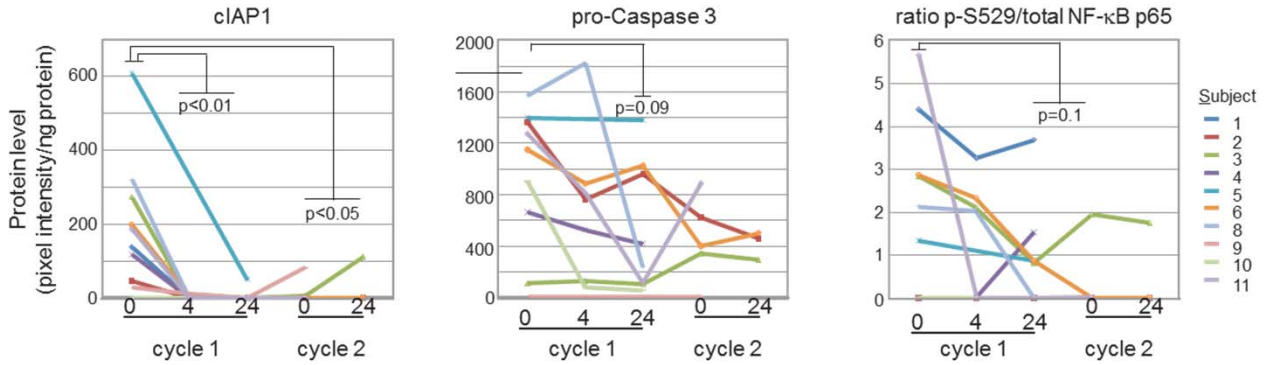
Treatments – Relapsed platinum resistant epithelial ovarian cancer (EOC), primary peritoneal cancer (PPC) or fallopian tube cancer (FTC) patients received Birinapant 47mg/m^2 IV on days 1, 8 and 15 of a 28-day cycle.

Sample type	Number collected	Planned analysis	Markers
Frozen tumor, fine needle aspirate	**11 pre, 7 cycle 2 (day 15)**	**Size-Simple Western**	**IAP1, IAP2, caspase 3, caspase 8, PARP, NFkB-p65, IkBa, NFkB-p52/100, cFLIP, RIP**
		Drug levels	--
Fixed tumor	11 pre, 7 post B	IHC	TNF, TRAIL, CD3, CD19, CD56, CD68
Plasma	11 (x6) cycle 1 PK	Drug levels	--
Plasma	11 (x2) pre/post B	Cytokines	TNF, TRAIL, IL-6, IL-8
PBMC	**11 pre (0, 4hr, 24hr), 10 cycle 2 (0, 4hr)**	**Size-Simple Western**	**IAP1, IAP2, caspase 3, NFkB-p65, IkBa, NFkB-52/p100,**
Whole blood	11 pre, 9 post B	T, B, NK cell counts	CD3, CD4, CD8, CD19, CD56, CD16

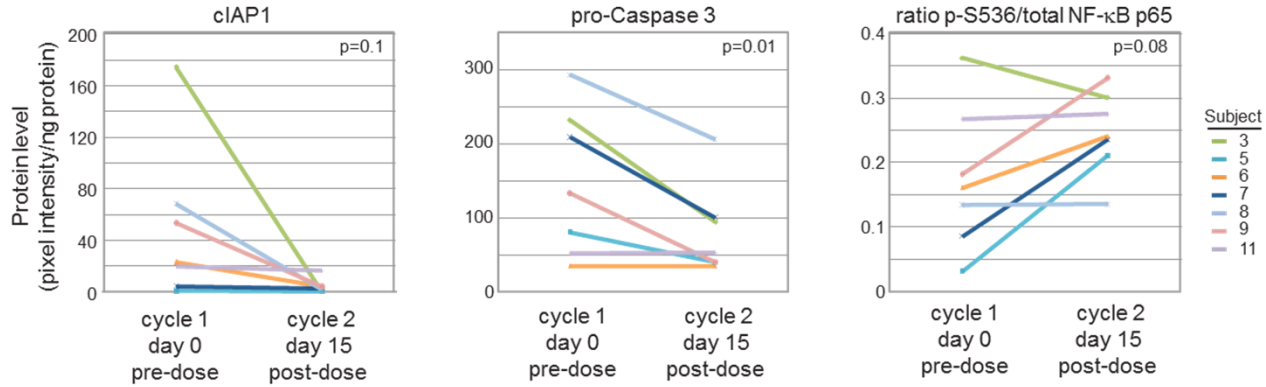
In collaboration with Dr. Christina Annunziata

Clinical application I: Phase II Study of SMAC-Mimetic Birinapant

PBMC sample analysis



Tumor core biopsy 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

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, cIAP1, 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: IκBα, Iκκα, NFκB p65 (pS536, pS529), NFκB1 p105/p50, NFκB2 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, PKCα, and PKCβII

Receptor tyrosine Kinase signaling: Shc 9pY239/240), Src (pY527, pY416), VEGFR

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

RNA regulation: S6 Ribosomal protein (pS235/236)

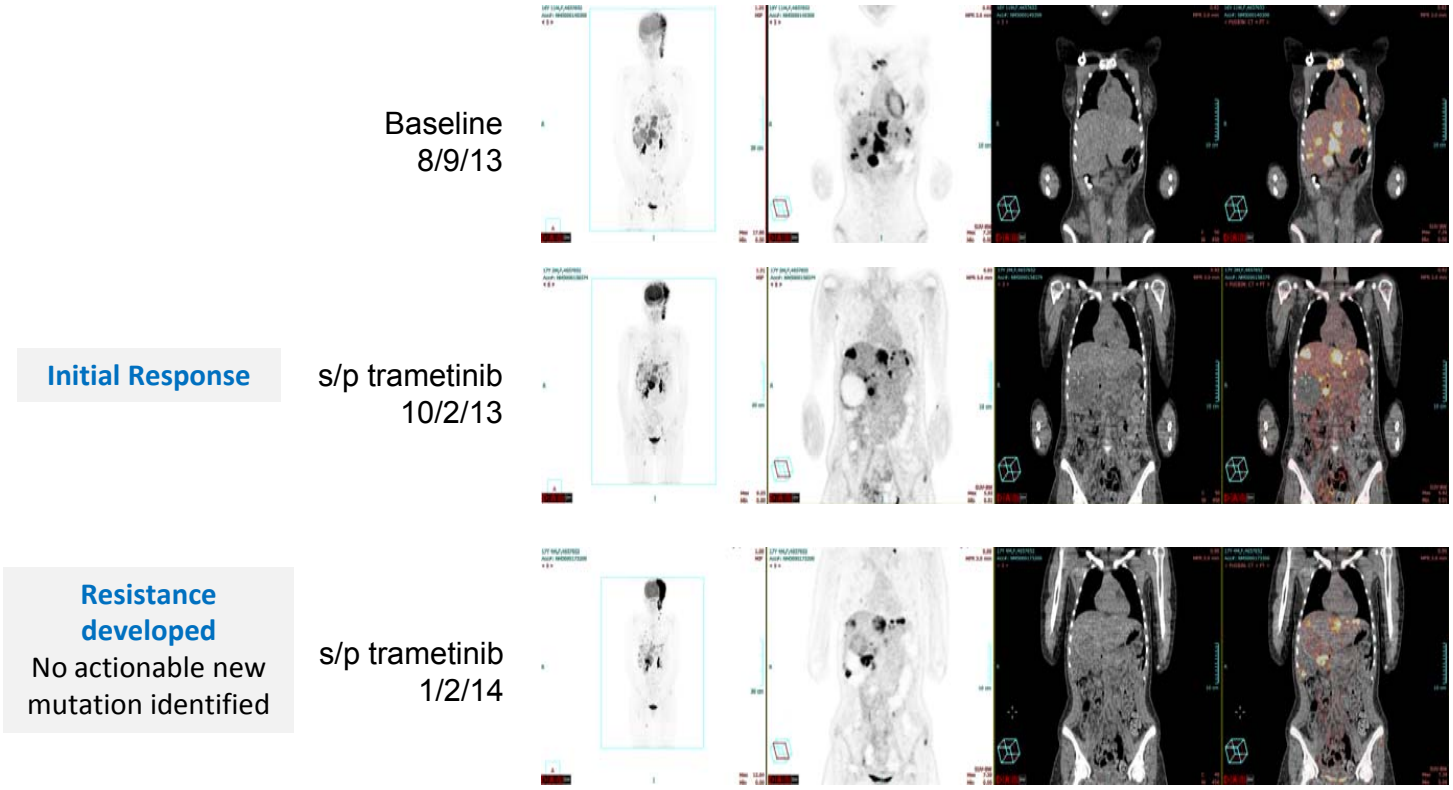
TGF-β/SMAD pathway: SMAD1, SMAD2 (pS465/467), SMAD3 (pS423/425), SMAD4, SMAD5

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

Ubiquitin-proteasome pathway: Ubiquitin

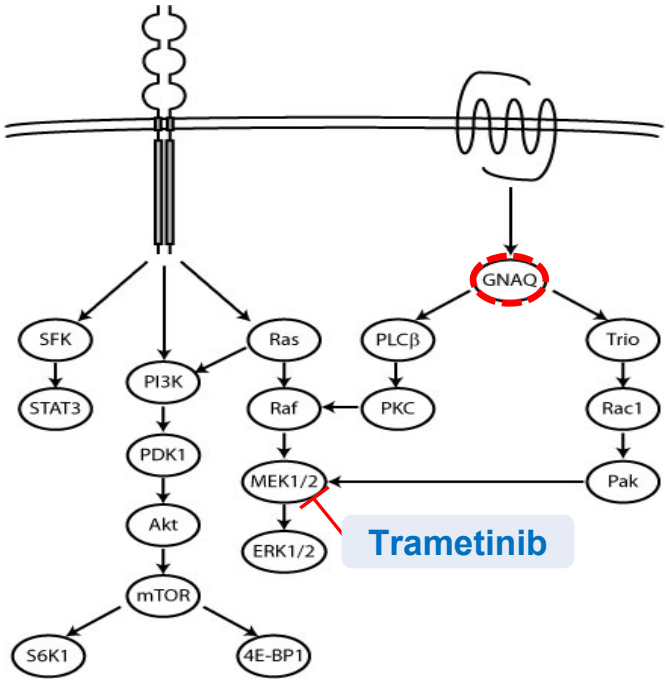
Wnt Signaling: β-Catenin

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



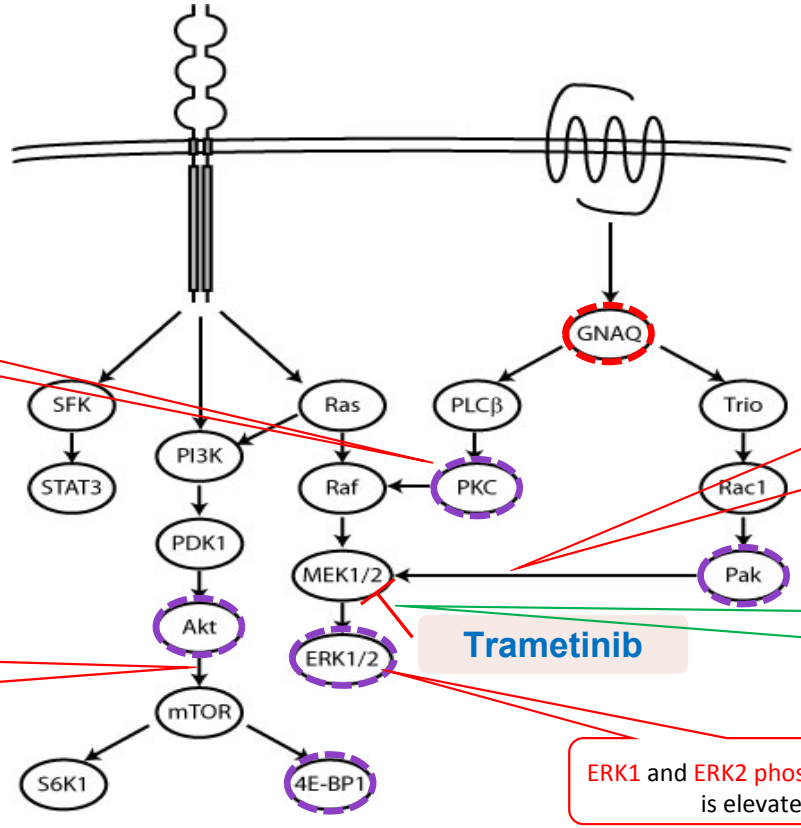
45 signaling molecules

- 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



PKC delta (PKC delta pS299 & pY311) is hyper-phosphorylated

MEK1 phosphorylation by p21-activated kinases (MEK1 pS298) is increased.

MEK1/2 activity in Trametinib resistant melanoma is probably mediated by Pak-dependent phosphorylation

MEK1 and MEK2 are not hyper-phosphorylated in the activation loop (MEK1/2 pS218/22) or on inhibitory sites (MEK1 pT386, MEK2 pT394).

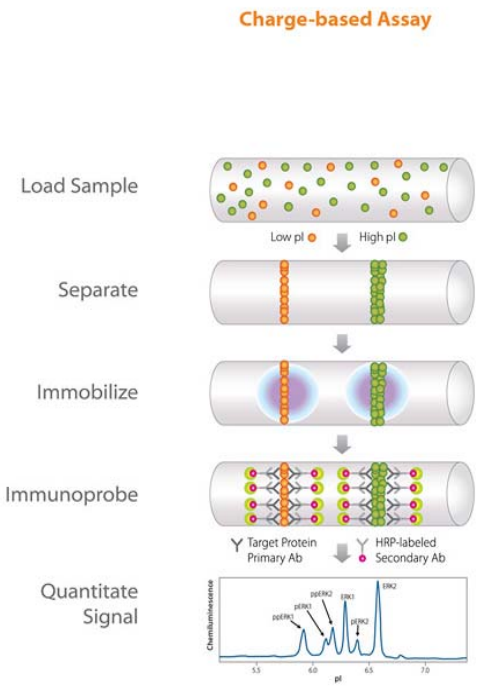
PI3K/Akt/mTOR pathway (AKT pS473 & pT308, 4EBP1 pT37/46, p70 S6 kinase pT389) is activated

ERK1 and ERK2 phosphorylation is elevated

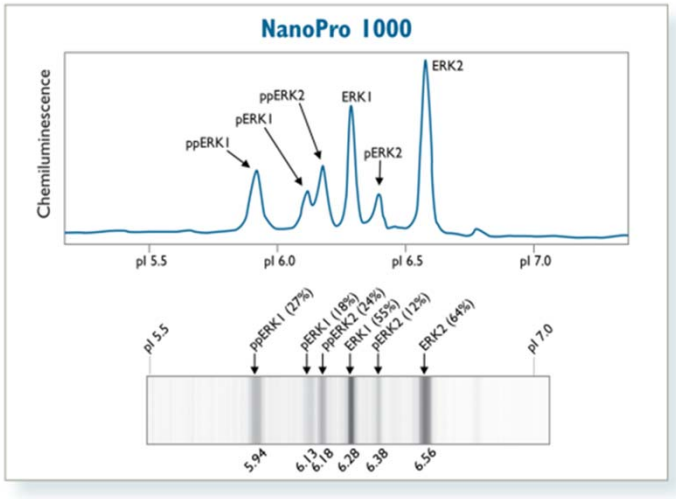
Potential treatment targets

- **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**

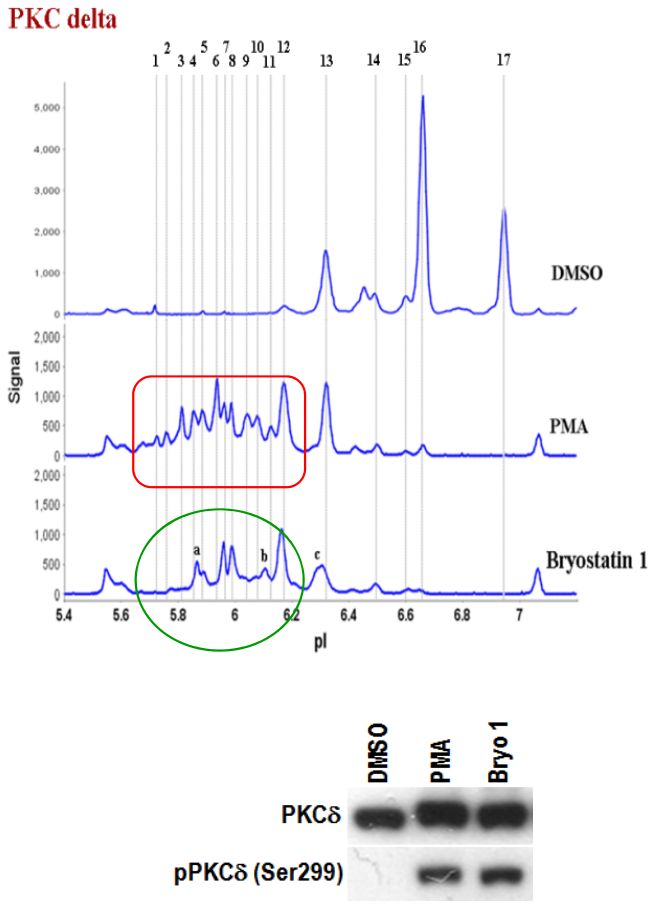
The IEF immuno-assay



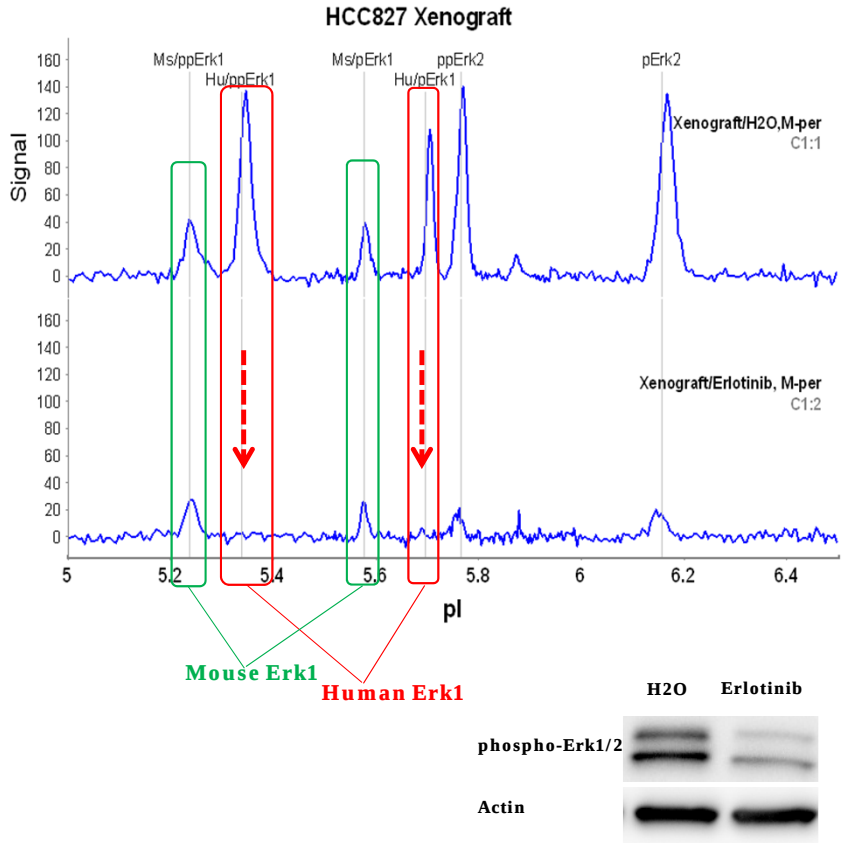
- Use isoelectric-focusing to separate proteins by charge
- Distinguish and detect different post-translationally modified states of a protein without using modification-specific antibodies



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.**

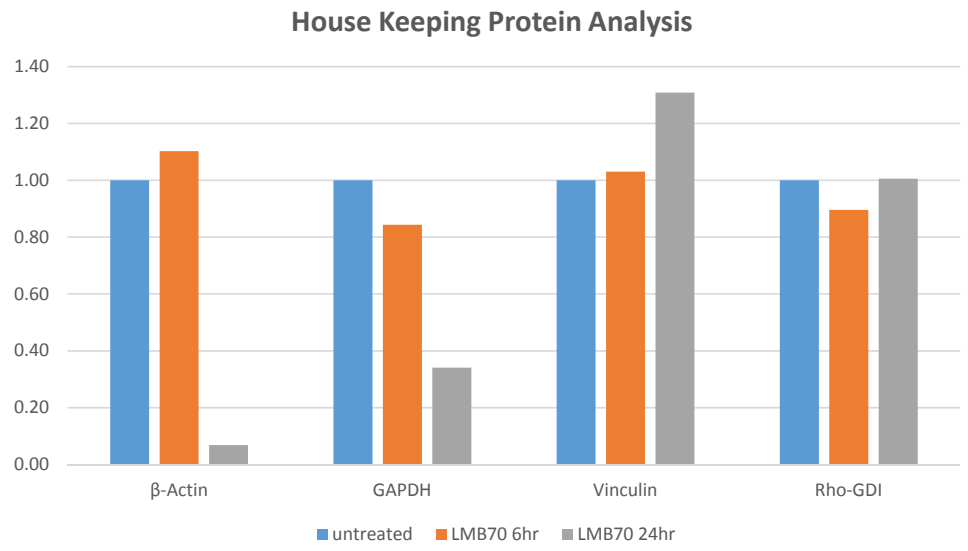
3) Signals obtained using antibodies specific for phosphorylated epitopes should be normalized to the total protein level of the target protein"

http://www.jbc.org/site/misc/ifora.xhtml

House keeping protein analysis

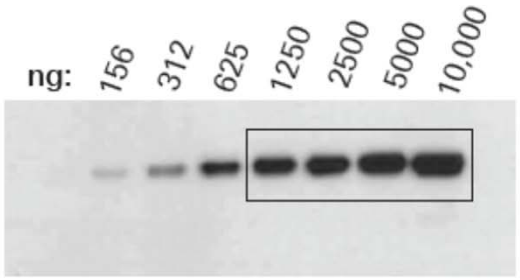
Simple Western house keeping protein assays:

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

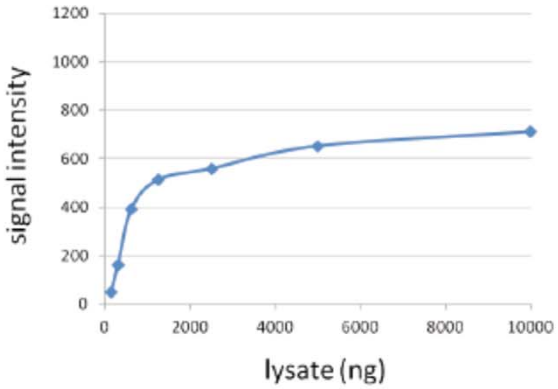


Impact of protein abundance and signal saturation

Band intensity and signal saturation affects analysis accuracy



LI-COR Biosciences, Western Blot Normalization

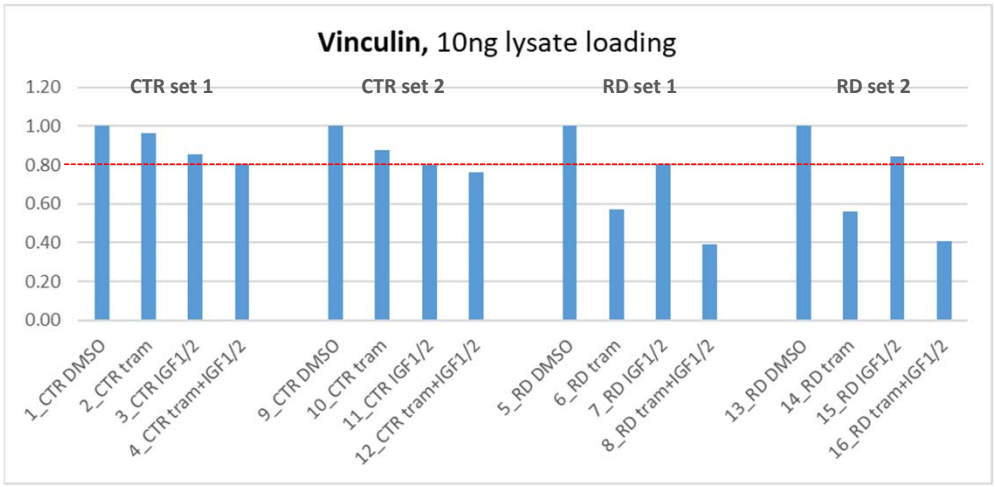


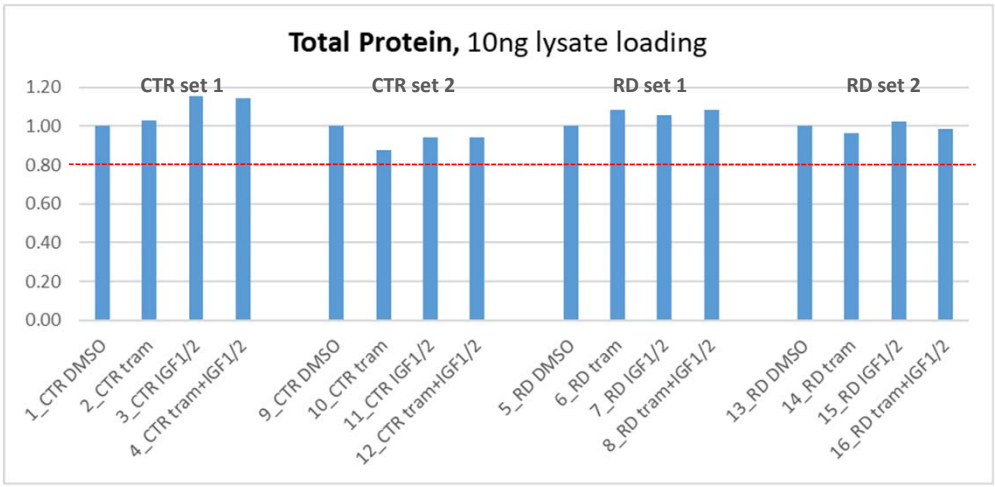
- Many housekeeping proteins and structural proteins used as internal loading controls are highly abundant, but target proteins are often expressed at much lower levels
- **The impact of protein abundance and saturation on Western blot normalization is often overlooked**

House keeping vs total protein analysis

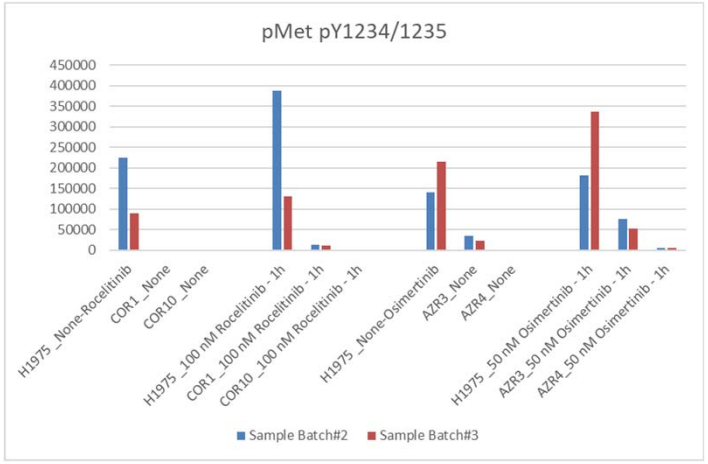
Simple Western assay for total protein analysis:

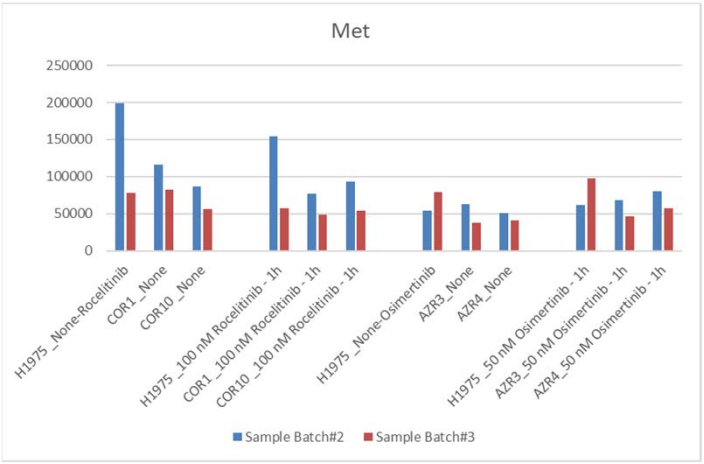
- Total protein assay is performed by labeling lysate proteins with biotin followed by HRP conjugated streptavidin detection
- The process is automated, and signals are captured by CCD camera and quantified with Compass software as Simple Western immunoassays

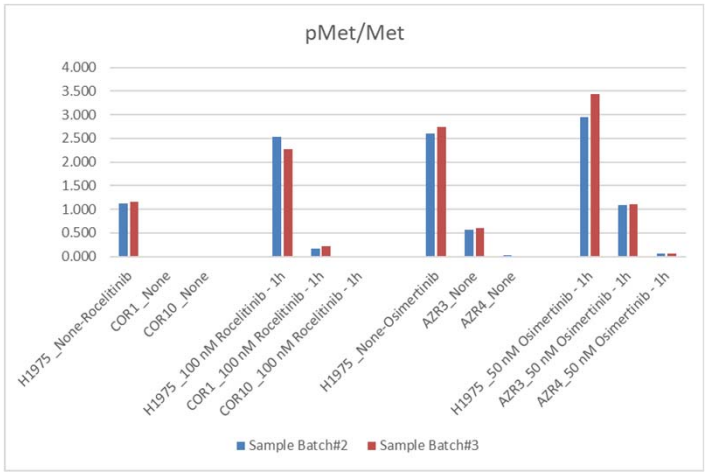


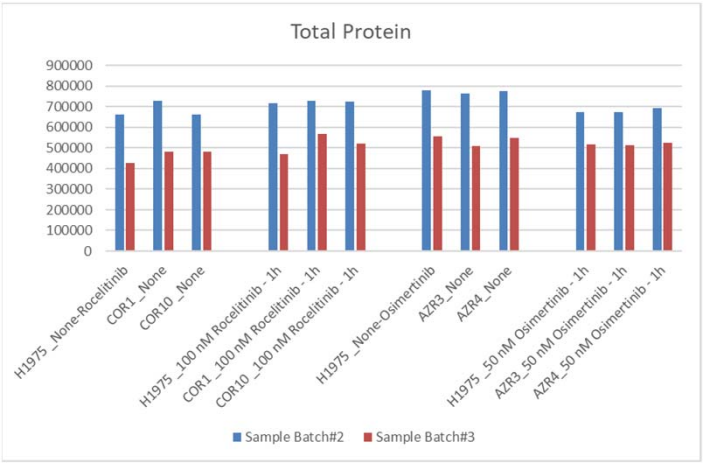


Normalize phospho-signal with pan-target-protein signal









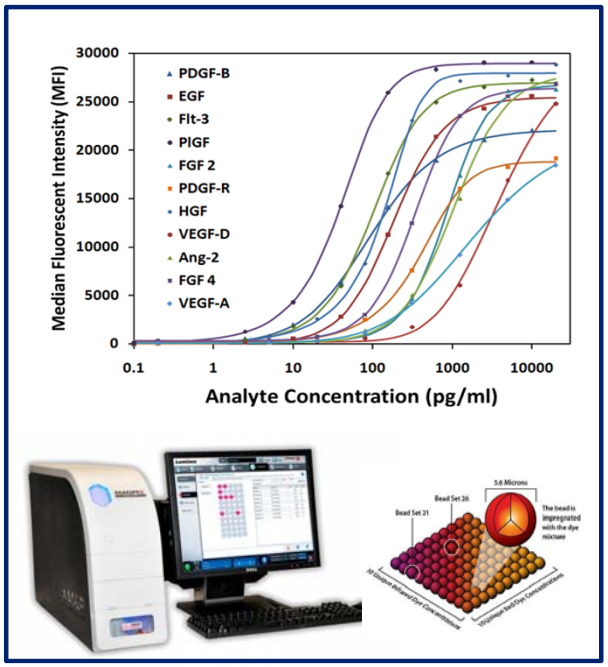
The Simple Western™ technology applications

- **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

Bio-Rad: https://www.**bio-rad**.com/webroot/web/pdf/lsr/literature/Bulletin_6335.pdf

Millipore: http://www.**emdmillipore**.com/US/en/life-science-research/protein-detection-quantification/Immunoassay-Platform-Solutions/milliplex-multiplex-assays-using-luminex/UjGb.qB.8WQAAAE_rn8RHeN.,nav?isCountryEMD=yes

R&D systems: https://www.**rndsystems**.com/products/human-xl-cytokine-discovery-luminex-high-performance-assay

xMAP kit finder: 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

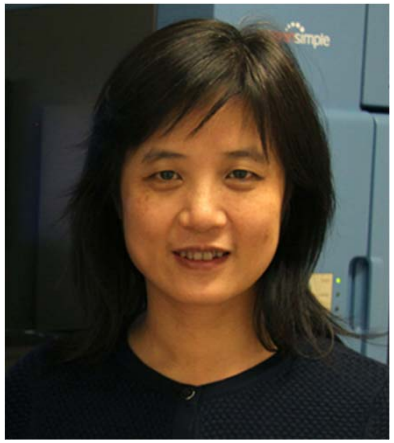
Operations

- 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

kedein@mail.nih.gov

240.760.6922



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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**-**D**etection by ind**EX**ing
- 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

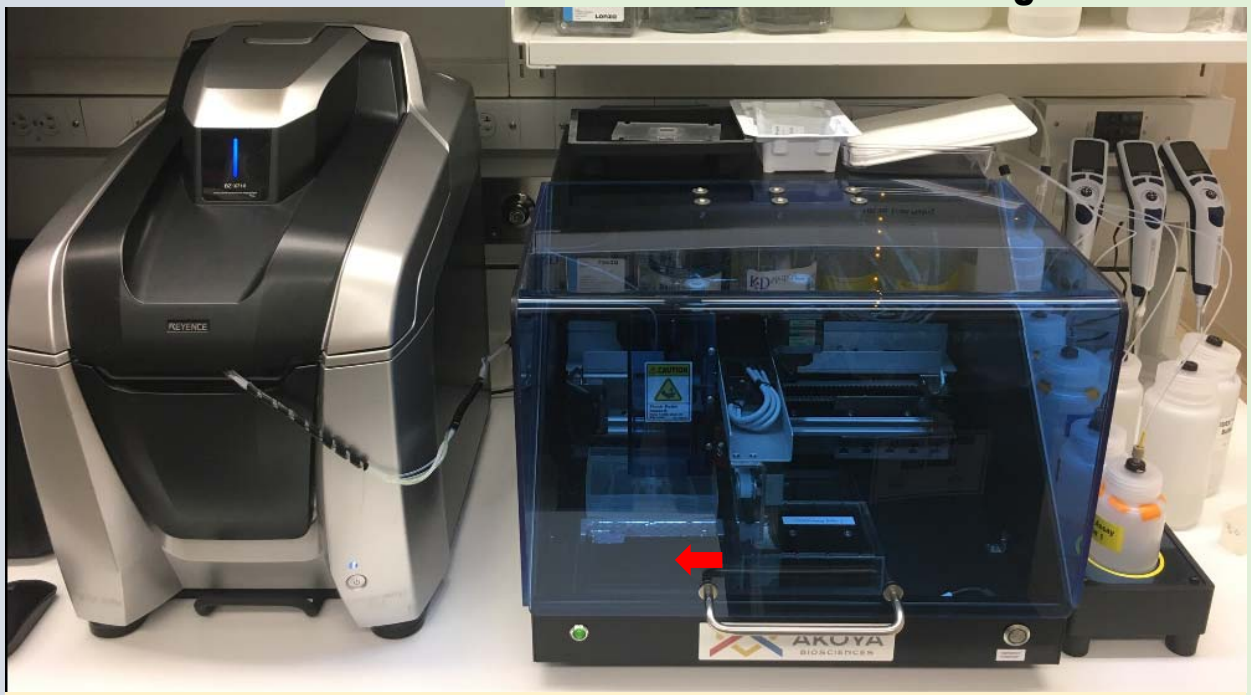
20X objective
Later versions could work with 40X

4 detection channels:
DAPI, fam, CY3, CY5

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

Keyence microscope

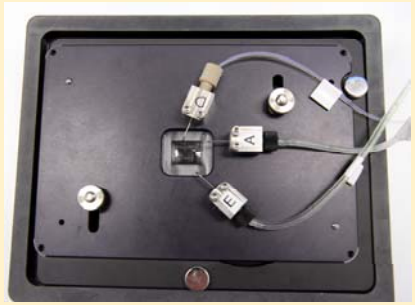
Microfluidics for automated buffer exchange



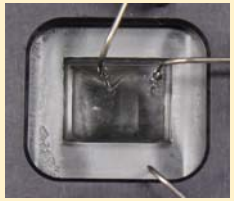
3 buffers with different amount of DMSO

Dyes for each cycle are prepared in 96 well plate

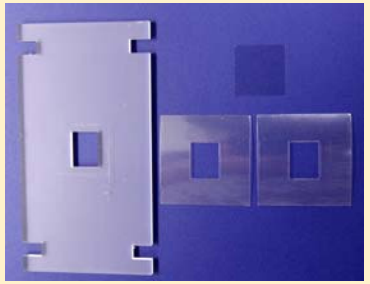
Tissue chamber



Sample on the coverslip



The coverslip is currently mounted using double side tape



CODEX-ready ANTIBODIES

Current human antibody panel for fresh frozen tissues

Target	Clone
CD11c	B-ly6
Ki67	B56
CD104	450-9D
CD19	HIB19
CD45	HI30
CD8	SK1
Podoplanin	NC-08
CD15	HI98
CD7	CD7-6B7
CD9	HI9a
CD90	5E10
CD3	UCHT1
Pan-Cytokeratin	AE-1/AE-3
CD4	
CD38	HB-7
CD31	WM59
CD22	HIB22
CD279	EH12.2H7
CD278	C398.4A
CD57	HCD57
CD21	Bu32
CD40	HB14
Collagen IV	
HLA-DR	

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

Target	Clone
CD11c	HL3
CD71	C2
CD79b	HM79B
CD16/32	2.4G2
CD21/35	7G6
F4/80	T45-2342
IgM	II/41
CD90	G7
CD5	53-7.3
CD45	30-F11
CD4	RM4-5
Ly6c	HK1.4
CD8a	53-6.7
MHC II	M5/114.15.2
CD106	429(MVCAM.A)
TCRb	H57-597
CD45R/B220	Ra3-6B2
CD24	M1/69
CD11b	M1/70
CD44	IM7
Ly6G	1A8
IgD	11-26c.2a

B cell markers:
B220, IgD, IgM, CD79

T cell markers:
TCRb, CD4, CD8A, CD5

Myeloid markers:
CD11b, LY6C, LY6G, CD11c, F4/80, CD16/32

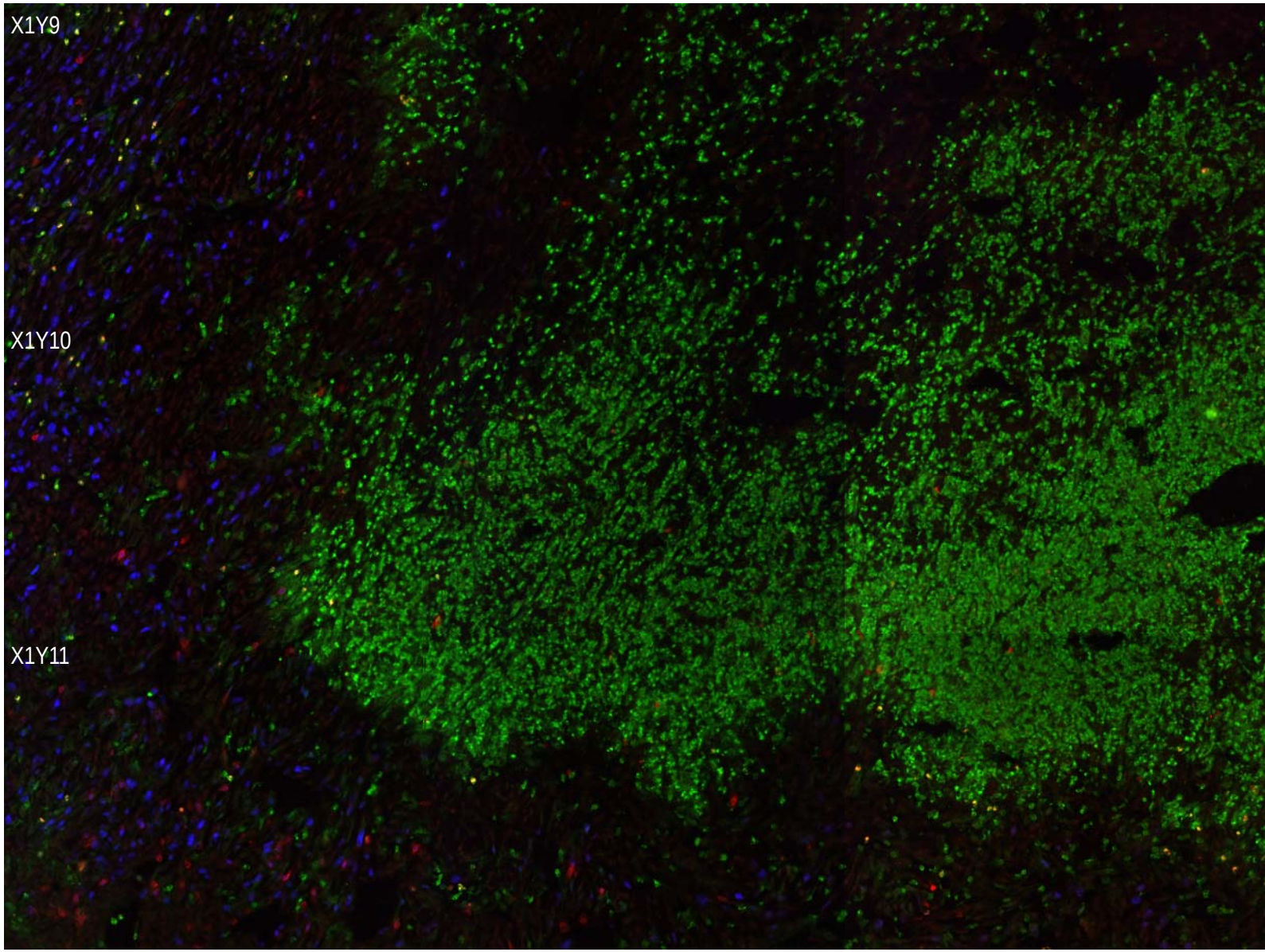
Vascular markers:
CD106

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

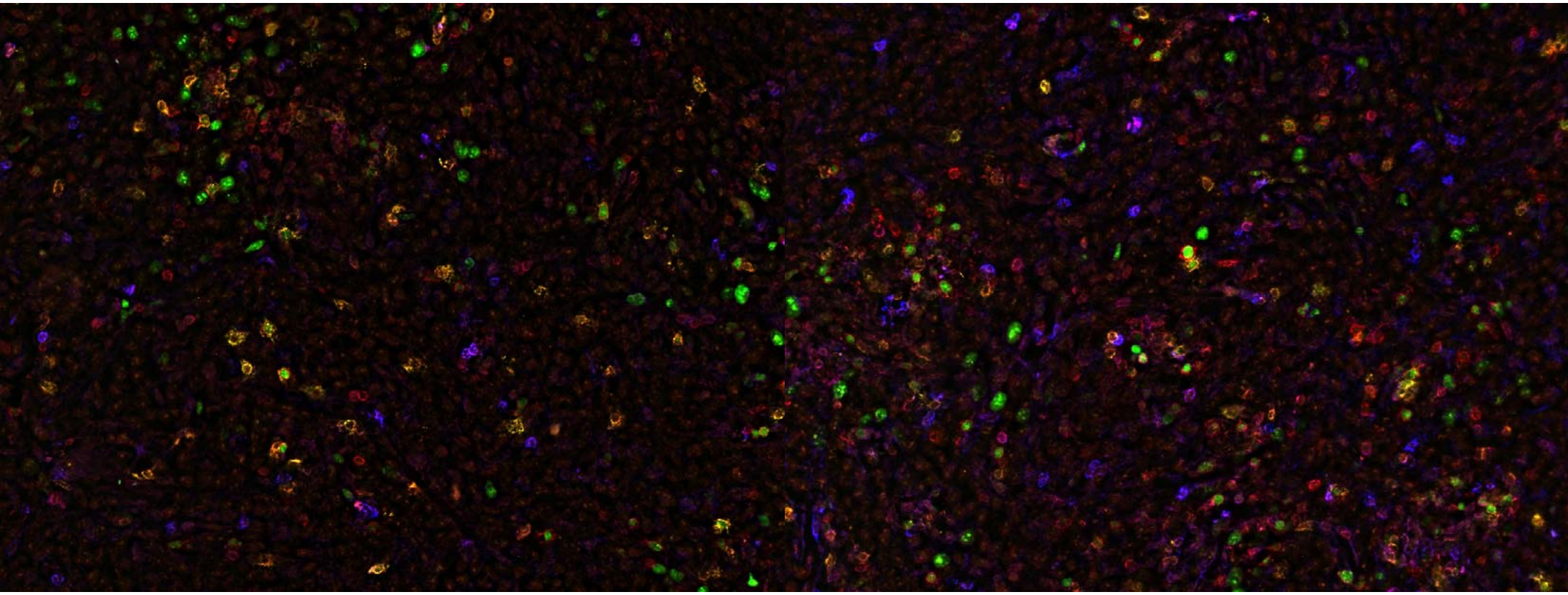


Collaboration with **David Wink** (Cancer and Inflammation Program), **Stephen Lockett**, **David Scheiblin** (OMAL)

4T1 tumor region stained with CD11b CD45 CD8a Ki67

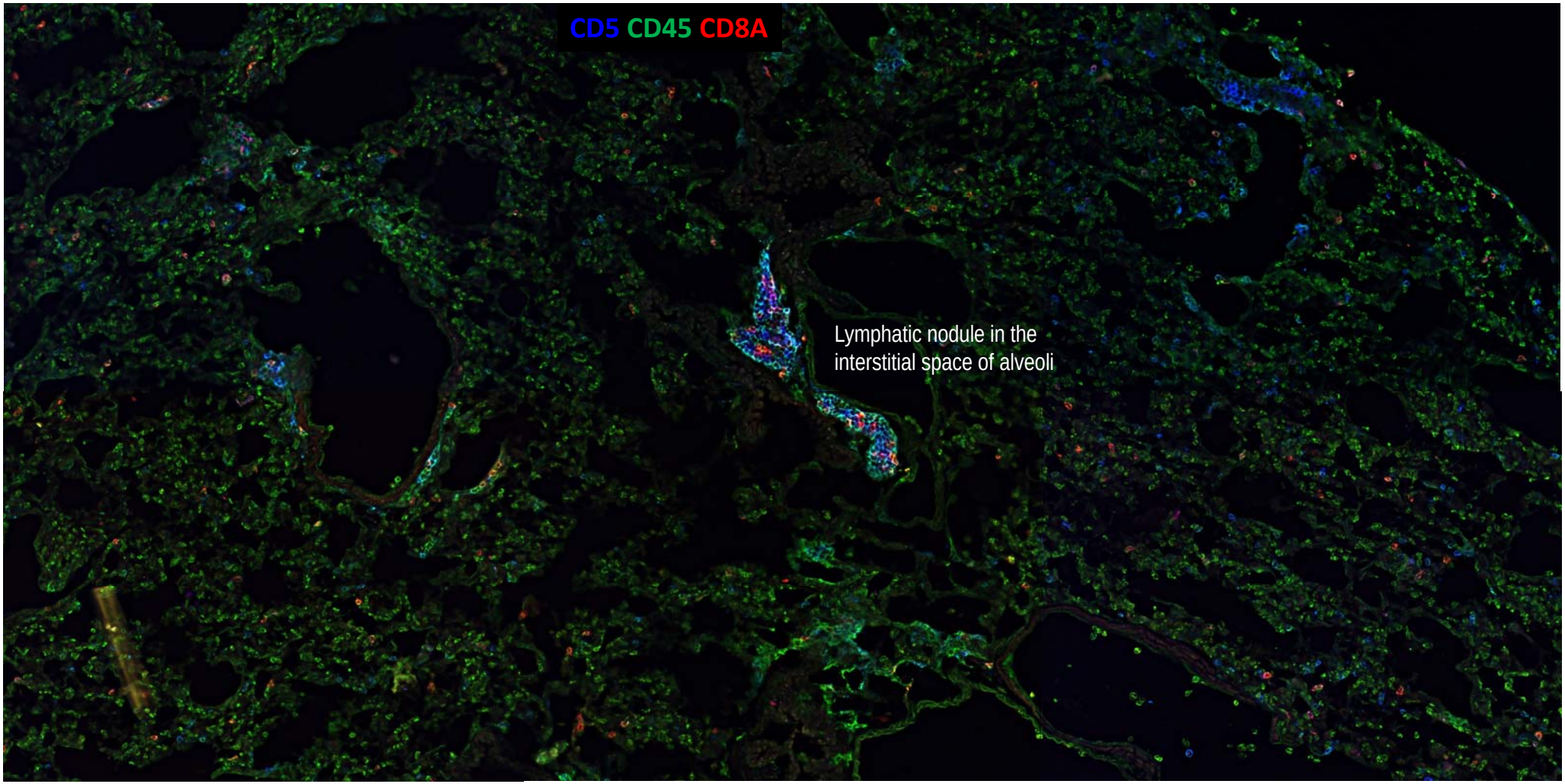
X4Y5

X4Y6



06.29.2018

Staining pattern in the tumor bearing mouse lung suggests specificity of the antibodies

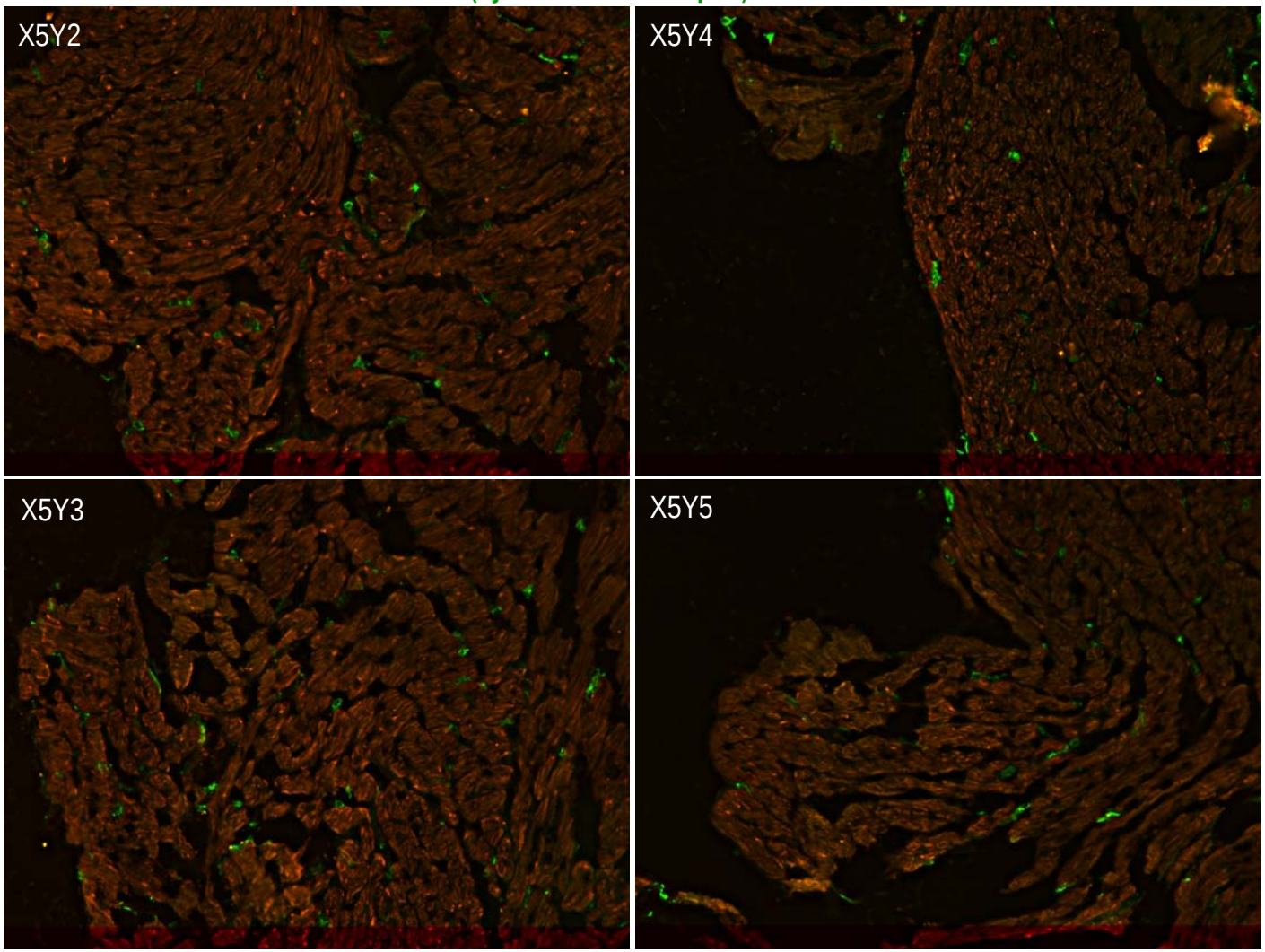


Collaboration with **Alex Wu**, **Patricia Steeg** (Women's Malignancies Branch) and **Lalage Wakefield** (LCBG)

CD44 staining in immune cells present between heart muscle fibers (mouse)

Reg1	2	3	4	5	6	7	8	9
18	17	16	15	14	13	12	11	10
19	20	21	22	23	24	25	26	27
36	35	34	33	32	31	30	29	28
37	18	19	40	41	42	43	44	45
54	53	52	51	50	49	48	47	46
55	56	57	58	59	60	61	62	63
72	71	70	69	68	67	66	65	64
73	74	75	76	77	78	79	80	81

CD44 (hyaluronic acid receptor) fam blank



Collaboration with **Meera Murgai, Sabina Kaczanowska and Rosandra Kaplan** (Pediatric Oncology Branch)

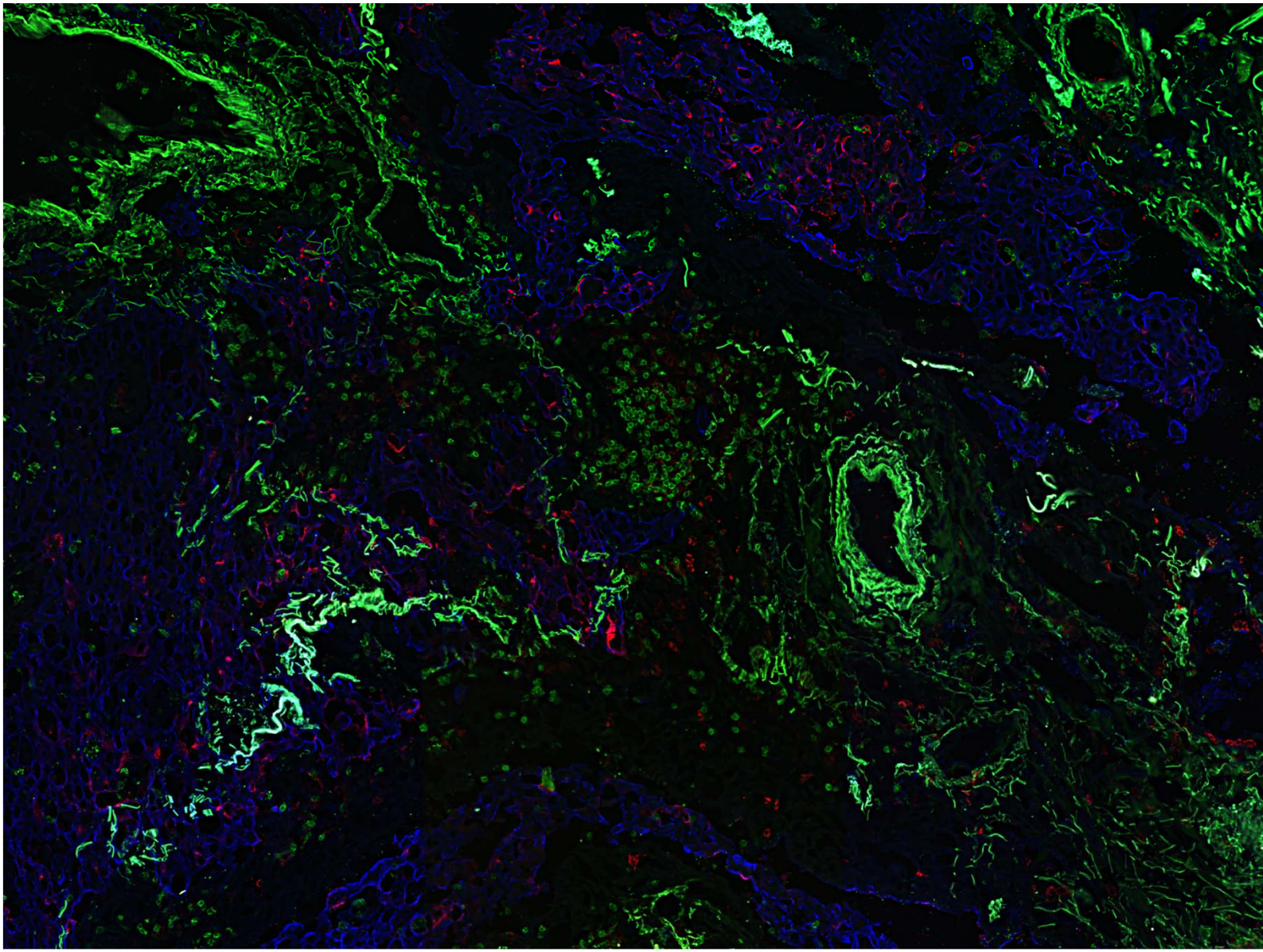
Adenocarcinoma of the lung
Human
(3 x 3 tiles show after deconvolution)

Staining:

CD3 (T cells)
CD19 (B cells)
Pancytokeratin

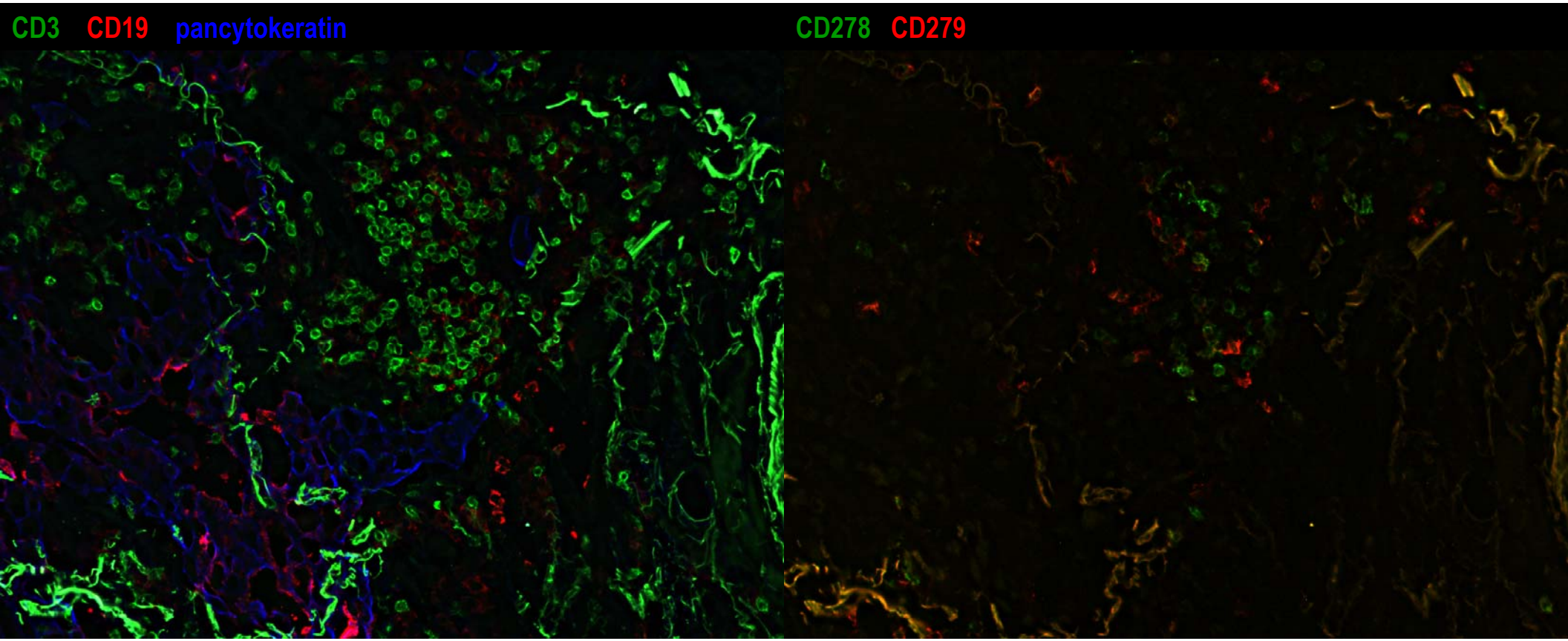
Also detected:

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



Collaboration with **Bríd Ryan** (LHC)

Adenocarcinoma of the lung (zoomed in image)

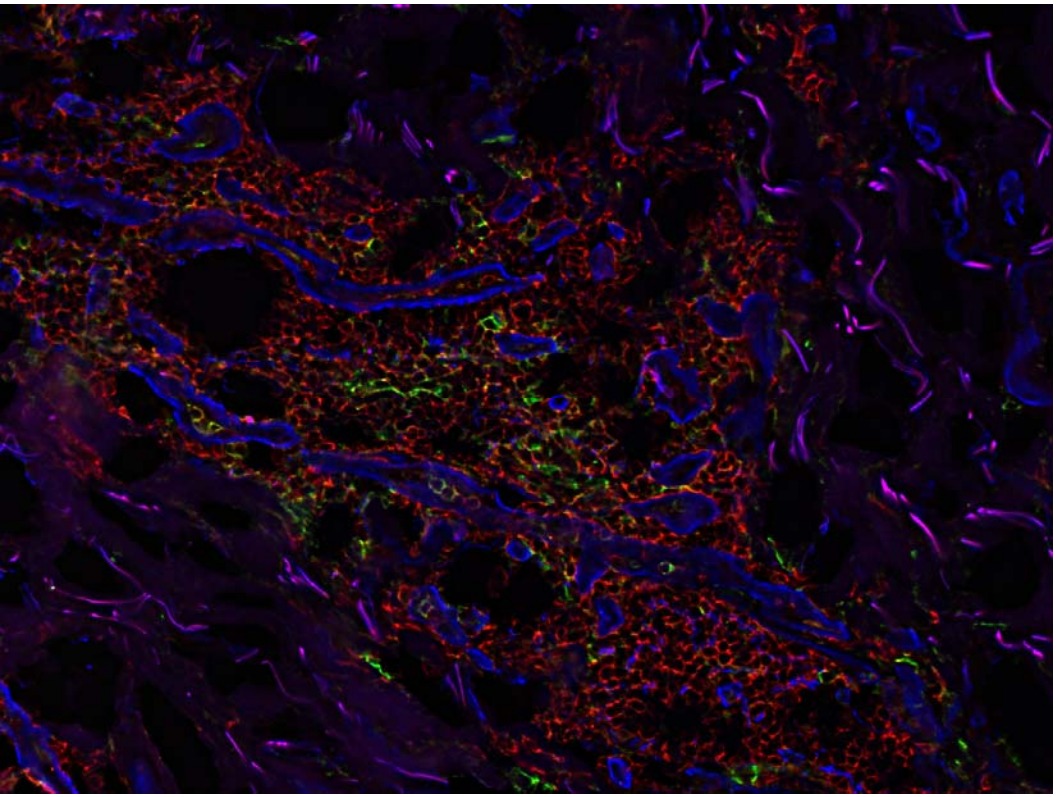


Collaboration with **Bríd Ryan** (LHC)

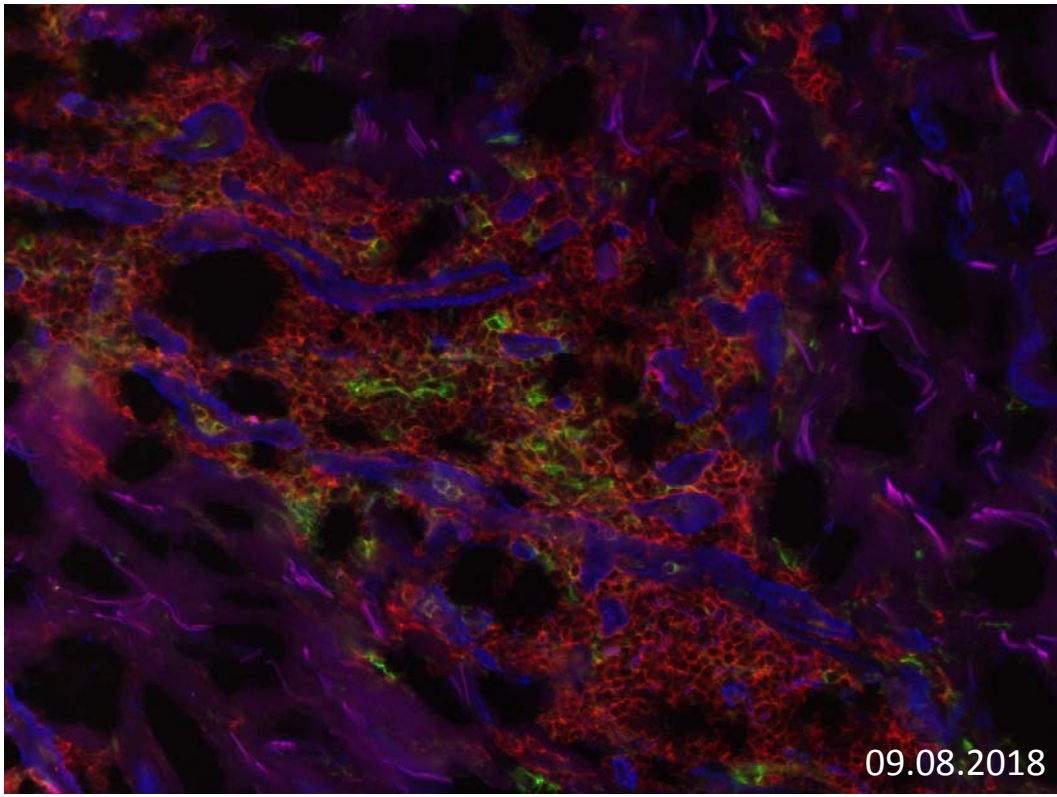
CD45^{+} immune infiltrate in human mesothelioma

CD45
CD11c
collagen IV

With deconvolution



Without deconvolution



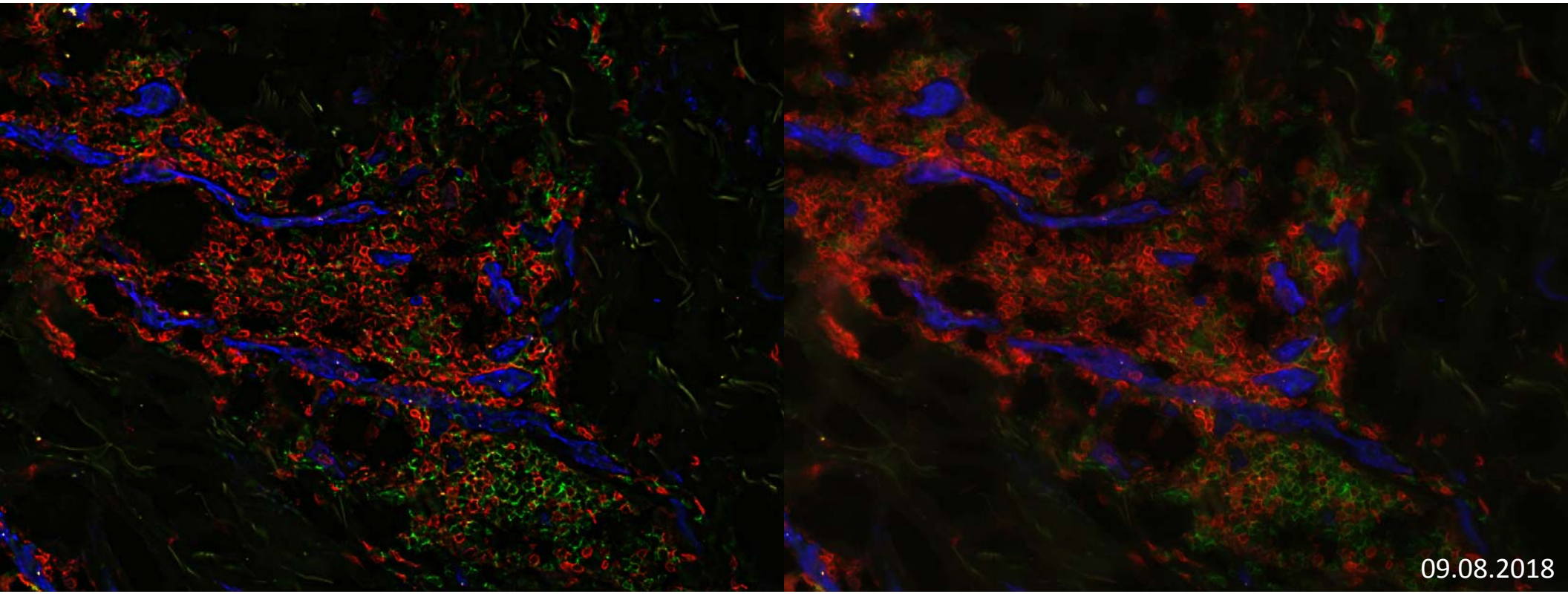
Collaboration with Qun (Queena) Jiang (TGMB)

CD3 (T cells)
CD22 (B cells)
CD31 (vasculature)

Majority T cells, relatively separate B cells (human mesothelioma)

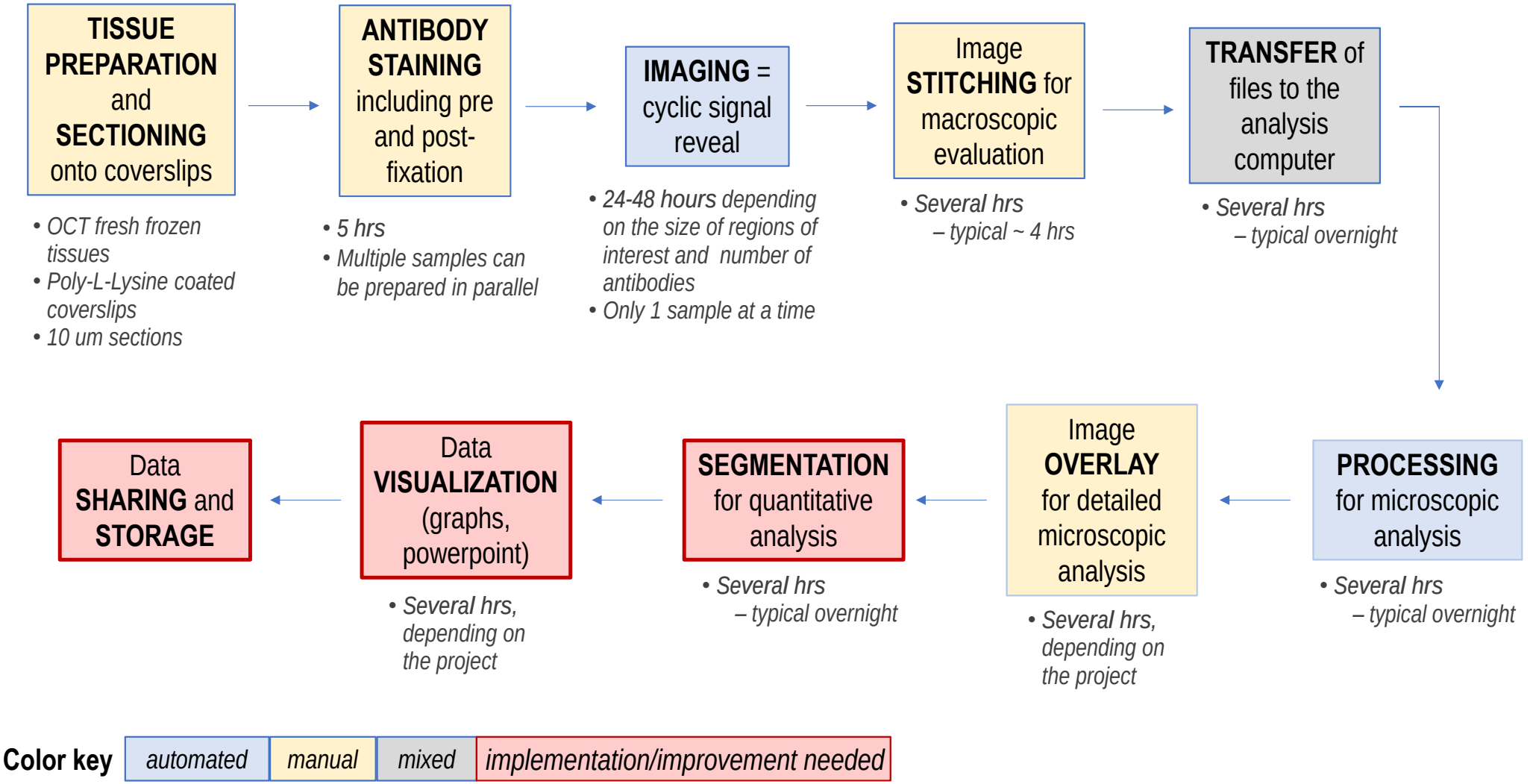
With deconvolution

Without deconvolution



Collaboration with Qun (Queena) Jiang (TGMB)

CODEX workflow



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 PHL, especially for difficult tissues)
- staining the tissue and running CODEX by CPTR

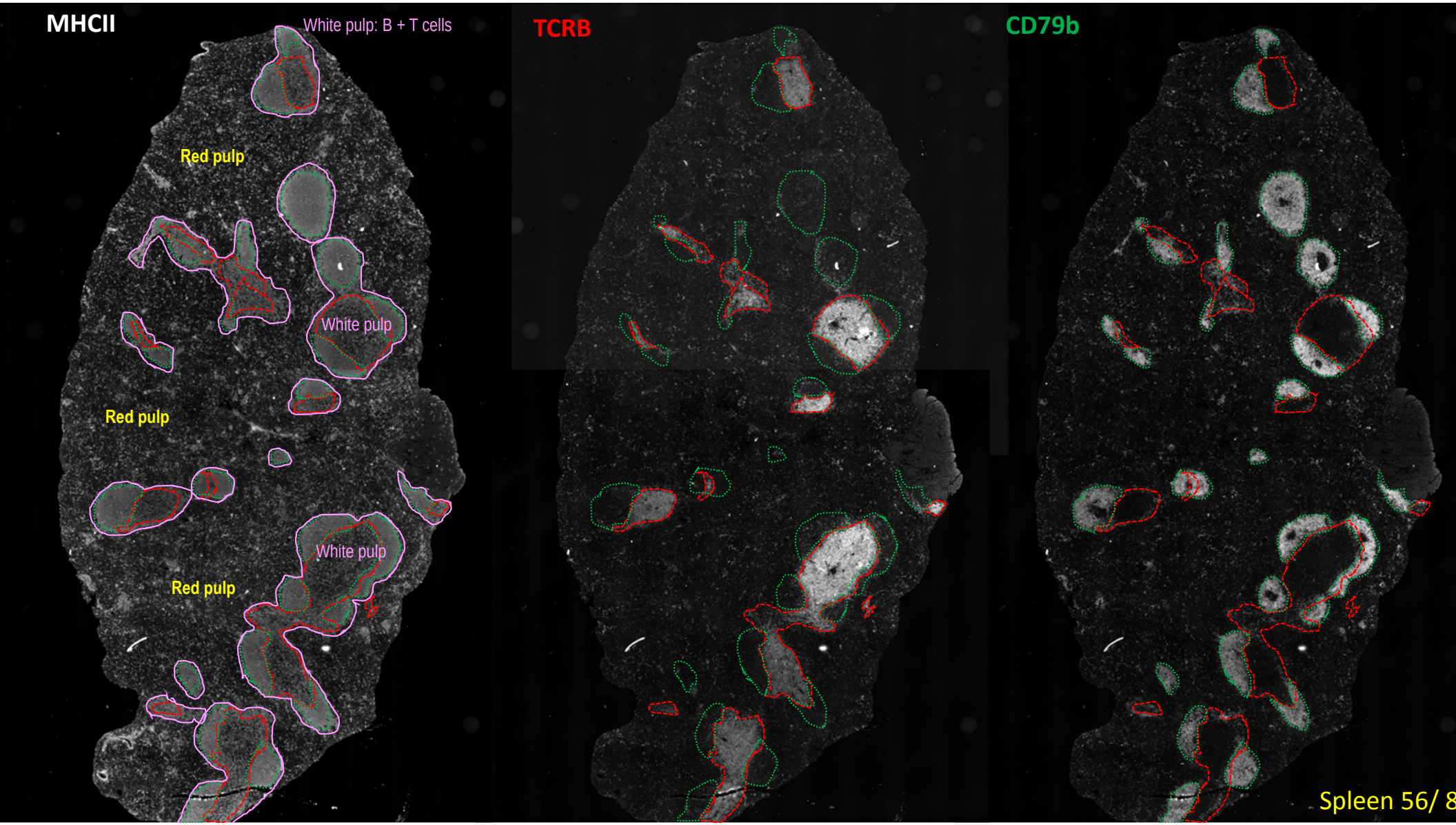
Data analysis:
- Primary data analysis by CPTR: image processing, annotation using Akoya pipeline, basic segmentation with Akoya software
- Secondary and tertiary analysis by investigator in collaboration with CPTR and the bioinformatics support

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

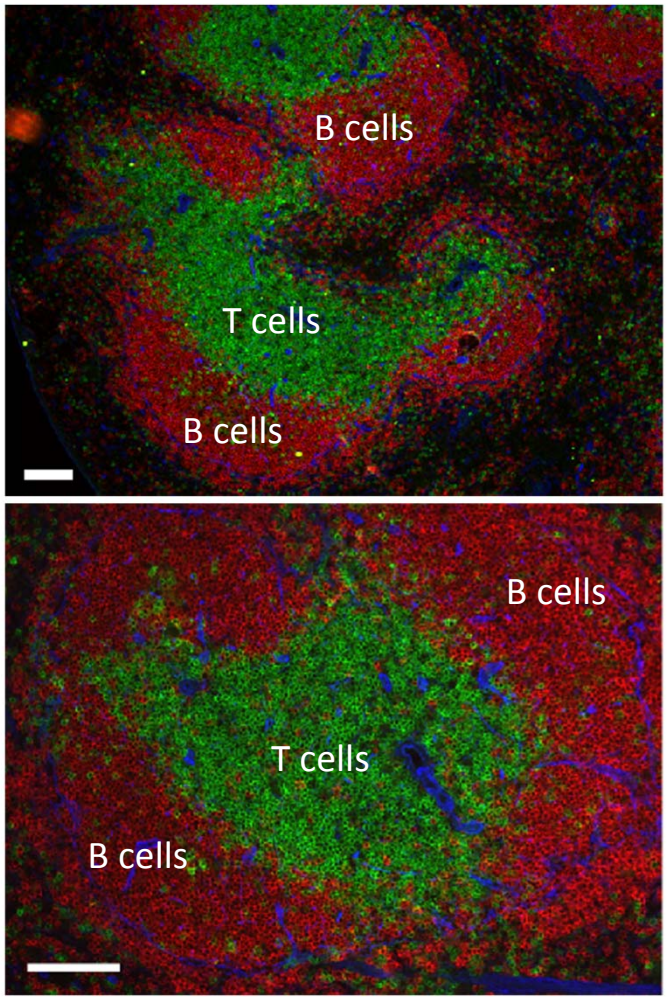
Procedure for getting access to the CODEX technology and details about the upcoming service *(contd.)*

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

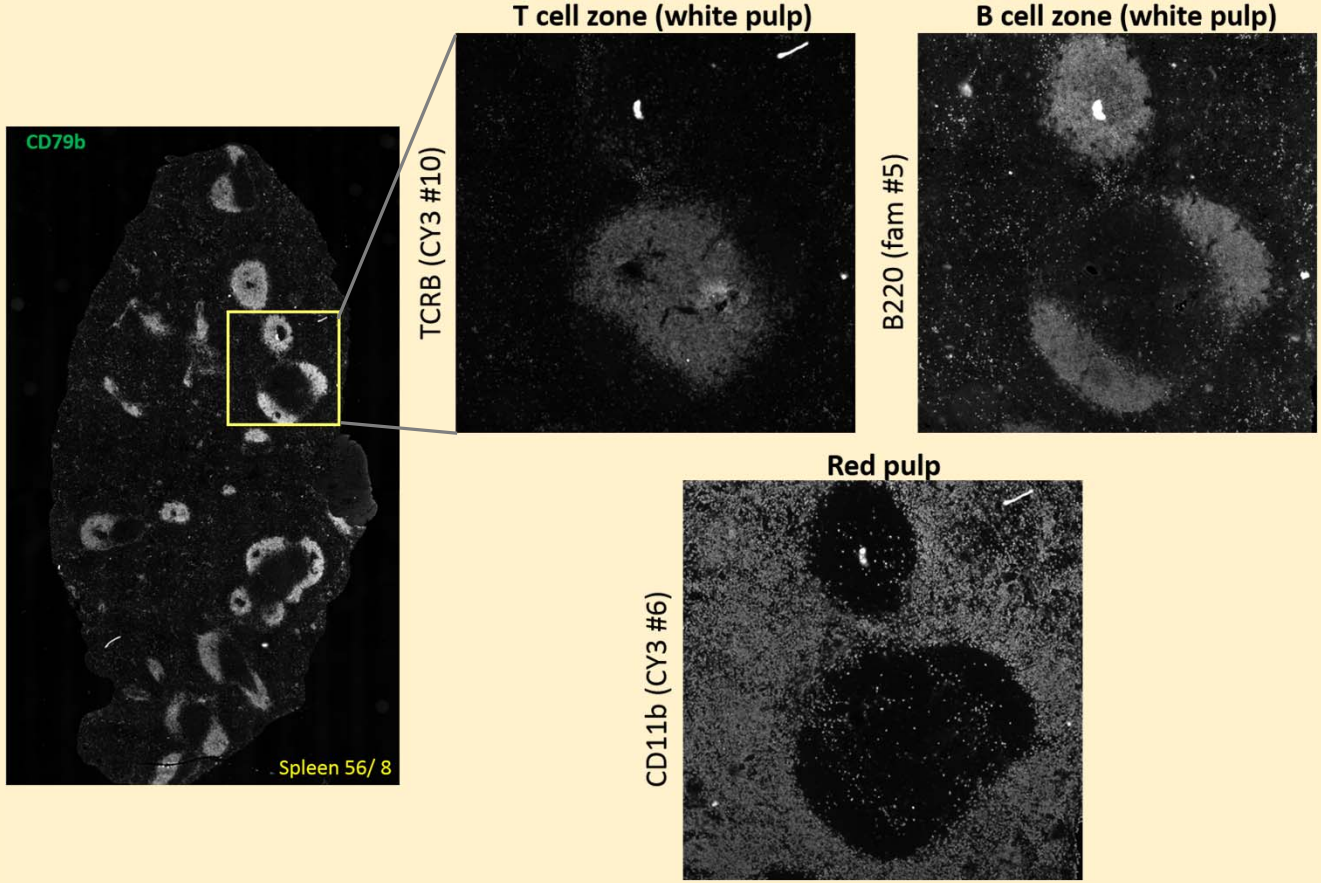

MHCII
White pulp: B + T cells
Red pulp
White pulp
Red pulp
White pulp
Red pulp
TCRB
CD79b
Spleen 56/ 8

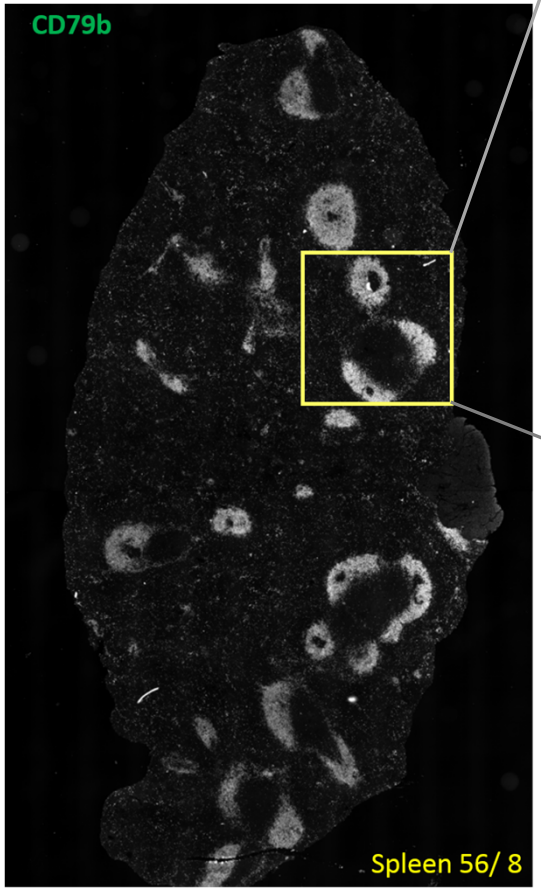
Literature: Staining of T and B cells in the spleen of C57BL/6 mice (OCT fresh frozen)



PLoS One. 2011;6(9):e24772.

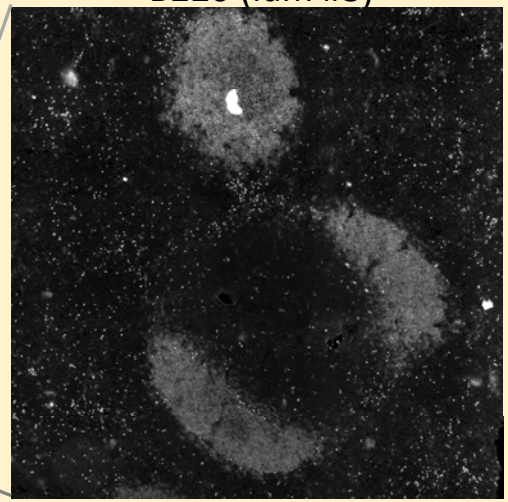
The pattern of CODEX ab staining matches the one described in the literature



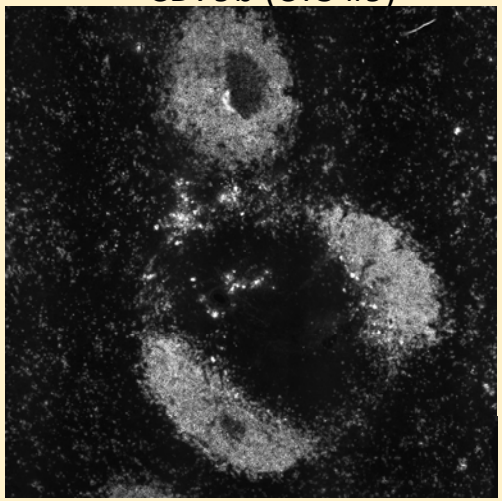


Markers identifying B cells (selected region)

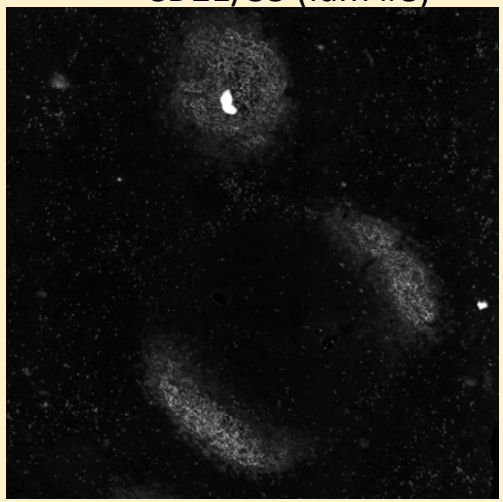
B220 (fam #5)



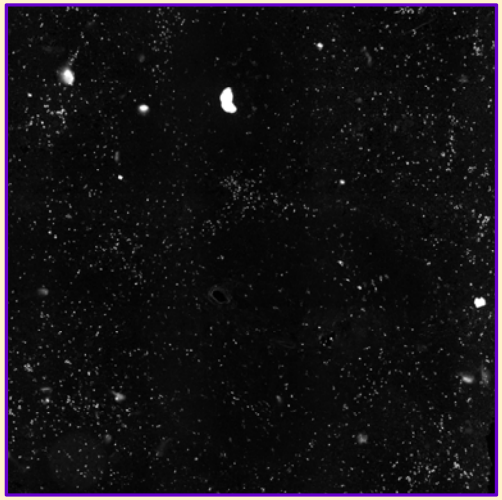
CD79b (CY5 #9)



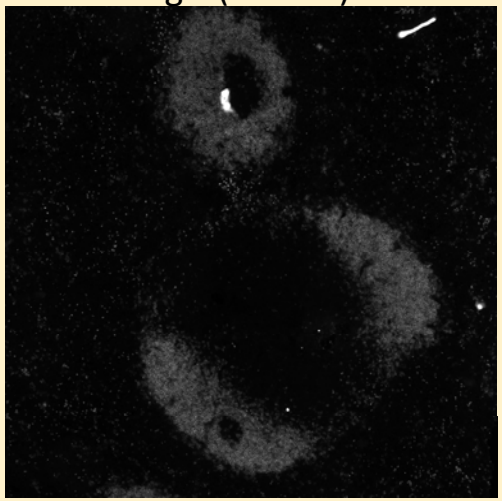
CD21/35 (fam #8)



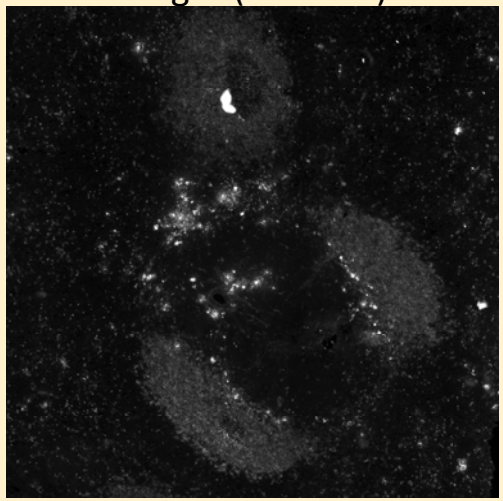
Blank (fam)



IgD (CY3 #9)

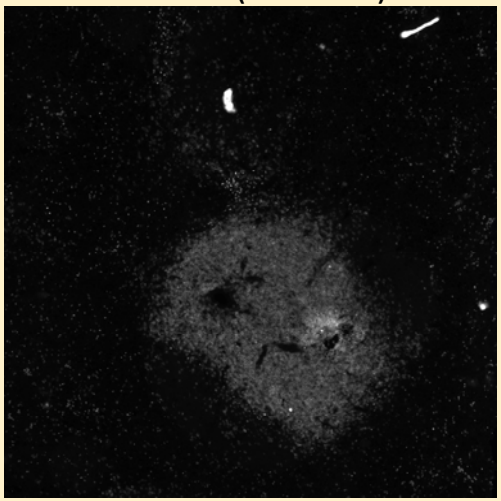


IgM (fam #10)

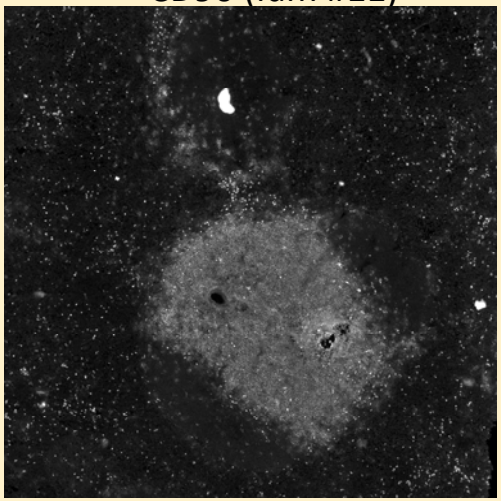


Markers identifying T cells (selected region)

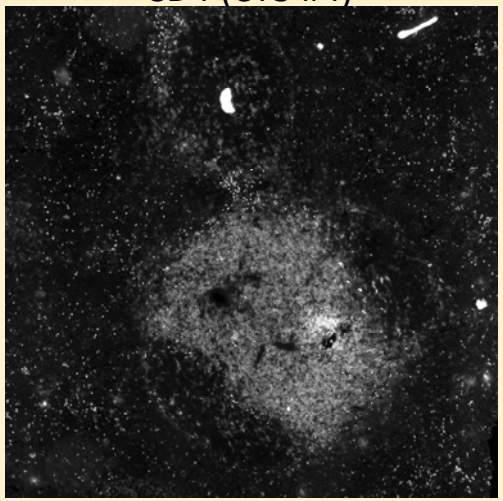
TCRB (CY3 #10)



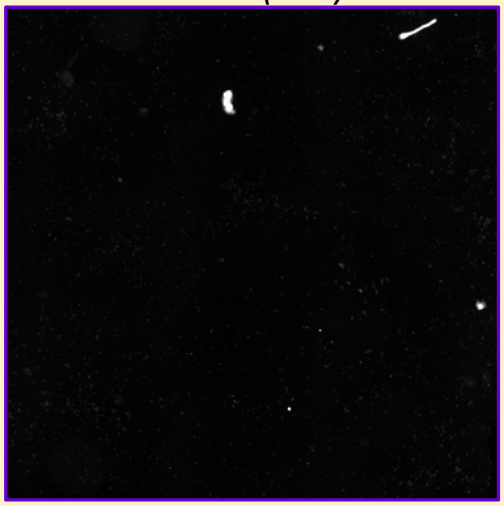
CD90 (fam #11)



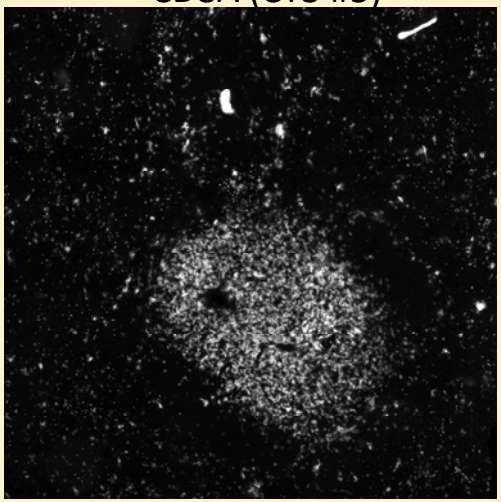
CD4 (CY3 #4)



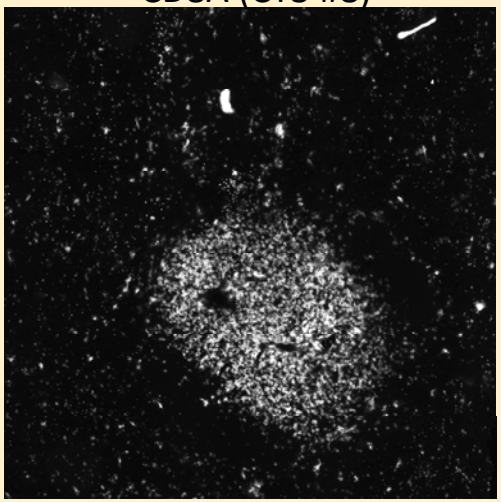
Blank (CY3)



CD8A (CY3 #5)



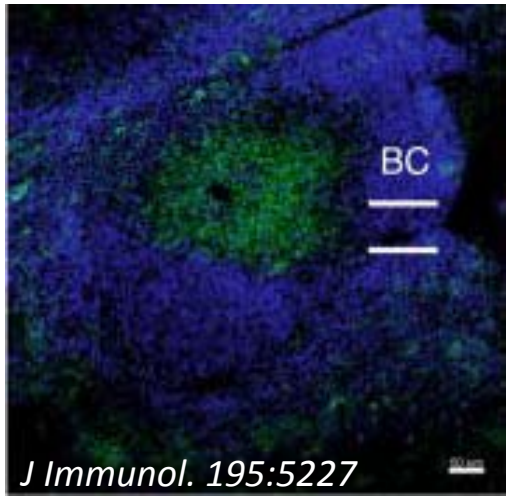
CD8A (CY3 #8)

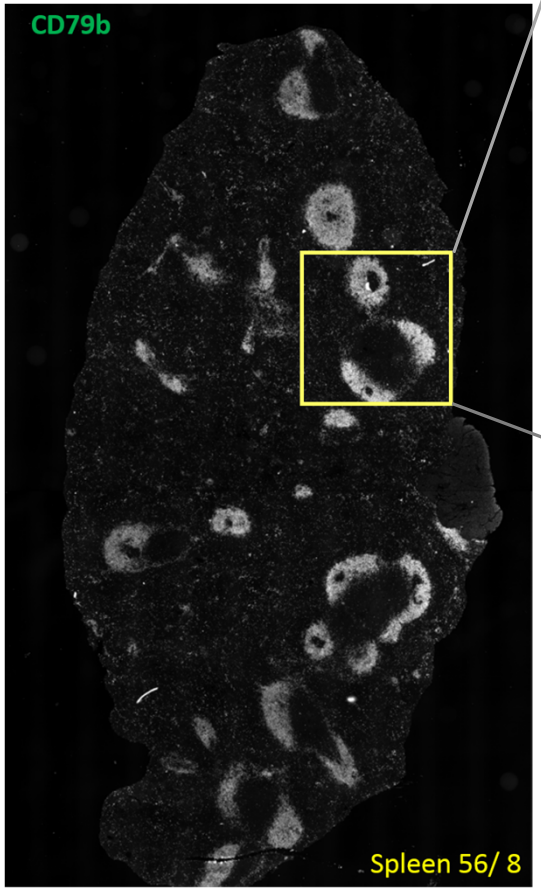


Literature: staining of mouse spleen

(BC: B cell region)

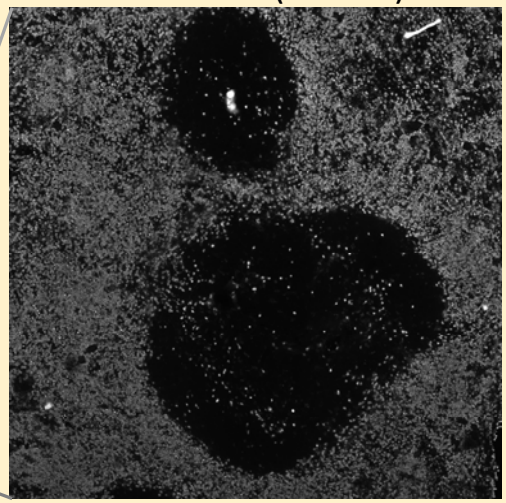
CD8 B220



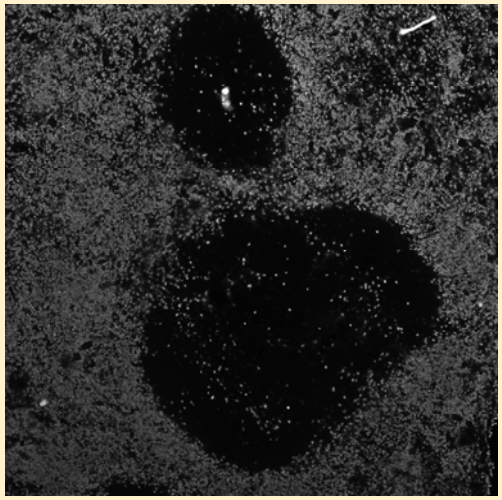


Markers staining cells in the red pulp (selected region)

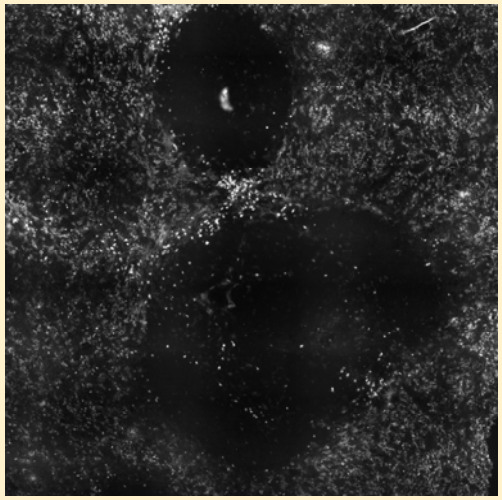
CD11b (CY3 #6)



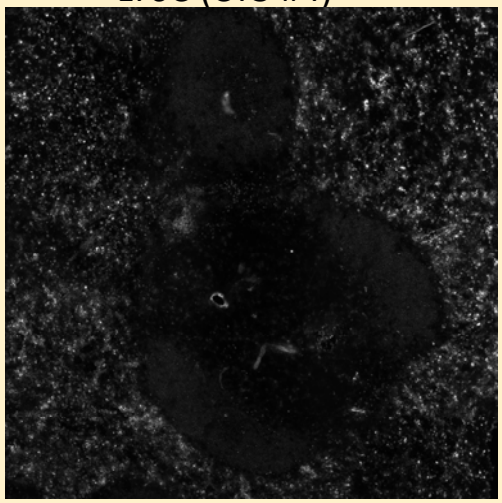
CD11b (CY3 #11)



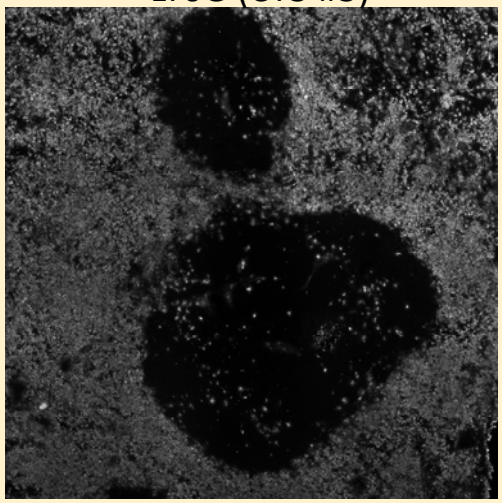
F4/80 (CY5 #6)



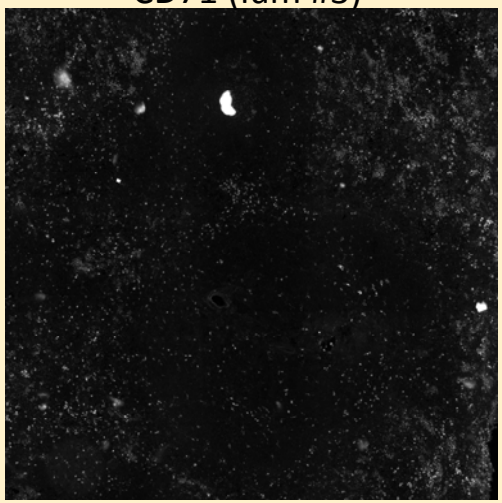
LY6C (CY5 #4)



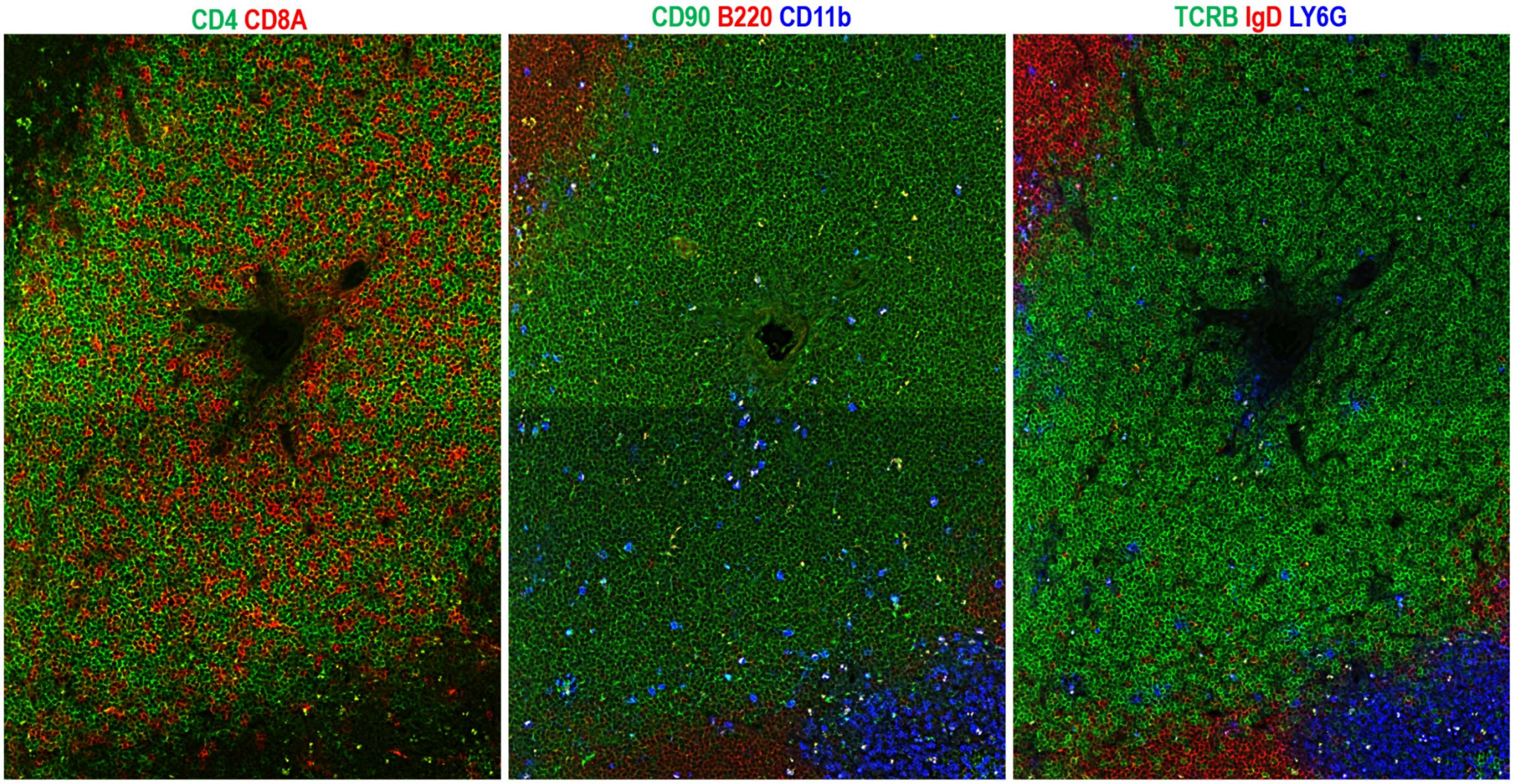
LY6G (CY5 #5)



CD71 (fam #3)

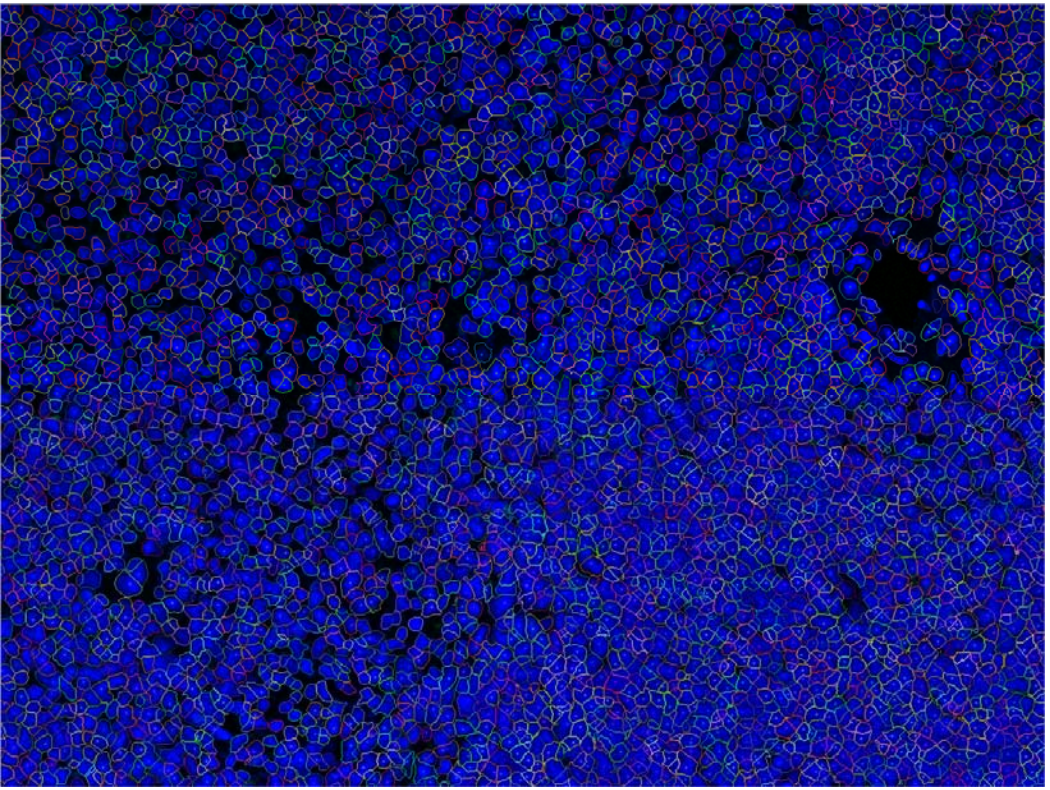


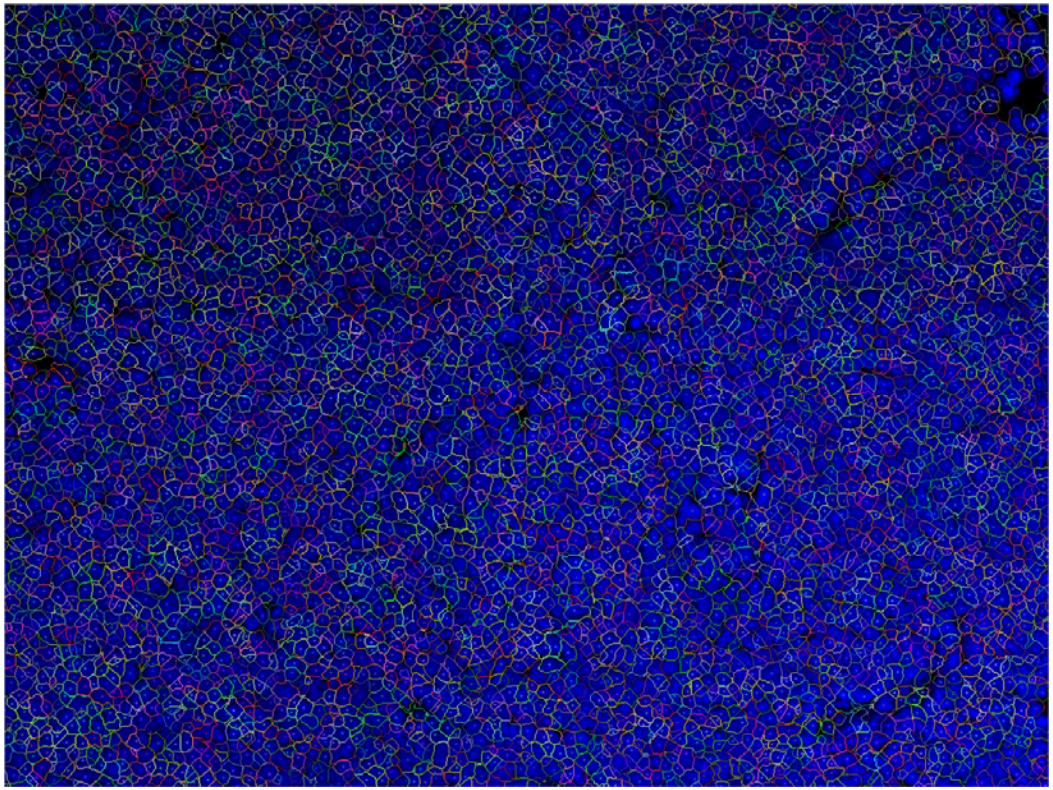
Specificity of staining: CD4/CD8A, CD90/B220/CD11b, and TCRB/IgD/LY6G stain different cells in the spleen as expected



Spleen 56 slide 15; Reg2 X8Y6Y7; 08.02.2018

The good image segmentation on spleen using the current Akoya software enables quantitative analysis at single cell level

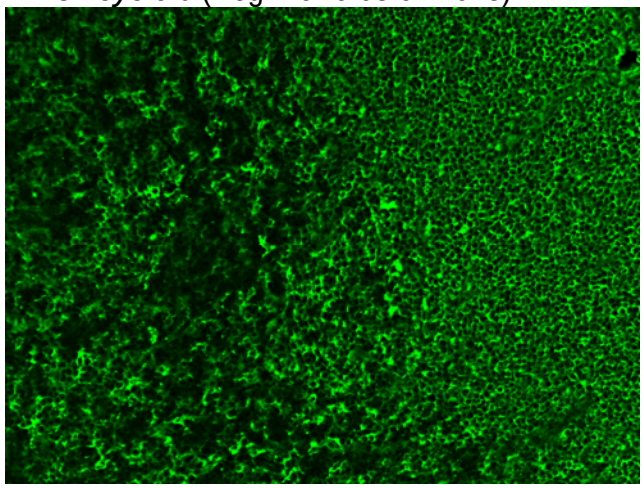




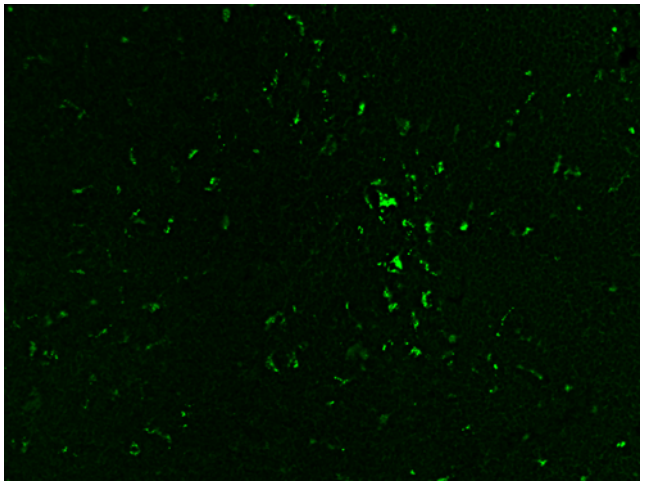
The specific signal is washed off with DMSO

Fam channel

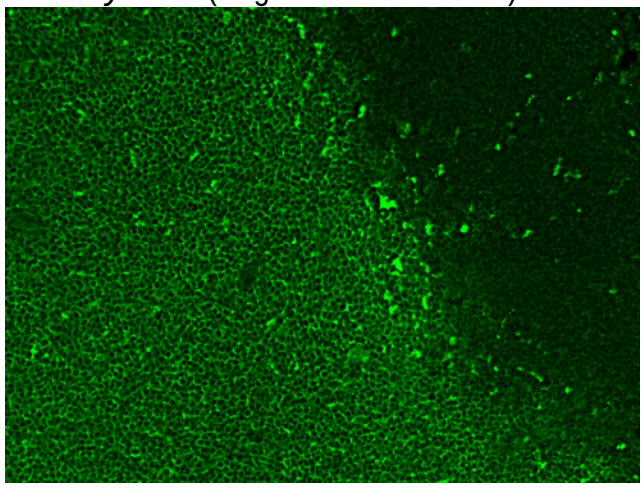
MHCII *cycle 6* (Reg1X9Y9 08.02.2018)



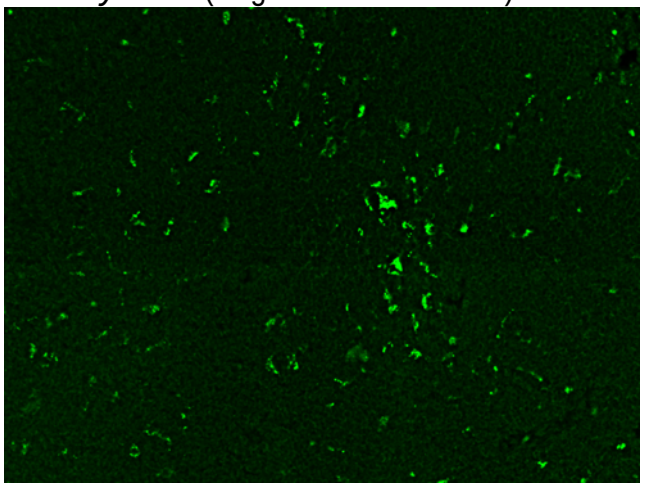
blank *cycle 7* (Reg1X9Y9 08.02.2018)



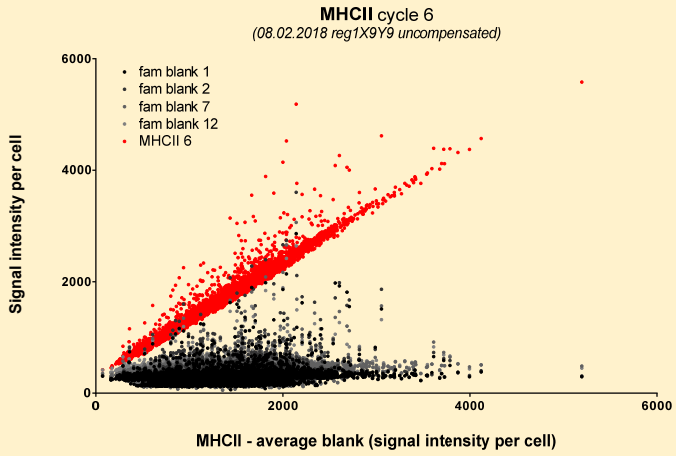
CD90 *cycle 11* (Reg1X9Y9 08.02.2018)

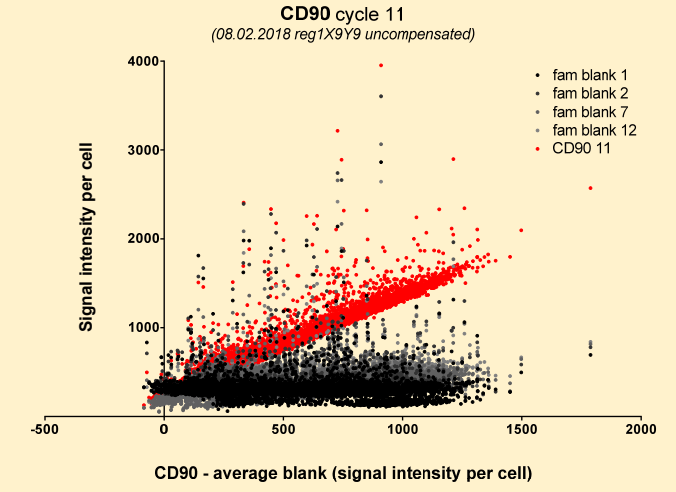


blank *cycle 12* (Reg1X9Y9 08.02.2018)



Separation of specific signal from background

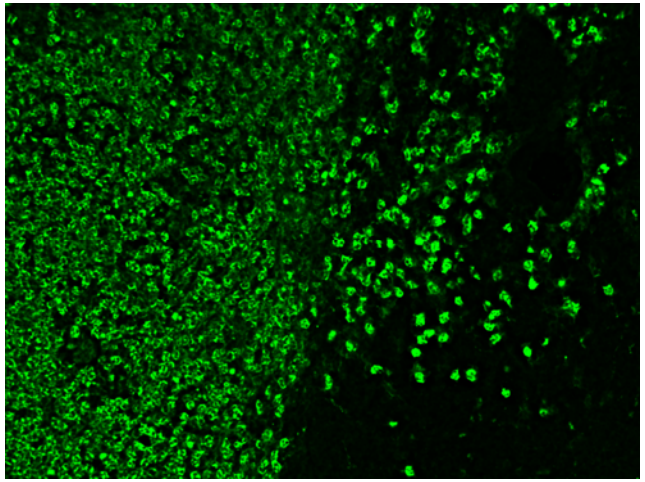




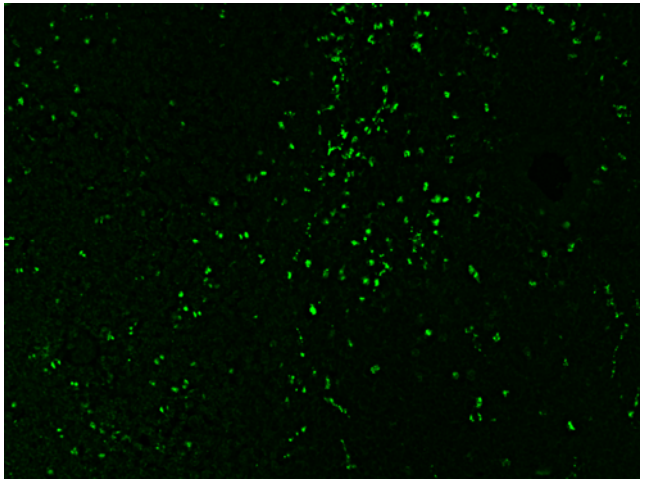
CY3 channel

The specific signal is washed off with DMSO

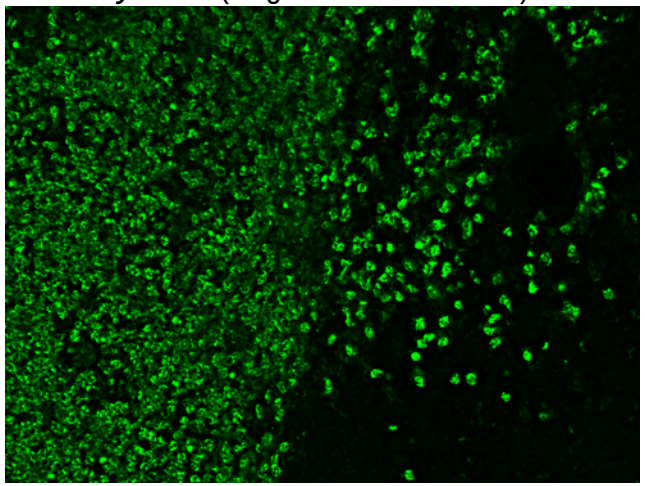
CD11b *cycle 6* (Reg1X8Y9 08.02.2018)



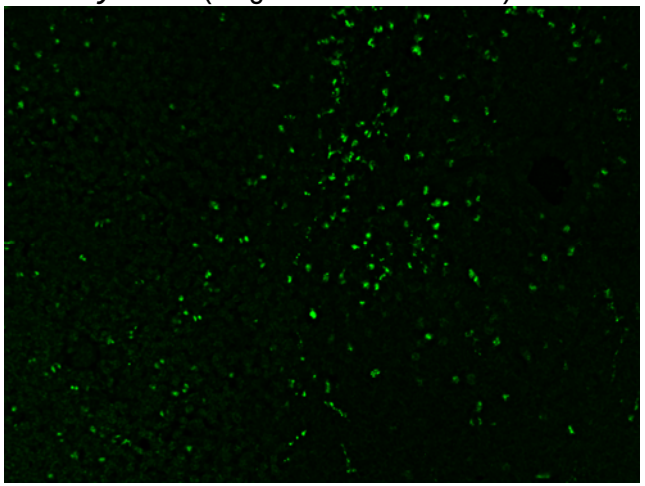
blank *cycle 7* (Reg1X8Y9 08.02.2018)



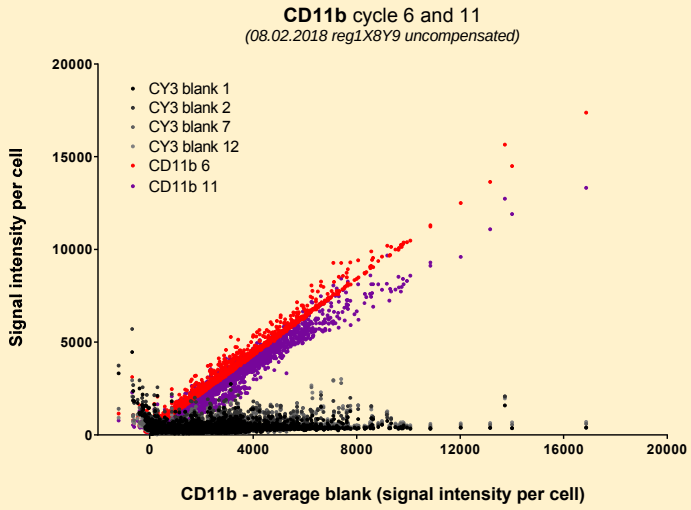
CD11b *cycle 11* (Reg1X8Y9 08.02.2018)



blank *cycle 12* (Reg1X8Y9 08.02.2018)



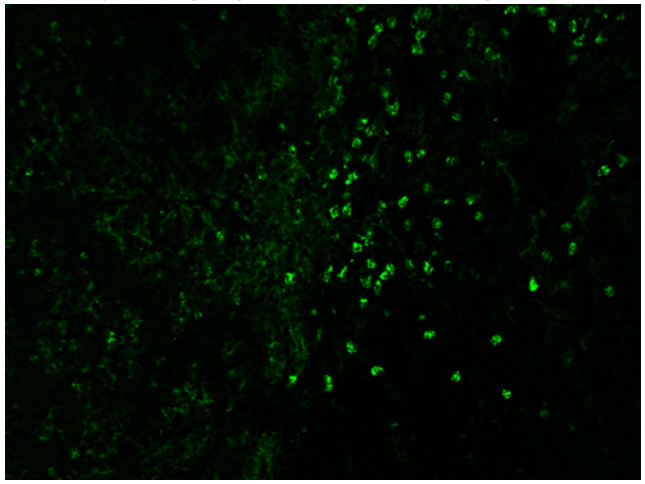
Separation of specific signal from background



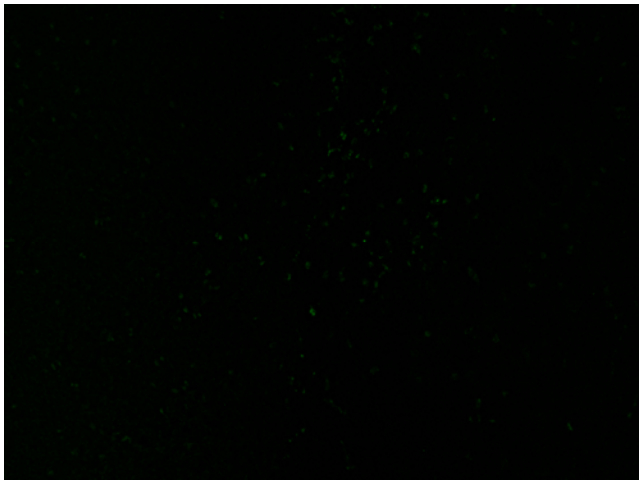
The specific signal is washed off with DMSO

CY5 channel

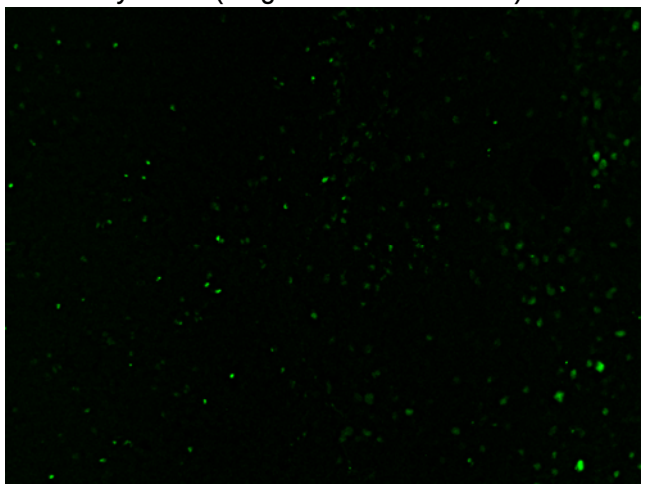
F4/80 cycle 6 (Reg1X8Y9 08.02.2018)



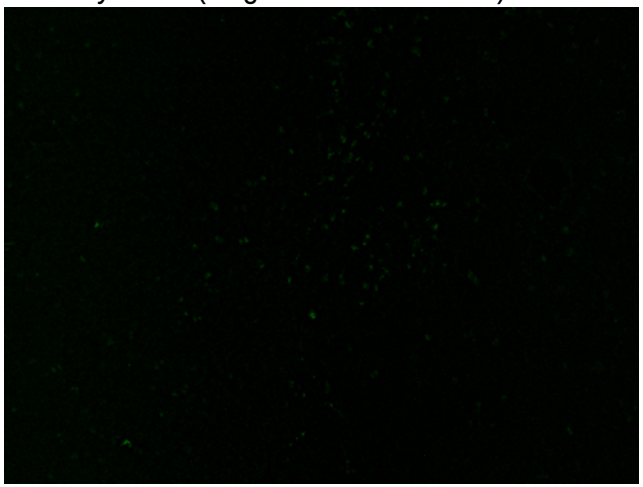
blank cycle 7 (Reg1X8Y9 08.02.2018)



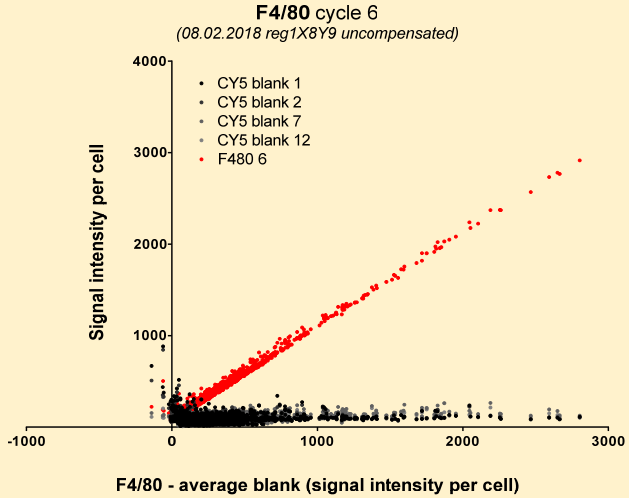
CD11b cycle 11 (Reg1X8Y9 08.02.2018)

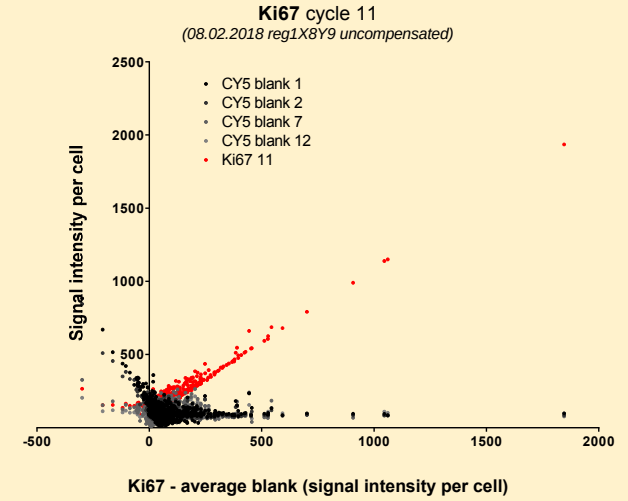


blank cycle 12 (Reg1X8Y9 08.02.2018)

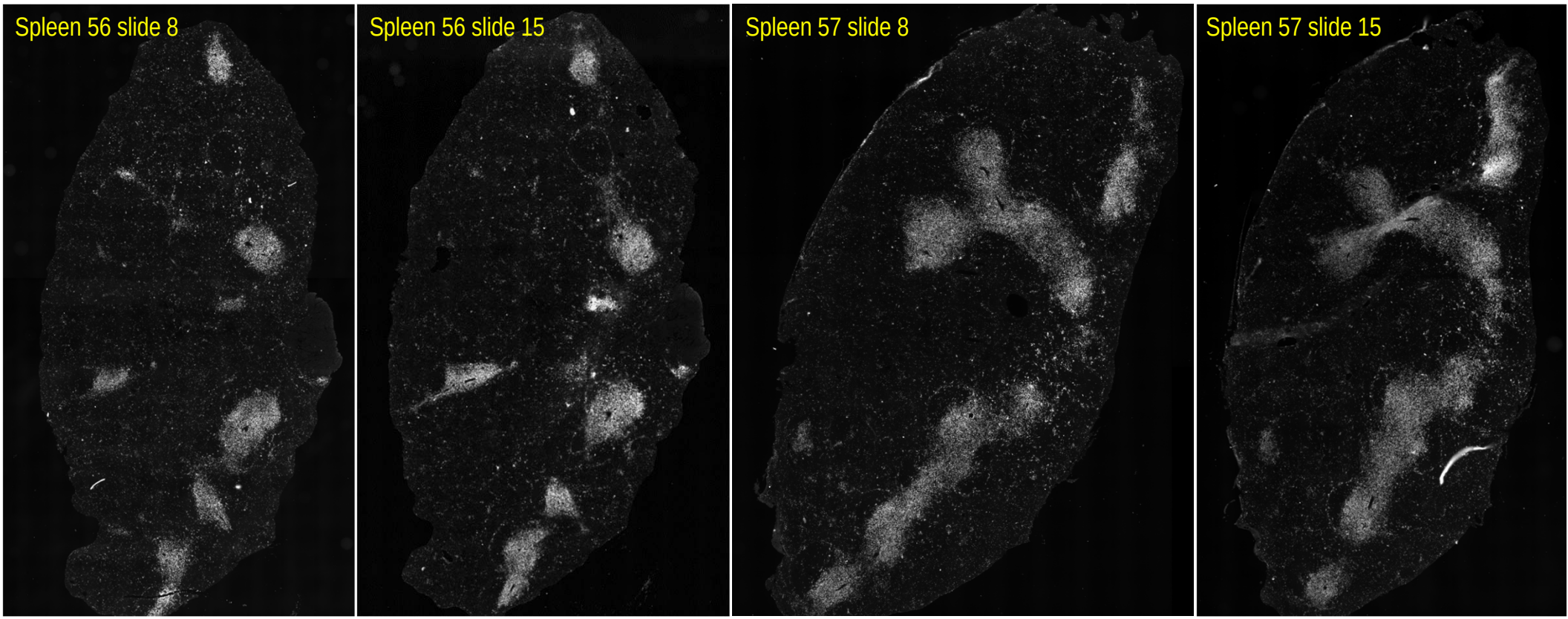


Separation of specific signal from background

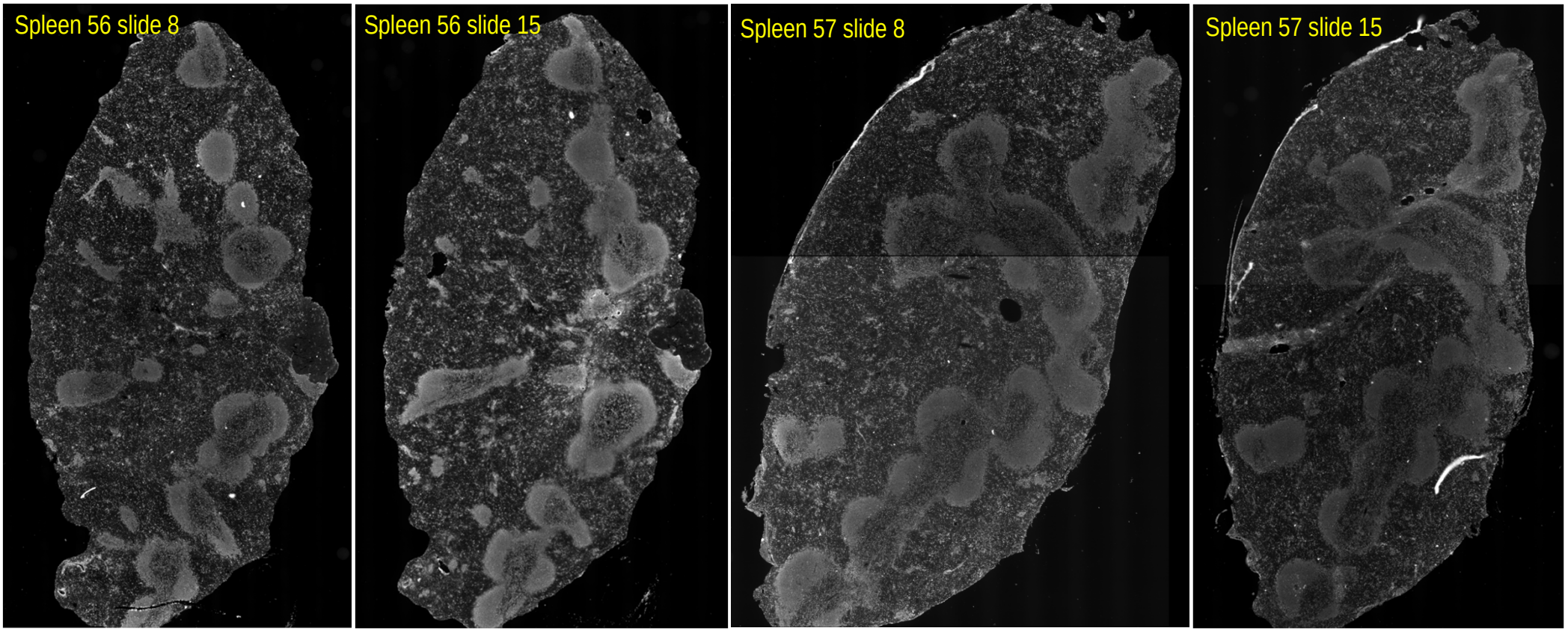




Reproducibility of staining between different spleen samples: CD8A



Reproducibility of staining between different spleen samples: MHCII



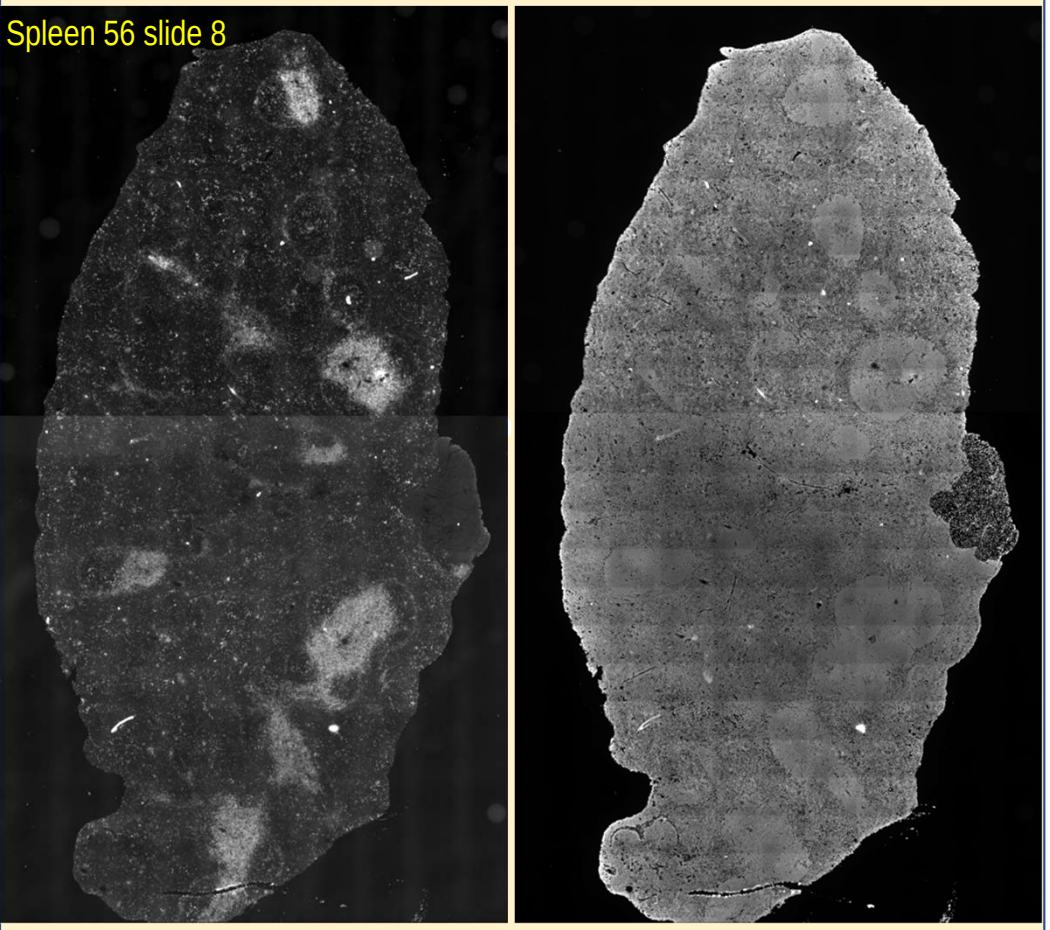
Similar CD4 staining pattern for CODEX and immunofluorescent staining (macroscopic comparison)

CODEX

CD4

DAPI

Spleen 56 slide 8



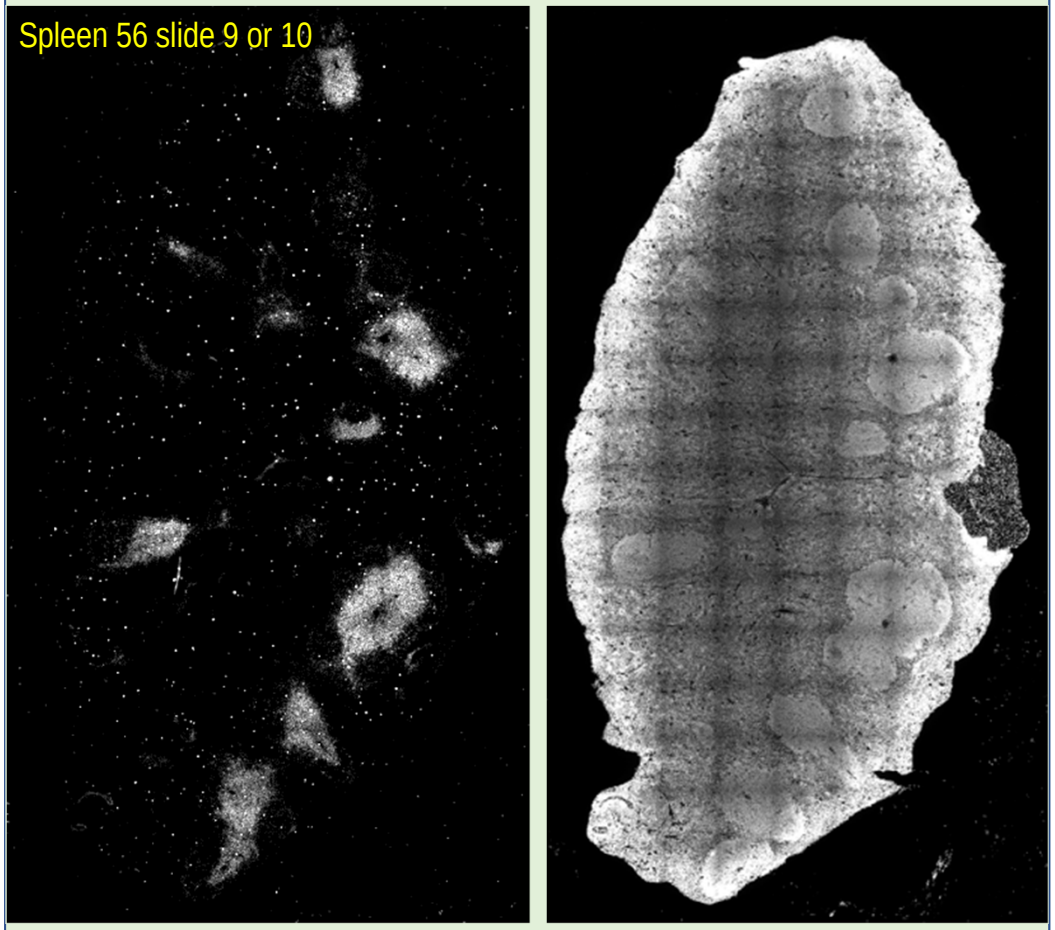
Immunofluorescent staining

Using CD4 RM4-RUO AF488 BD Bioscience antibody (same clone as CODEX)

CD4

DAPI

Spleen 56 slide 9 or 10

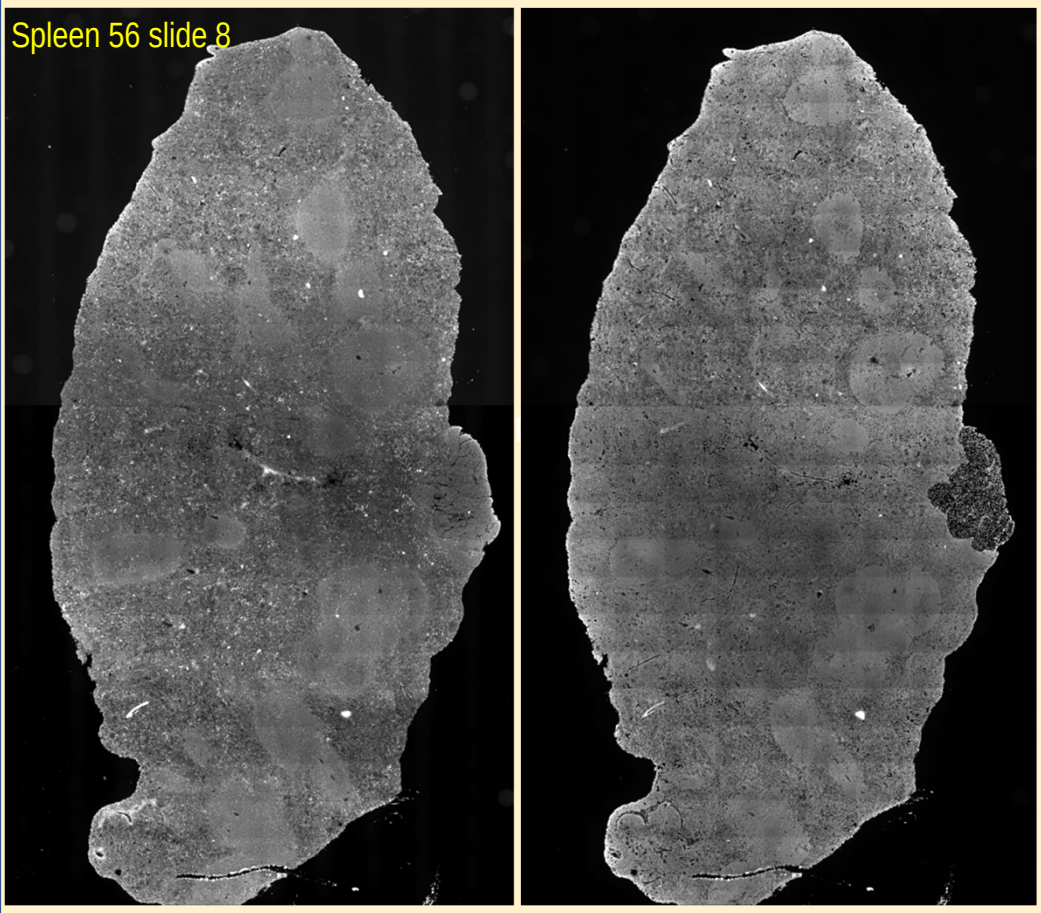


Staining performed by David Scheiblin (OMAL) and imaged with widefield microscope

Comparable CD45 staining pattern for CODEX and immunofluorescent staining (macroscopic comparison)

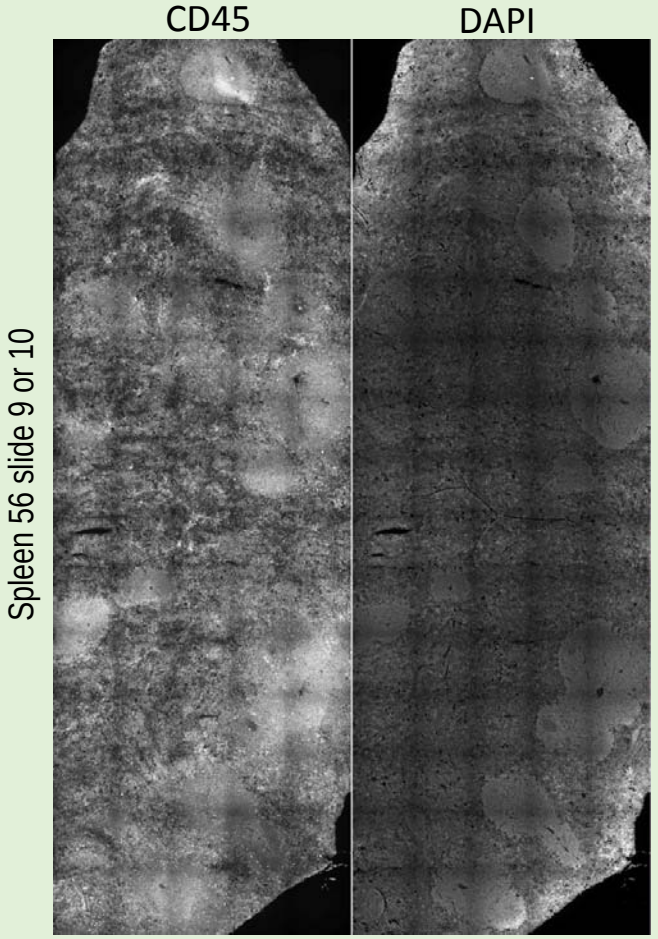
CODEX

CD45 DAPI



Immunofluorescent staining

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



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,

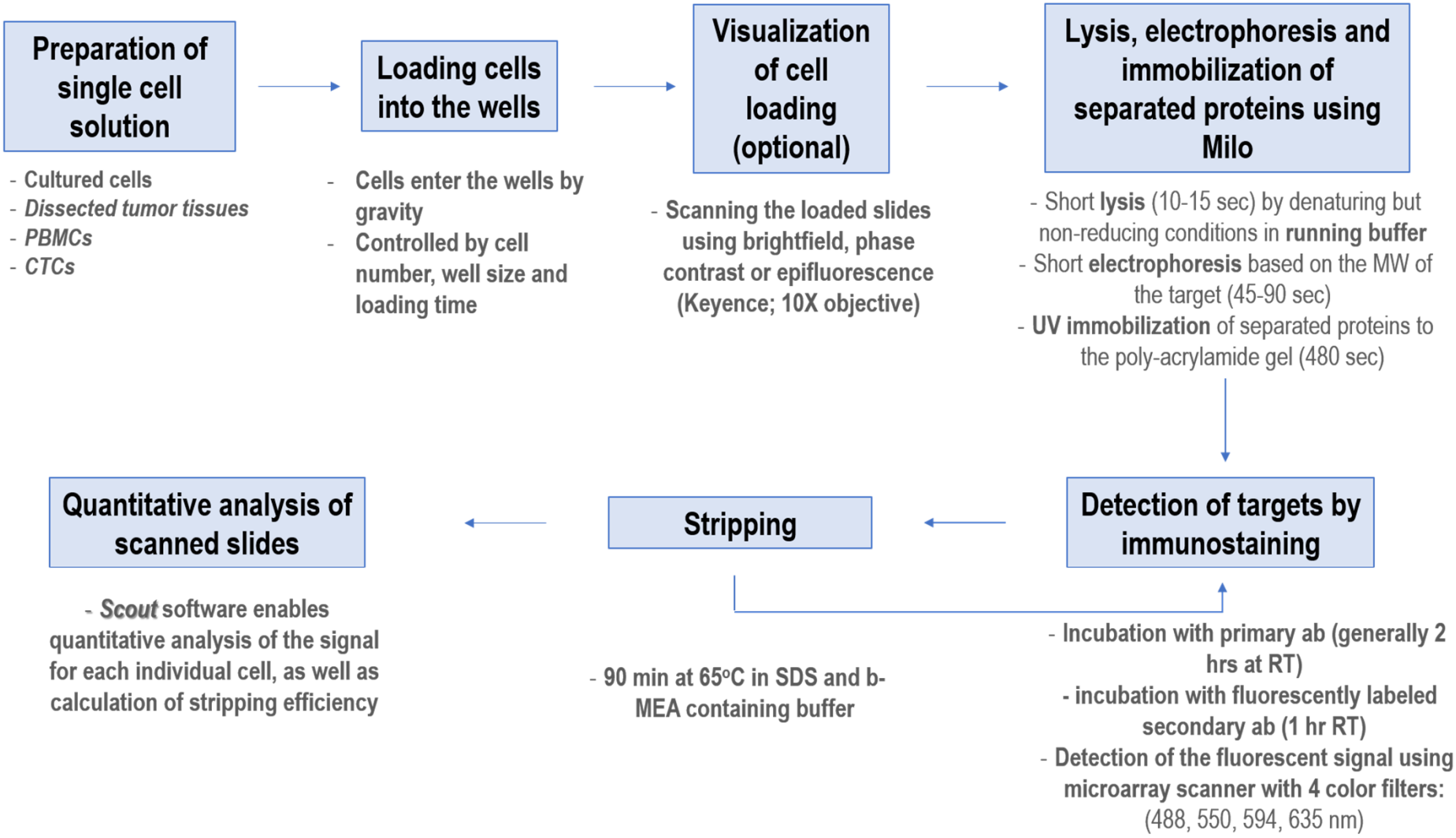
3.) measure activation of intracellular signaling pathway — including phosphorylated targets or transcription factors;

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

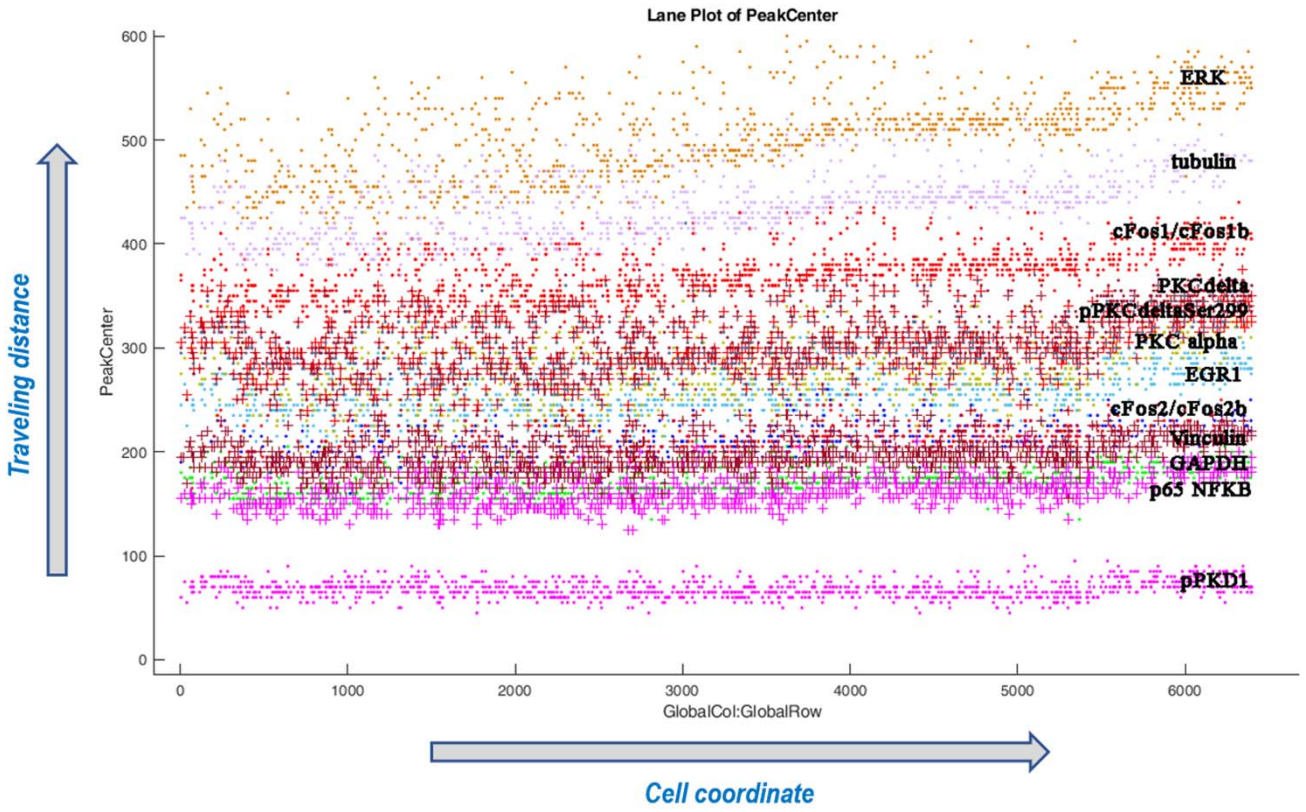


Some project examples

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

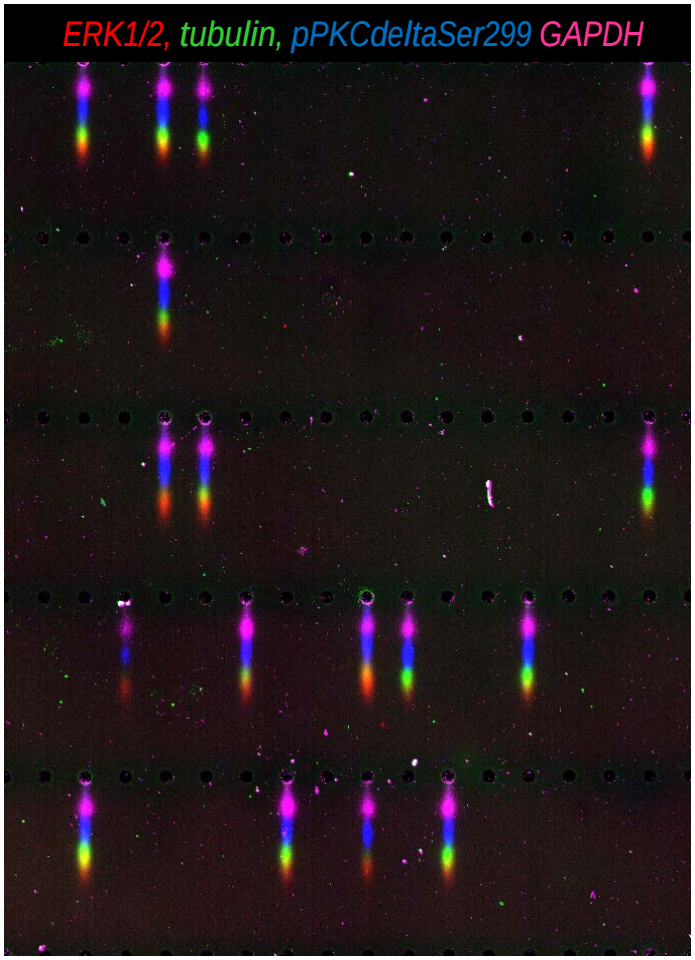
Successful separation and detection of 12 targets after multiple stripping steps using 4 color detection

#	Target	Characteristics	Antibody species
1.	cFos	Newly synthesized protein	mouse
2.	EGR1	Newly synthesized protein	Rabbit
3.	GAPDH	Housekeeping/loading control	Goat
4.	Beta Tubulin	Loading control	Mouse
5.	pPKCdeltaSer299	Phosphorylation/activation	Rabbit
6.	ERK total	Loading control	Mouse
7.	pPKD1	Phosphorylation/activation	Rabbit
8.	PKC alpha	Target protein	Rabbit
9.	PKC delta	Target protein	Rabbit
10.	cFos	Newly synthesized protein (repeat)	Rabbit
11.	P65 NFKB	Transcription factor	Mouse
12.	PKC delta	Target protein (repeat)	Rabbit
13.	Vinculin	Loading control	Mouse

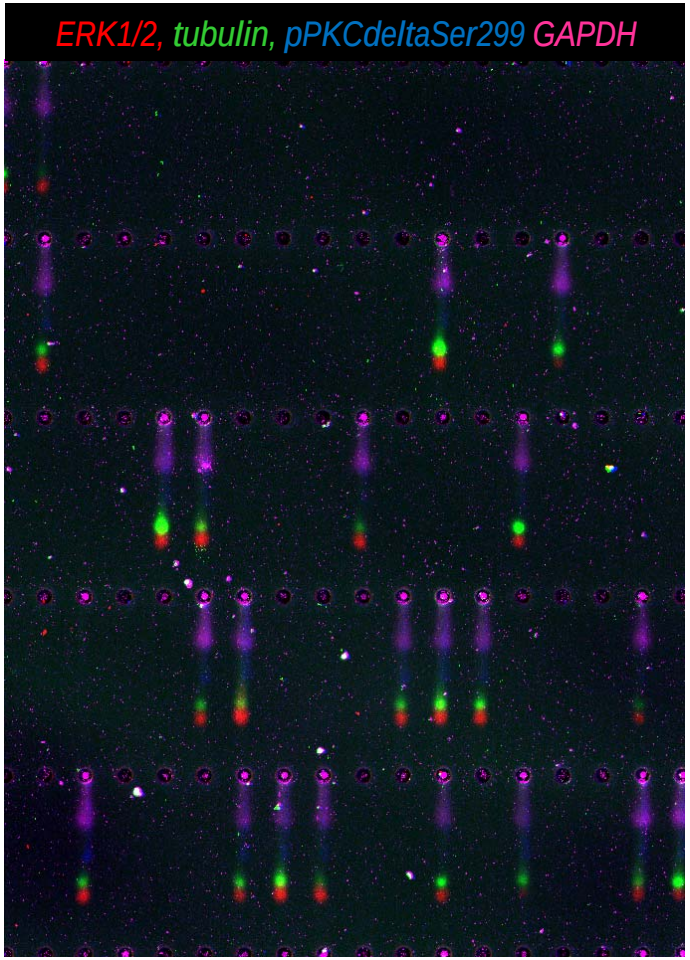


Good separation of 4 targets with 40-130 kDa molecular weight range

Representative composite images

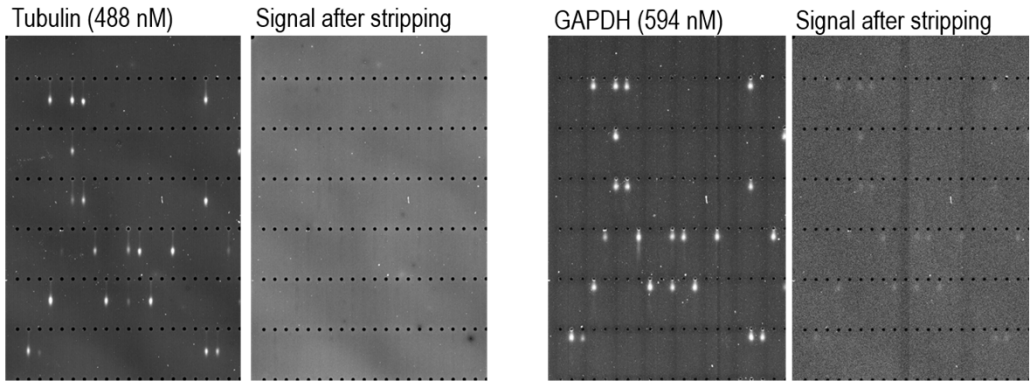


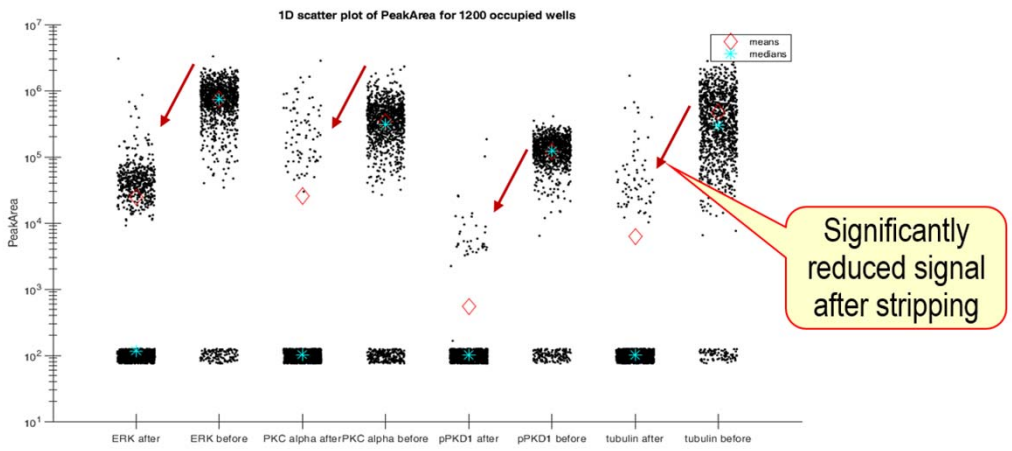
Treated LNCaP cells (PMA 1000 nM 60 min)



Control

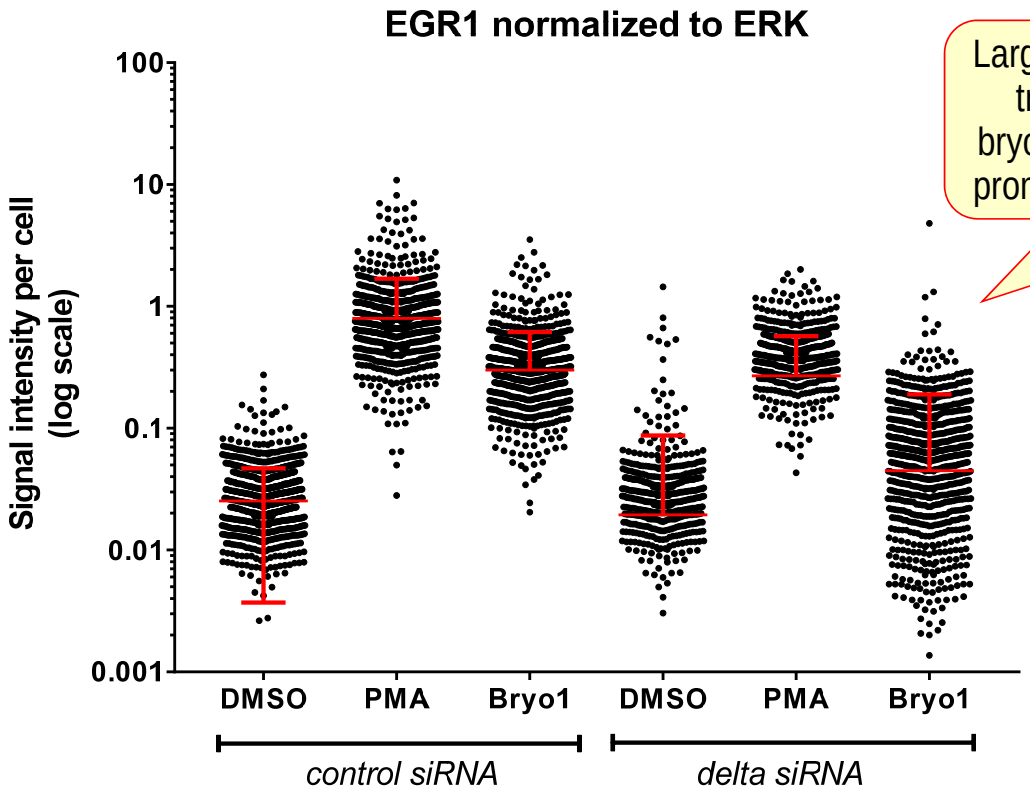
Stripping efficiency for different signals





Target	Wavelength (nm)	Median stripping efficiency (%)	95% of signal is between %-% efficiency
GAPDH	594	93.1	77.3 - 103.3
EGR1	635	88.9	80 - 95.3
Tubulin	488	100	77.1 - 128.3
pPKCdelta Ser299	635	89.8	74.9 - 111
ERK	635	96.4	80.9 - 109.4
pPKD1	532	100.6	76.5 – 110.9
PKC alpha	488	99.8	-11.2 – 129.1
PKC delta	532	96.7	6.7 - 110.0
P65 (mouse)	635	94.2	24.0 – 102.5
Vinculin (mouse)	532	101.8	65.3 - 108.5
PKC delta b	635	93	72.4 – 109.1
Cyclin A (rabbit)	635	90.4	68.1 - 117.7

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

Procedure for getting access to the single cell western 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
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;
   - Supporting data: it helps us understand the project better
   - Proposed experiments: think about experiments in phases
     - 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: CPTR with or without the investigator
   - **Immunostaining, scanning** the signal, **stripping**, **re-staining**: CPTR and/or the investigator depending on manpower and the project
   - **Data analysis** using Scout 2.0: CPTR 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 CPTR

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