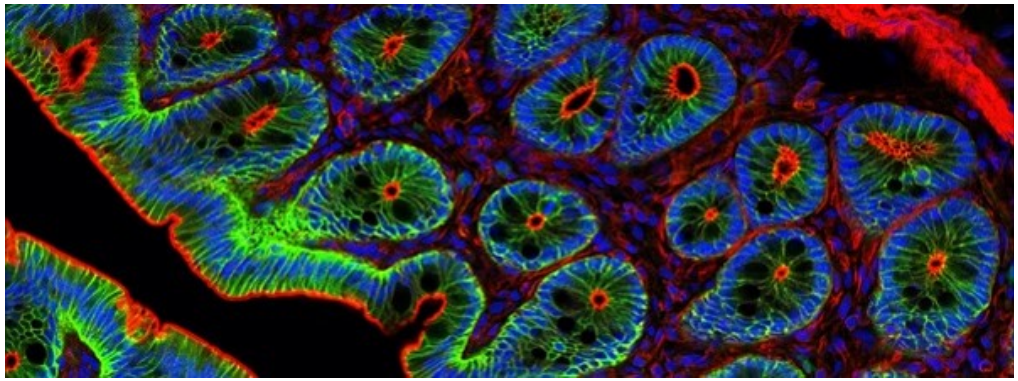
How Specific is Your Image?

Edward Verwayen

November, 2014



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Agenda

Part 1:

Introduction to Cell Signaling Technology

Introduction to immunofluorescence (IF)

Antibodies for IF: The importance of validation

Antibodies and products for IF

Part 2:

Studying cellular events: Protocol optimization

Troubleshooting and technical support

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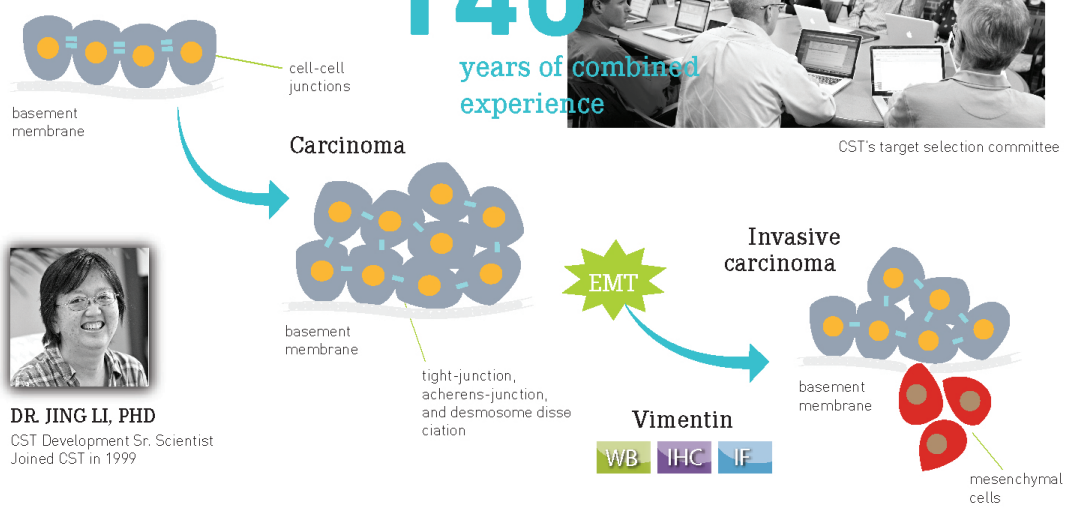
Target Selection and Initial Development

STEP 1

Target Selection:
Recognition of the Scientific Community's Need



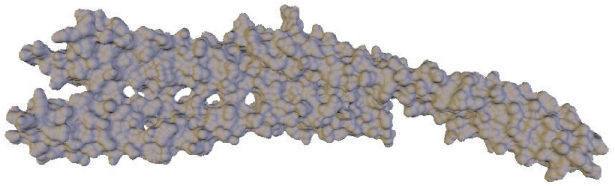
DR. JING LI, PHD
CST Development Sr. Scientist
Joined CST in 1999

140
years of combined experience

CST's target selection committee

STEP 2

Antigen Design & Clonal Expression



This 3-D molecular model of Vimentin can be viewed on Sketchfab.com

900+
clones screened by ELISA

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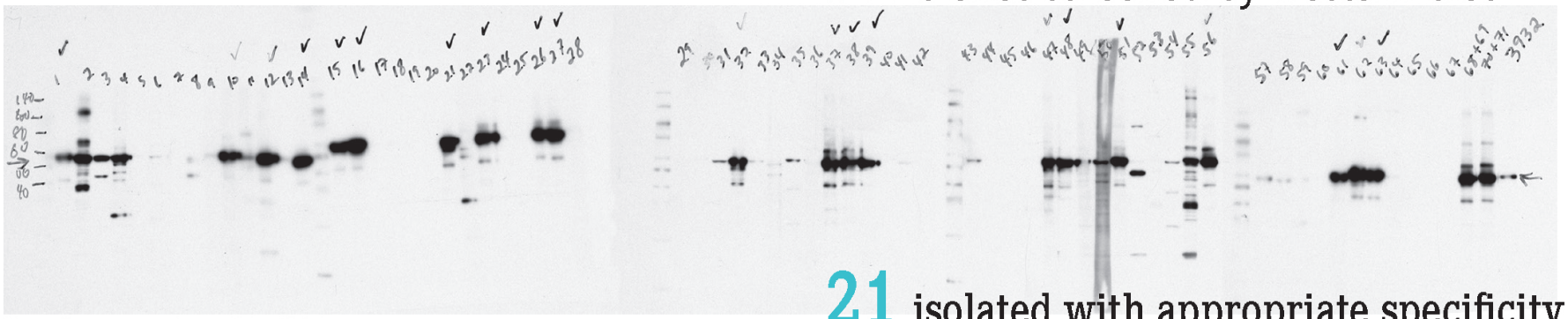
Screening for target specificity

STEP 3

Screening for Target Specificity



Western blots to assess the specificity of 71 clones.

71 clones screened by western blot

21 isolated with appropriate specificity

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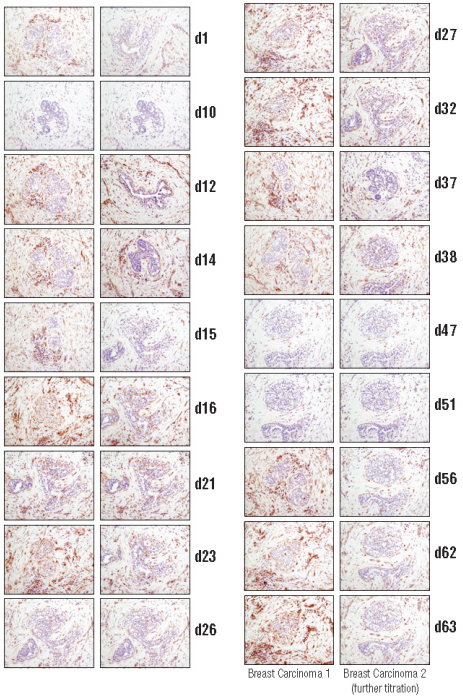
Screening for other applications

STEP 4

IHC Screening for Localization



21 clones screened by IHC and ICC

4 clones isolated with robust, clean and specific staining

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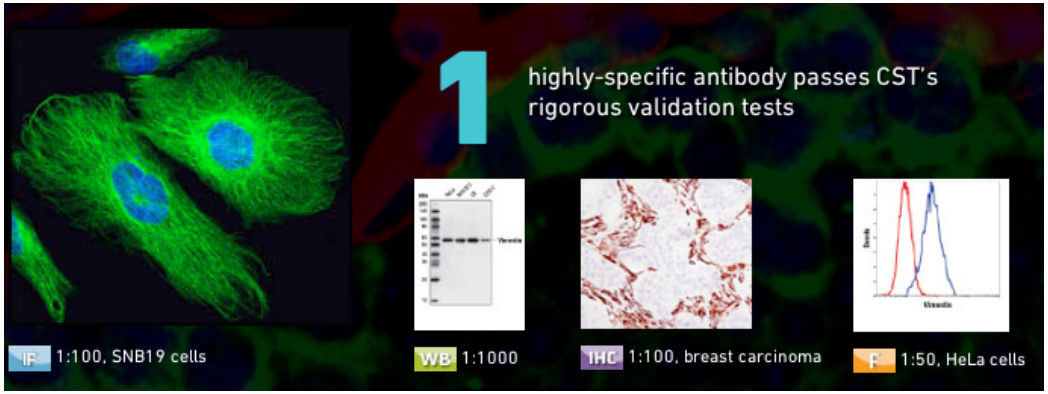
Final lot testing

STEP 5

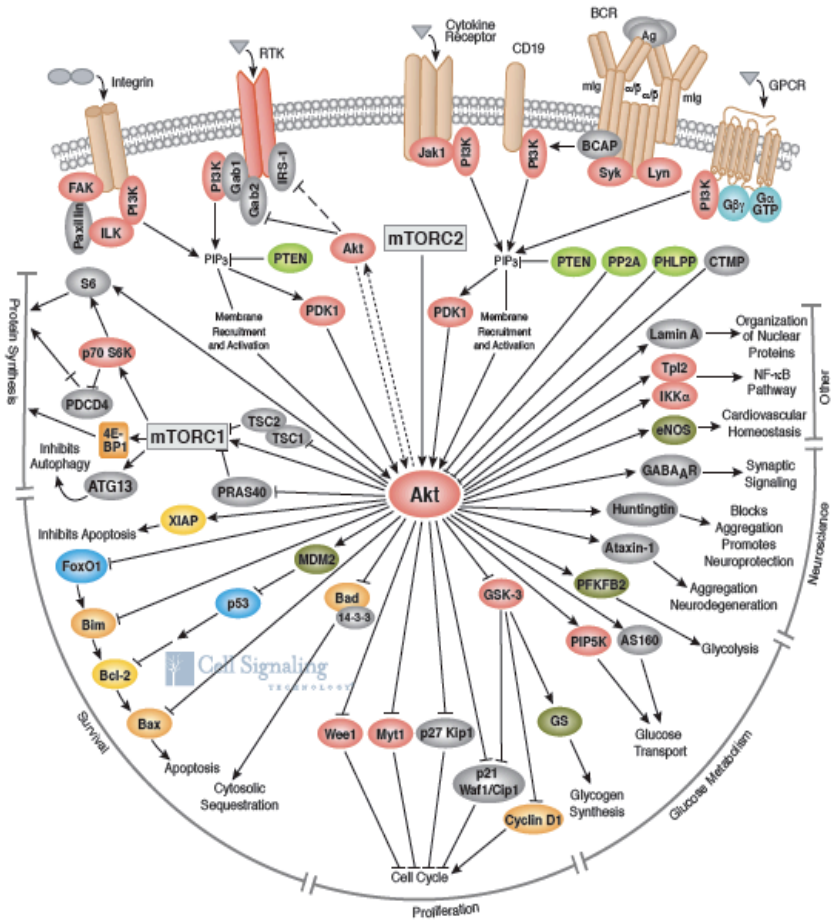
Final Lot Testing

1 highly-specific antibody passes CST's rigorous application validation tests

- ✓ WB Western Blotting
- IP Immunoprecipitation
- ✓ IHC Immunohistochemistry
- ✓ IF Immunofluorescence
- ✓ F Flow Cytometry
- ChIP Chromatin IP



Cell Signaling Technology™ Products



- Total and activated state polyclonal and monoclonal antibodies, secondary, conjugated and motif antibodies
- Antibody kits for WB, IF, ELISA, ChIP…
- Companion reagents
- Services: carrier-free, custom-formulated antibodies, bulk and proteomics services

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Immunofluorescence

An introduction

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What is immunofluorescence (IF)

IF can be performed using different starting materials

IF-IC

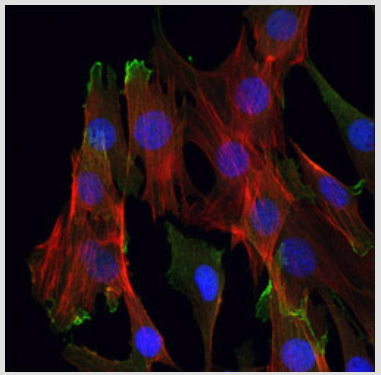
- Historically, immunocytochemistry (IC or ICC)
- Cultured cells, either adherent or in suspension

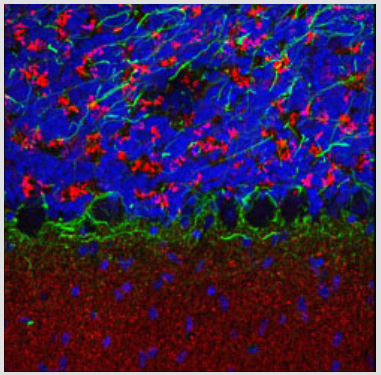
IF-F

- Frozen biological tissues
- Superior antigen preservation compared to IF-P

IF-P

- Paraffin embedded biological tissues or cell pellets
- Simple storage, superior tissue morphology





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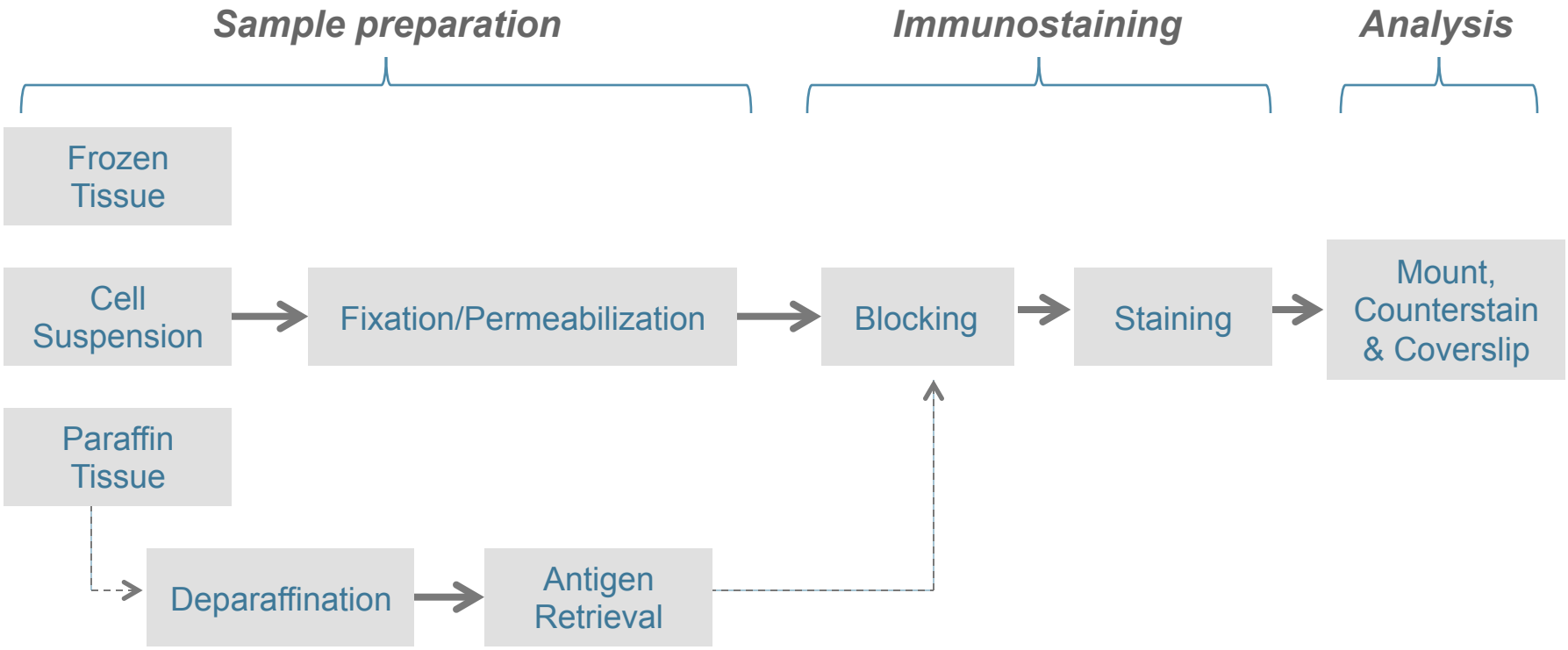
How does immunofluorescence work?



http://www.cellsignal.com/support/protocols

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Antibodies for IF

The importance of validation

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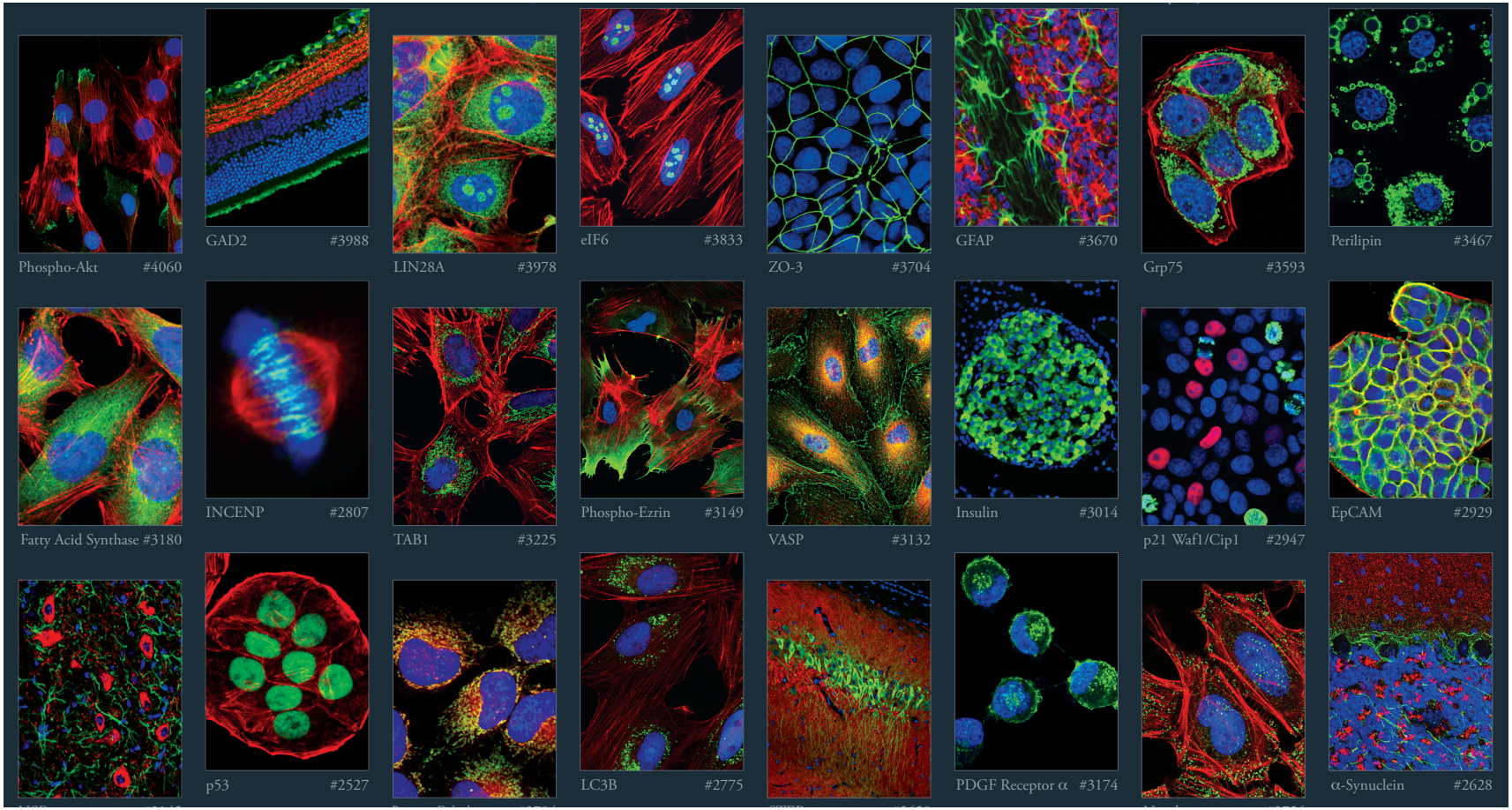
The beauty of high quality IF images



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The importance of antibody validation

Western blot functionality is no indication for antibody binding for other techniques, especially IHC or IF, where the antigen is in its native conformation

- Several publications addressing this issue:
  - Bordeaux *et al.*, Biotechniques, 48, 2010
  - Welsh *et al.*, Clin Cancer Res, 18, 2012
  - Blow, Biotechniques, 2013
- The keys to good validation:
  - Correct use of controls
  - Test antibody reproducibility
  - Test correct tissue expression and localization

All CST™ antibodies are validated for each relevant technique to help you achieve the best possible results

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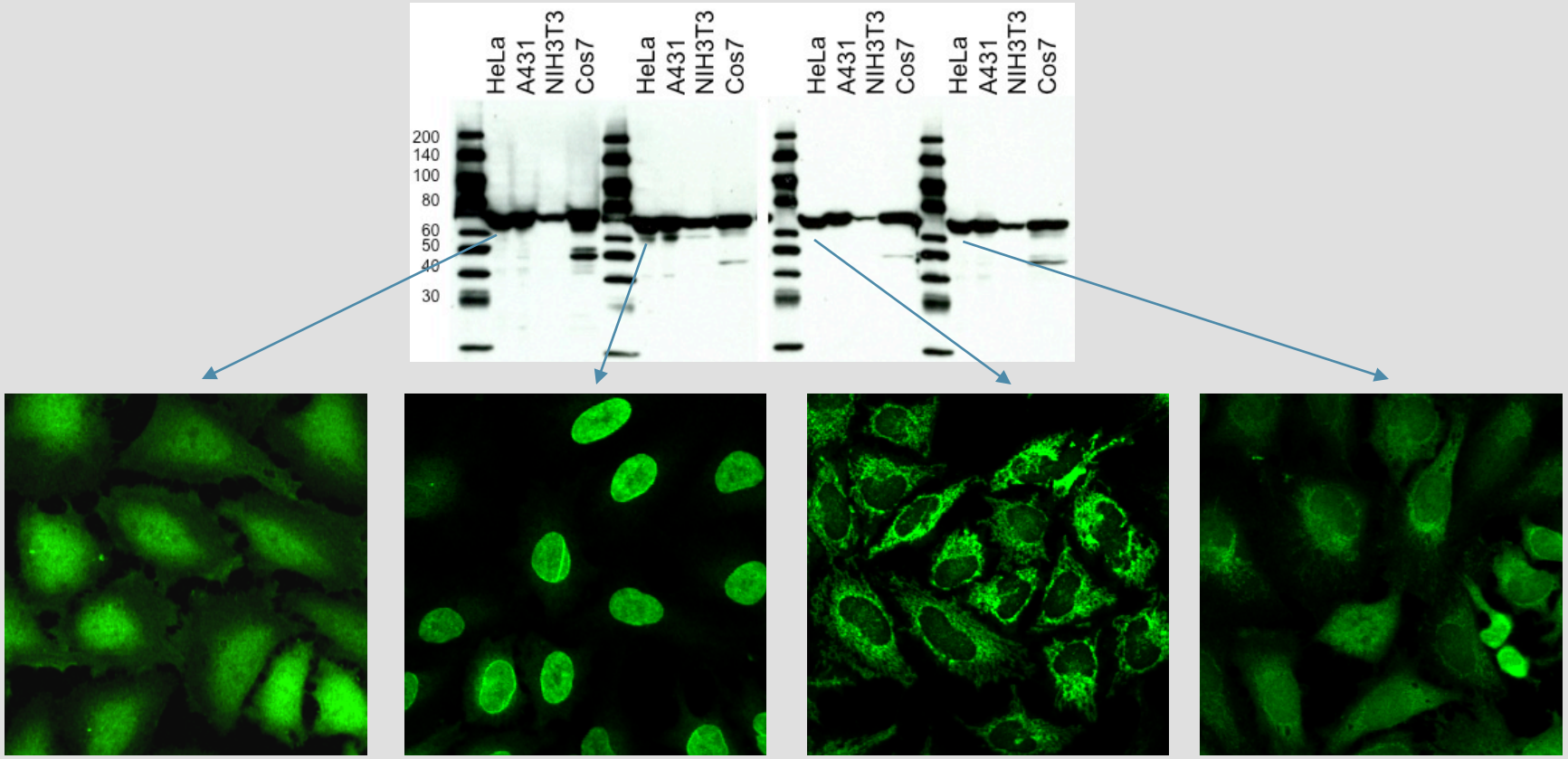
Validation in IF: Testing in multiple applications



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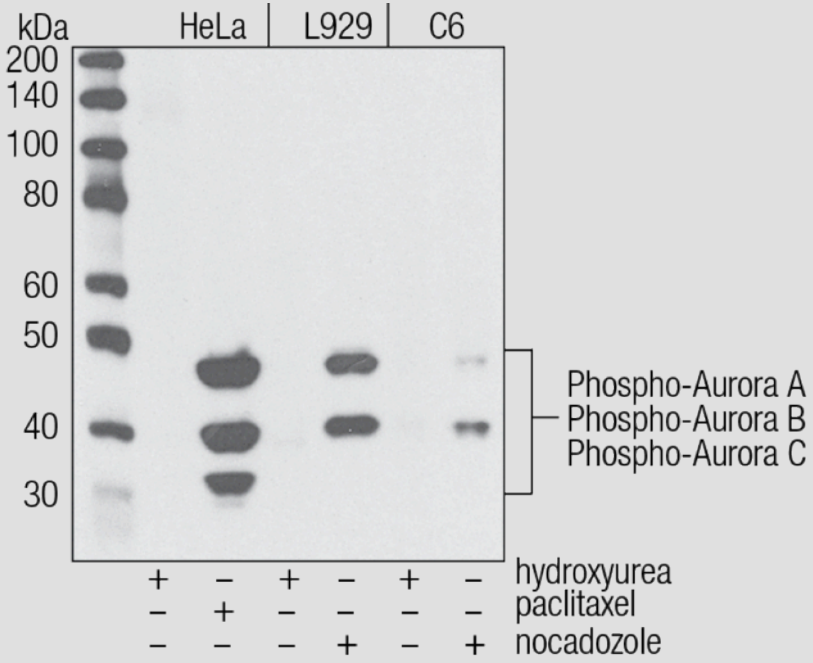
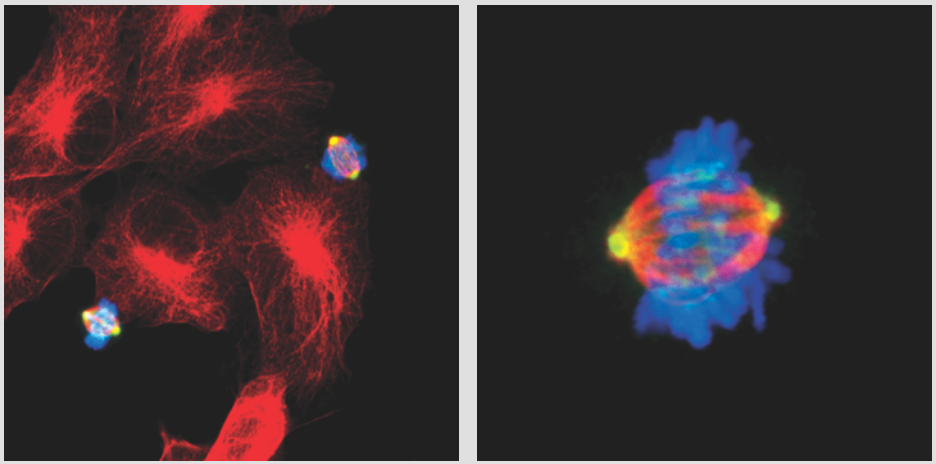
Antibody validation: Strong testing models

- **Comparison with Western Blot**



Phospho-Aurora A (Thr288)/Aurora B (Thr232)/Aurora C (Thr198) (D13A11) XP® Rabbit mAb #2914



GREEN = Phospho-Aurora A (Thr288)/Aurora B (Thr232)/ Aurora C (Thr198) (D13A11) XP® Rabbit mAb #2914

RED = β-Tubulin #2116

BLUE = Phospho-Histone H3 (Ser10) (6G3) Mouse mAb #9706

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CST validation experiments

Combination of several experiments to provide the highest quality and most thoroughly tested antibodies

- Test on positive and negative cells and tissues
  - siRNA treatment (knockdown models)
  - Phosphatase treatment
  - Knockout mouse models
- Verification of changes in expression, activation, or subcellular localization in response to known treatments
- Optimization of dilution, buffers and protocols
- Comparison of staining pattern to other well-established clones against the same target

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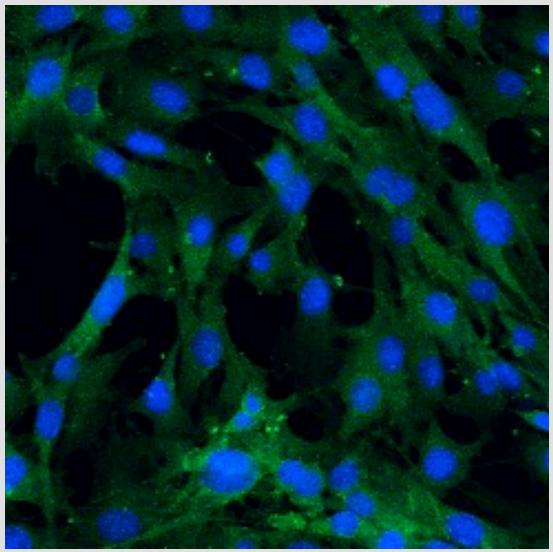
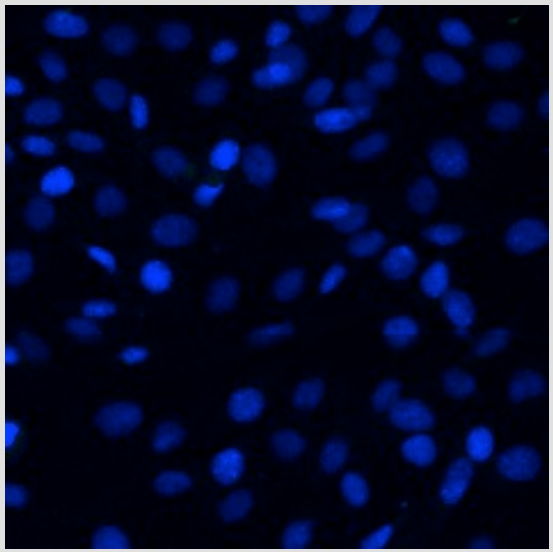
Validation in IF: Antibody specificity

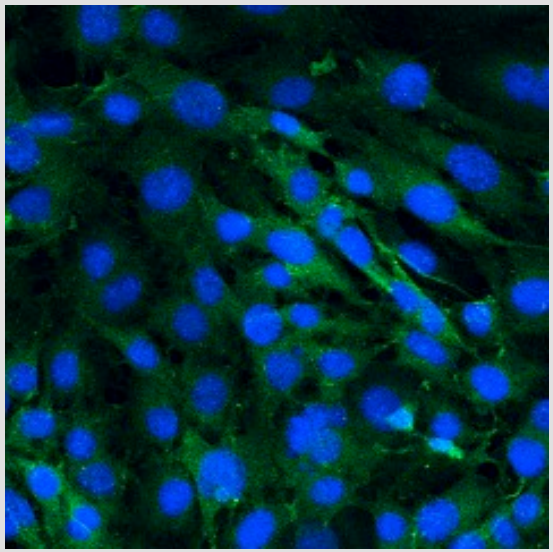
- **Use of knock out models**



MEF, GSK wild type



MEF, GSK3α KO



MEF, GSK3β KO

GREEN = GSK-3α (D80D1) XP® Rabbit mAb #4818
BLUE = DRAQ5® #4084 (nuclei)

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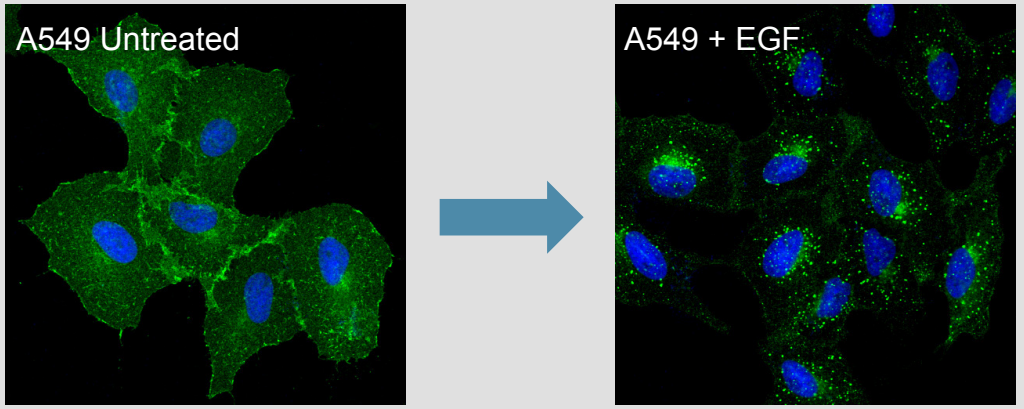
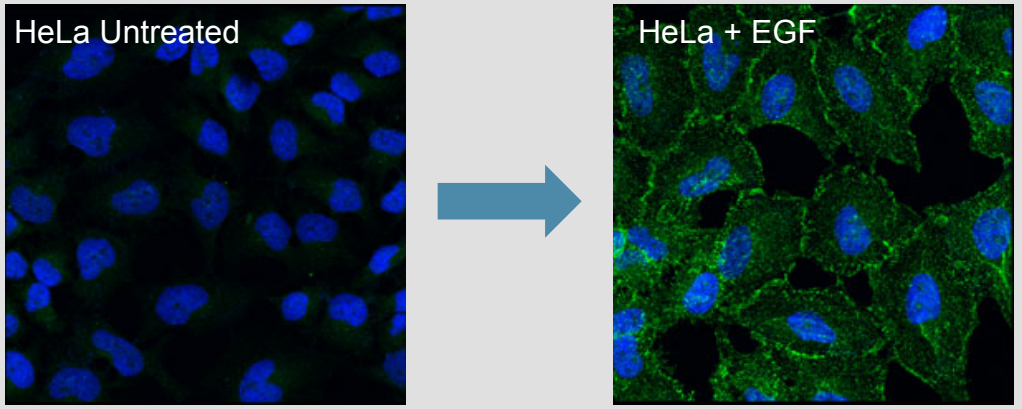
Validation in IF: Activation-state antibody specificity

EGF Receptor (D38B1) XP® Rabbit mAb #4267



Phospho-EGF Receptor (Tyr1068) (D7A5) XP® Rabbit mAb #3777



GREEN = antibody BLUE = DRAQ5® #4084 (nuclei)

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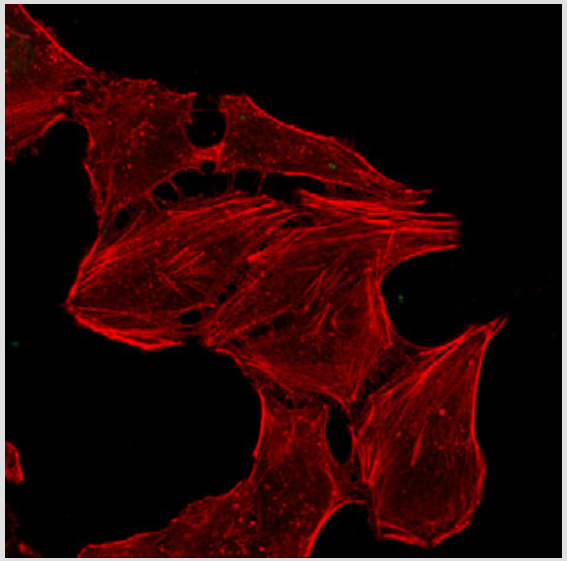
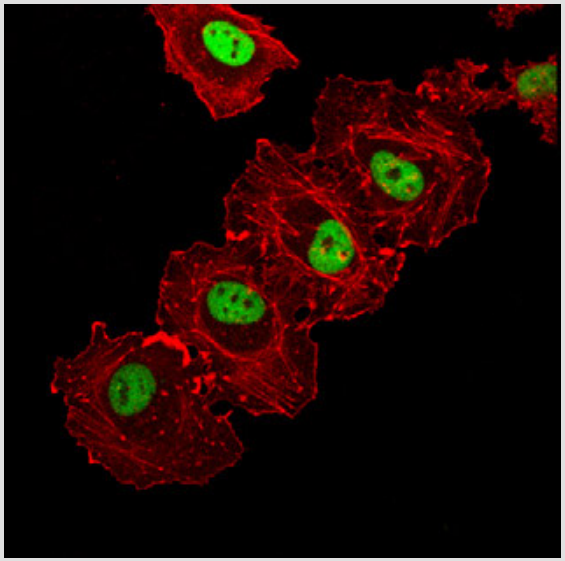
Validation in IF: Antibody specificity

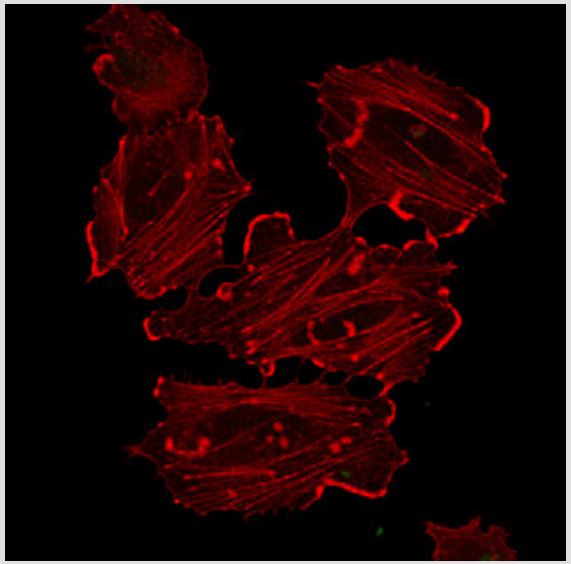
- **Use of activators and inhibitors**



Starved



TPA



TPA + λ-Phosphatase

GREEN = Phospho-c-Fos (Ser32) (D82C12) XP® Rabbit mAb #5348
RED = DY-554 phalloidin (actin filaments)

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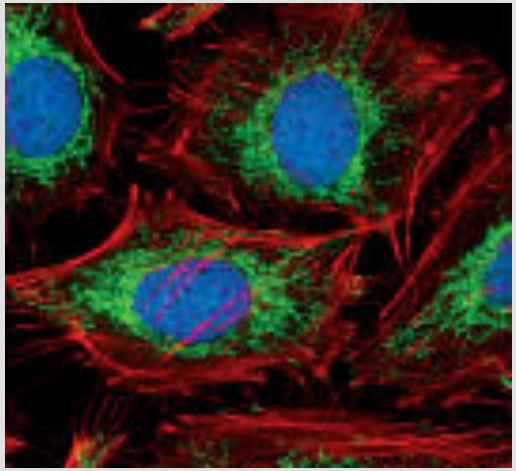
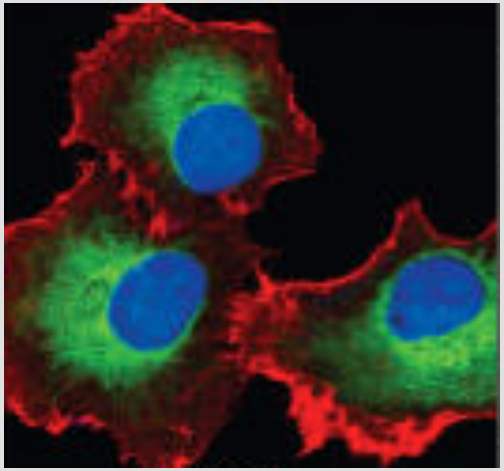
Antibody validation: Verification of subcellular location

Mitochondria



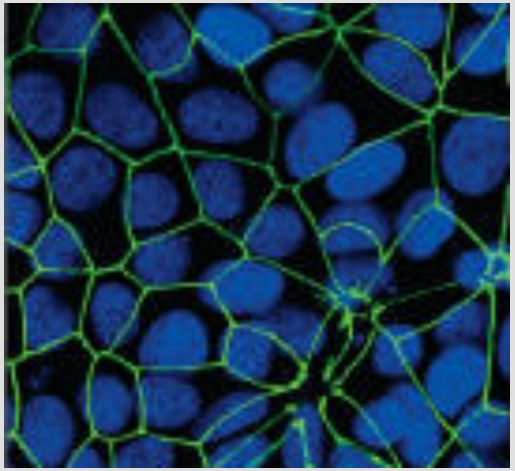
COX IV (3E11) Rabbit mAb #4850

Endoplasmic reticulum



ERp72 (D70D12) XP® Rabbit mAb #5033

Intercellular Junctions



ZO-3 (D57G7) XP® Rabbit mAb #3704

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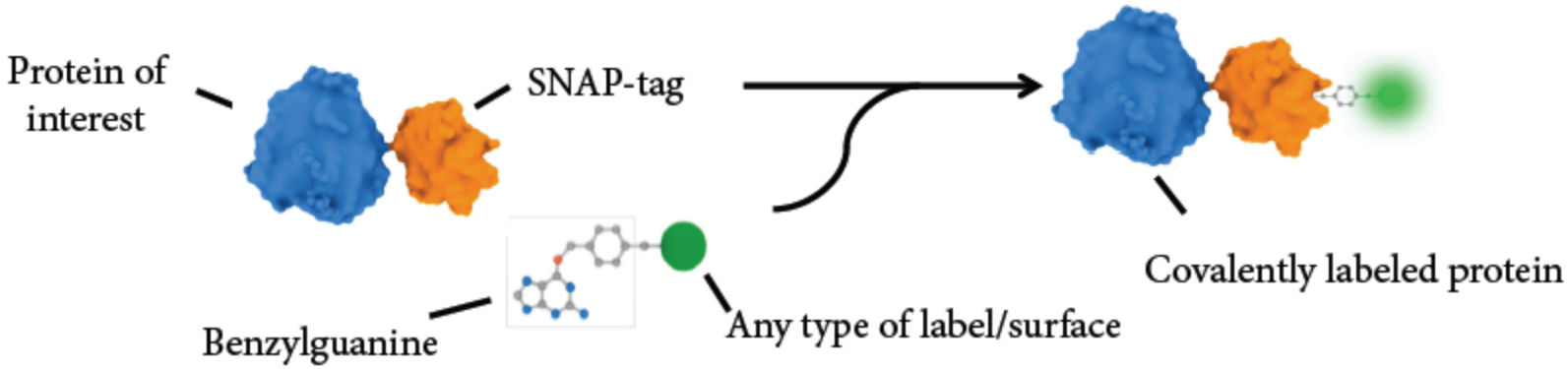
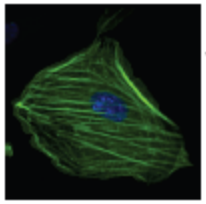
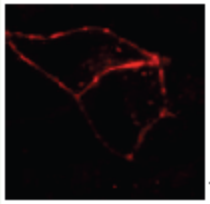
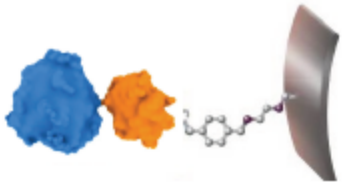
Overview of SNAP-tag Technologies

- **Overview** – fusion protein technology based on unique protein tags that can covalently self-label in a variety of contexts including: in live cells, fixed cells and in cell lysates.

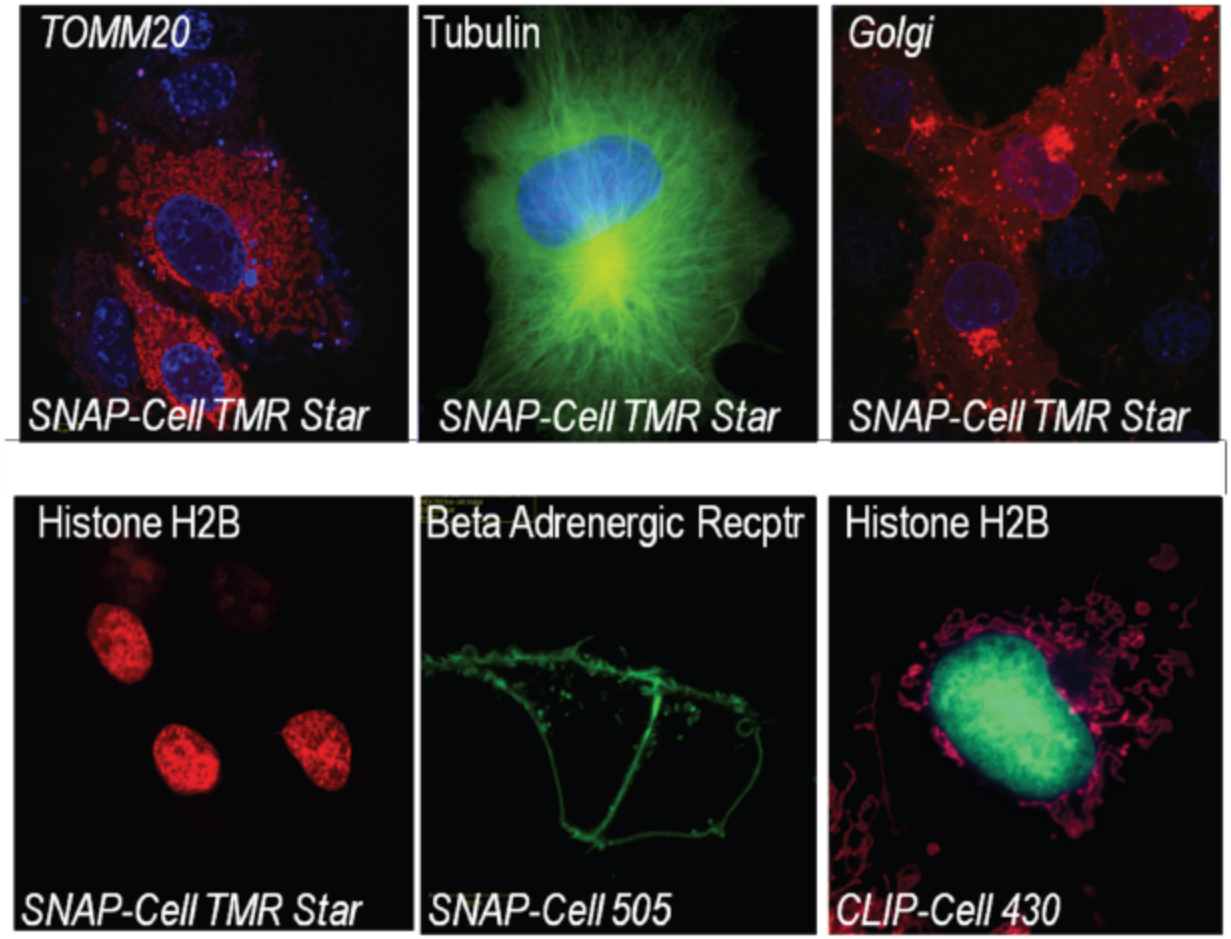
- **Applications** – wide range of validated applications including: cellular imaging, biochemical assays, pull-downs, etc.



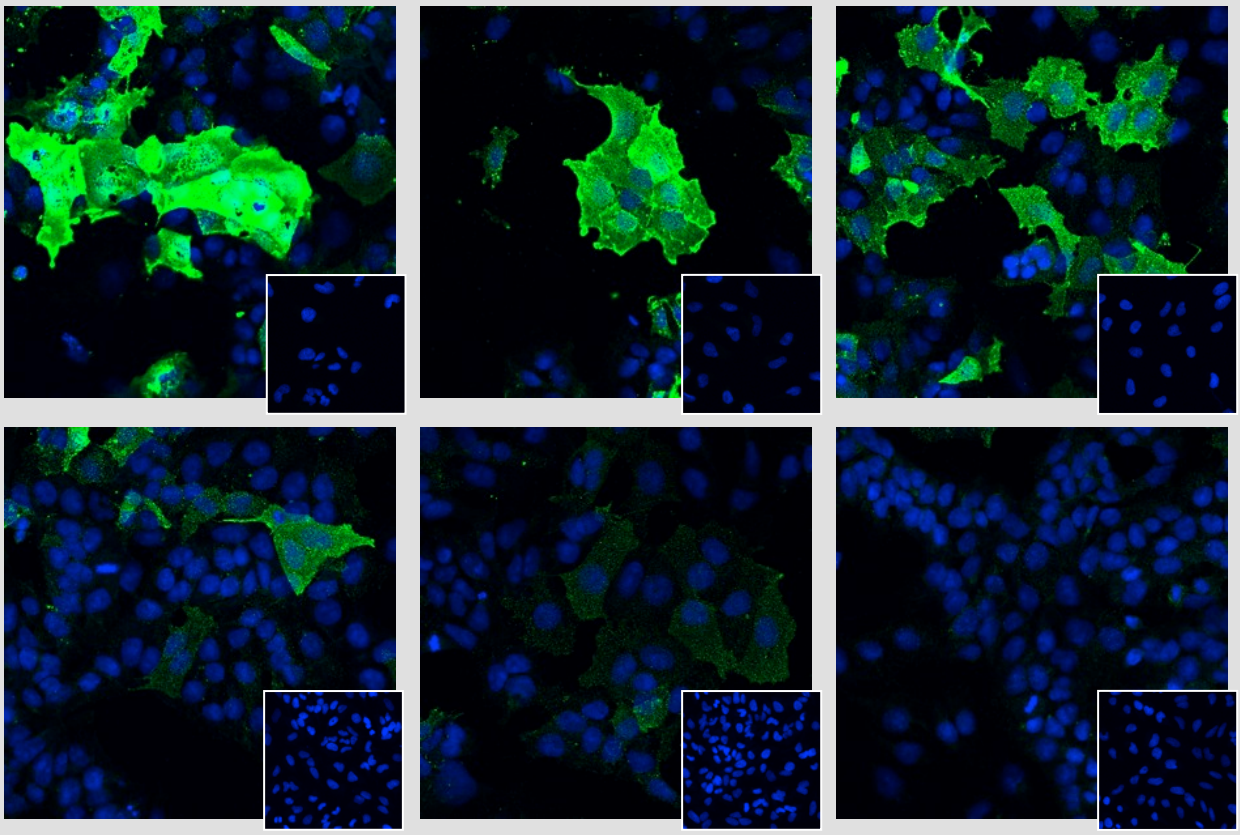
Functionalities "Encoded" by Each Label

"Label"		Product Name	Function/Application	
Cell-permeable fluorophores		SNAP-Cell CLIP-Cell	Visualizing proteins inside or on the surface of living or fixed cells	 *3T3 Cells* *SNAP-Actin* *SNAP-Cell 505*
Non cell-permeable fluorophores		SNAP-Surface CLIP-Surface CoA Labels	Visualizing proteins on the surface of living or fixed cells (ex. receptor internalization)	 *COS7 Cells* *SNAP-ADRβ2* *SNAP-Surface Alexa Fluor® 546*
Magnetic and non-magnetic beads		SNAP-Capture	Protein pull-downs	

Tags Localize Appropriately with Fusion Partner



Protocol optimization: Finding the right antibody dilution



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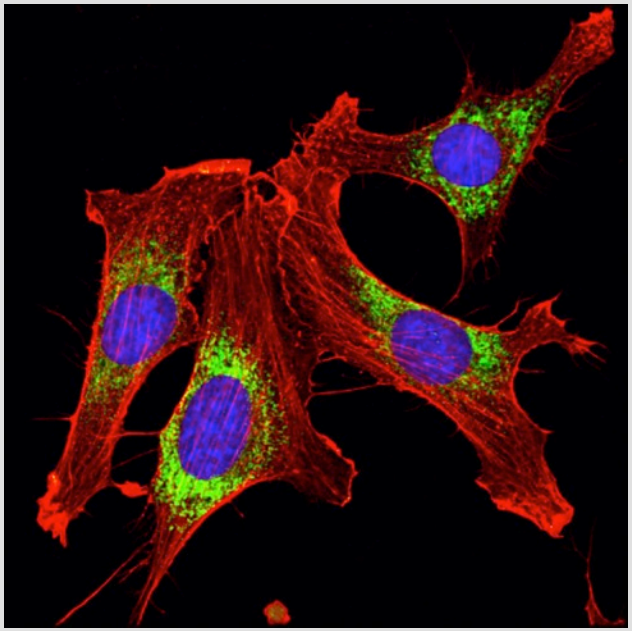
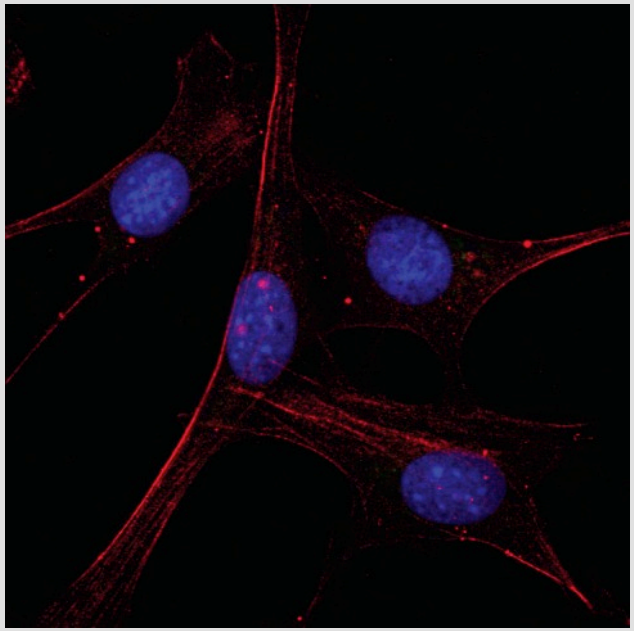
Protocol optimization: Fixation and permeabilization

- **Comparison of permeabilization reagents**

Methanol



Triton X-100



GREEN = PDI Antibody #2446 RED = β-Actin (BH10D10) #3700
BLUE = DRAQ5® #4084 (nuclei)

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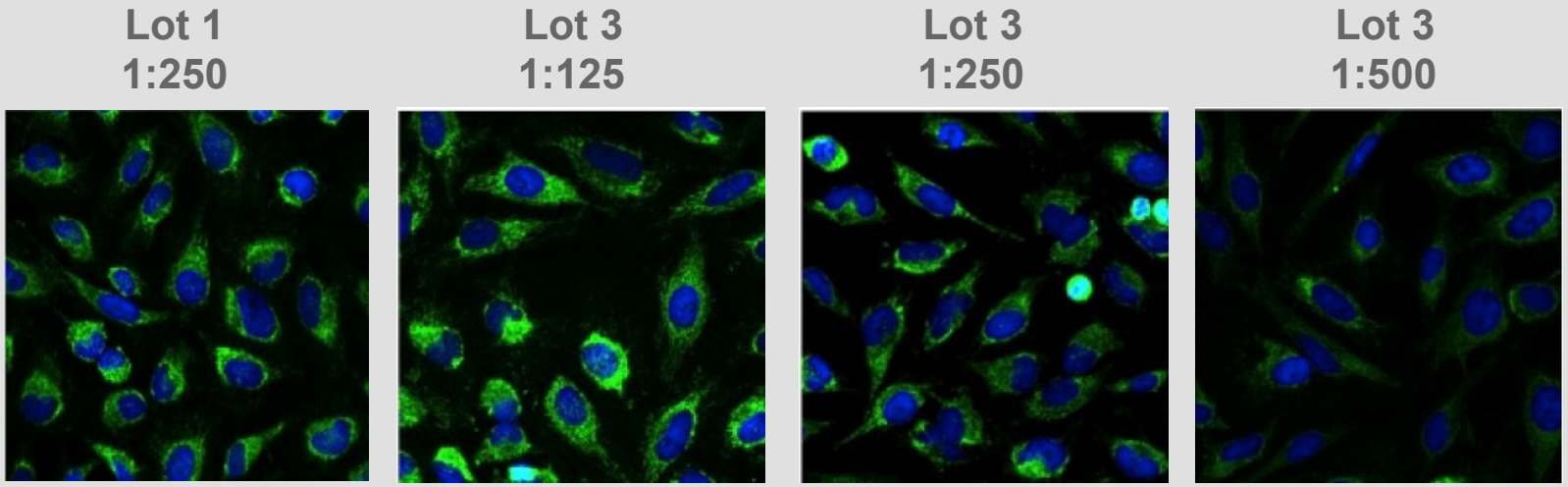
XP® antibodies display exceptional reproducibility

- Lot-to-lot comparison



GREEN = COX IV (3E11) Rabbit mAb #4850
BLUE = DRAQ5® #4084 (nuclei)

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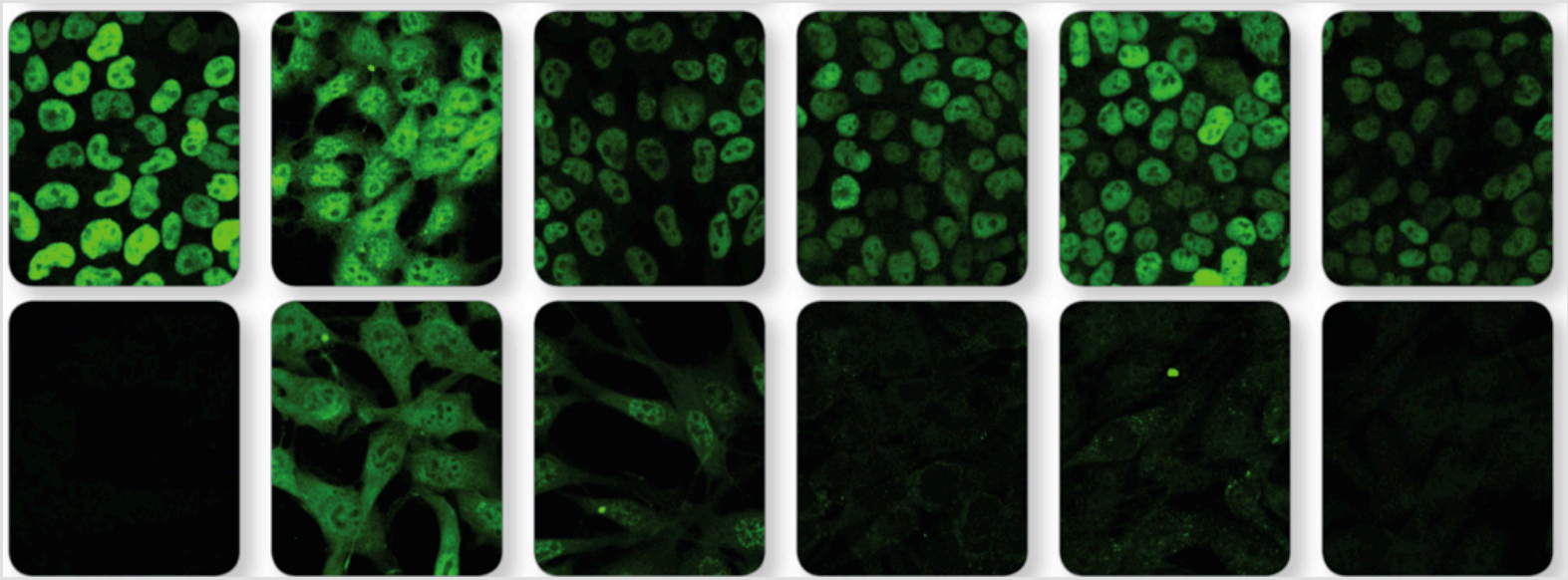
What does validation mean for you?

- **Competitor comparison**



Nanog (D2A3) XP® Rabbit mAb (Mouse Specific) #8822

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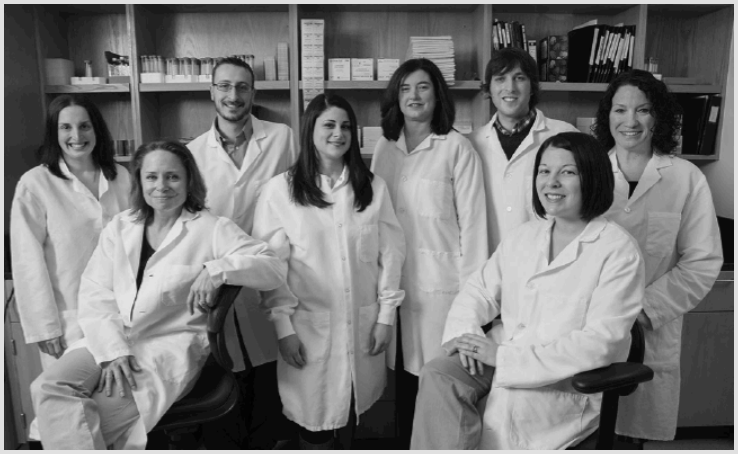
What does Antibody Validation mean for you?

The accuracy of IF images is dependent on the quality of the primary antibody used

- Enables you to choose the right antibody for immunostaining
- Publication-quality results that you can trust
- Ready-to-go protocol for each antibody
- Guaranteed antibody performance
- No more wasting of time and samples



CST IF group

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Antibody Products for IF

XP® Monoclonal Antibodies

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The History of Antibody development at CST

Conception of Modification Specific Antibodies

↓

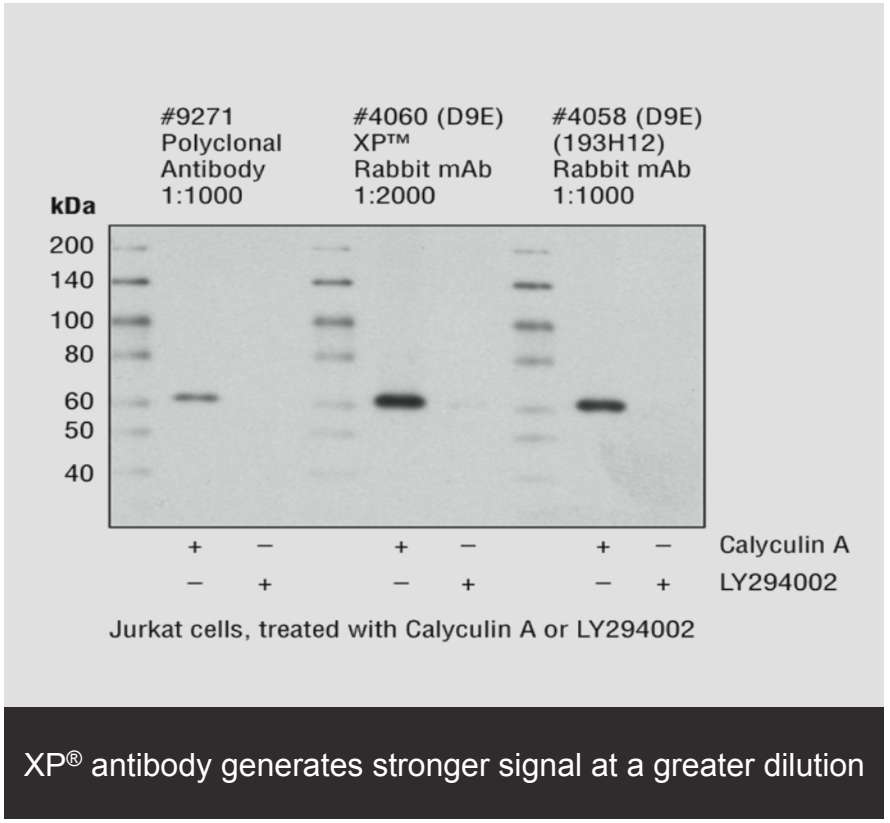
Development of Rabbit Polyclonal Antibodies

↓

Progression of Rabbit Monoclonal Technologies

↓

Emergence of XMT® Technology



XP® antibody generates stronger signal at a greater dilution

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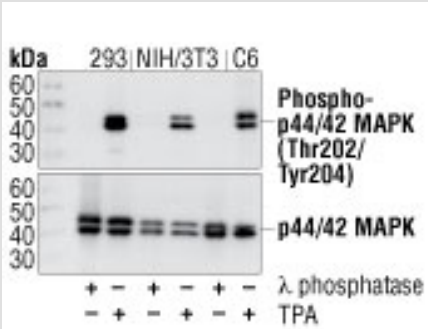
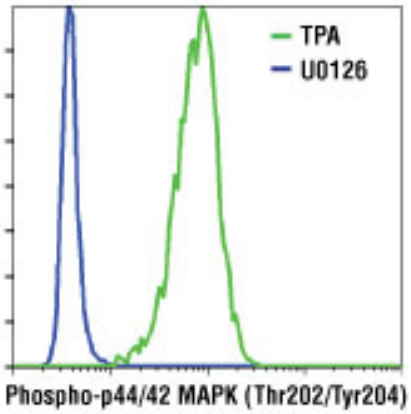
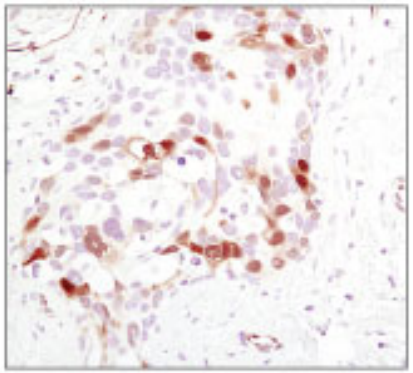
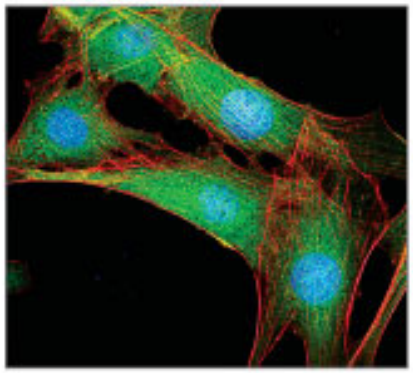
XP® antibodies performance in relevant applications

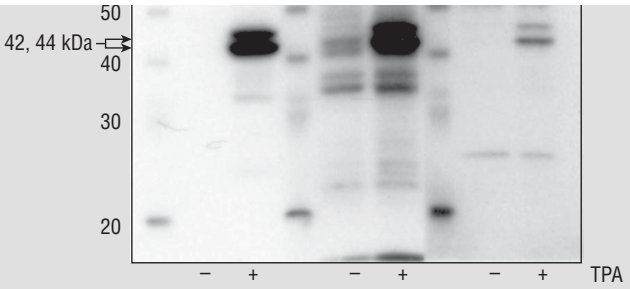
Phospho-p44/42 MAPK (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370

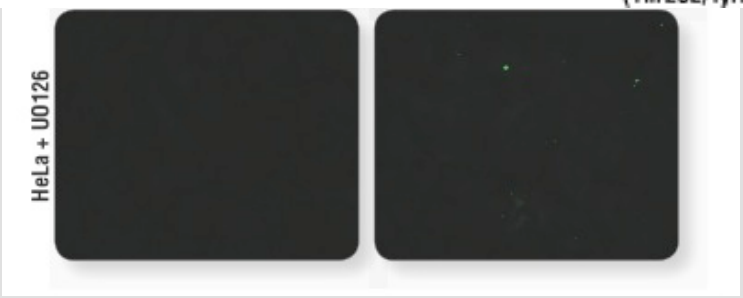












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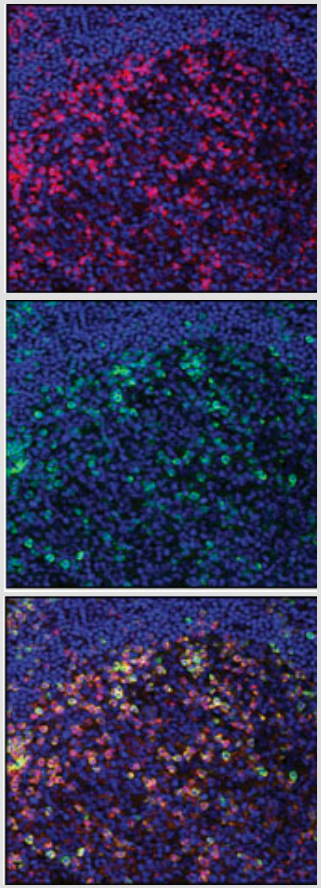
What does XP® bring to you?



Zap-70 (D1C10E) XP® Rabbit mAb #3165

- Validated for all relevant applications
- Best specificity and sensitivity
- Best stability
- High quality antibodies for challenging and clinically relevant targets
- www.cellsignal.com/technologies/xmt/index

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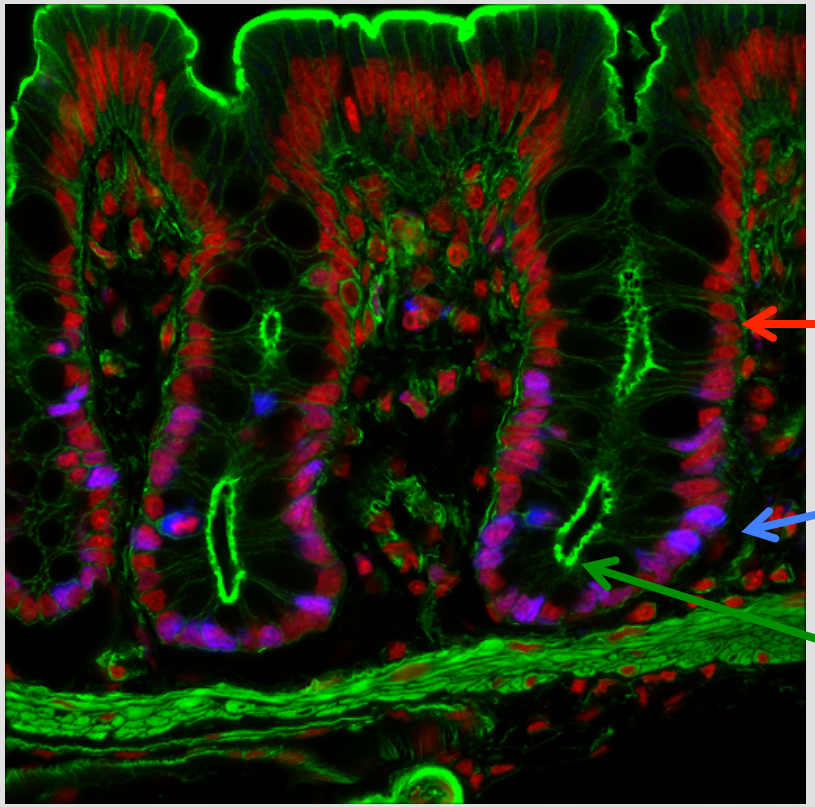
Antibodies validated for IF: Some examples



Analysis of cell proliferation in rat colon

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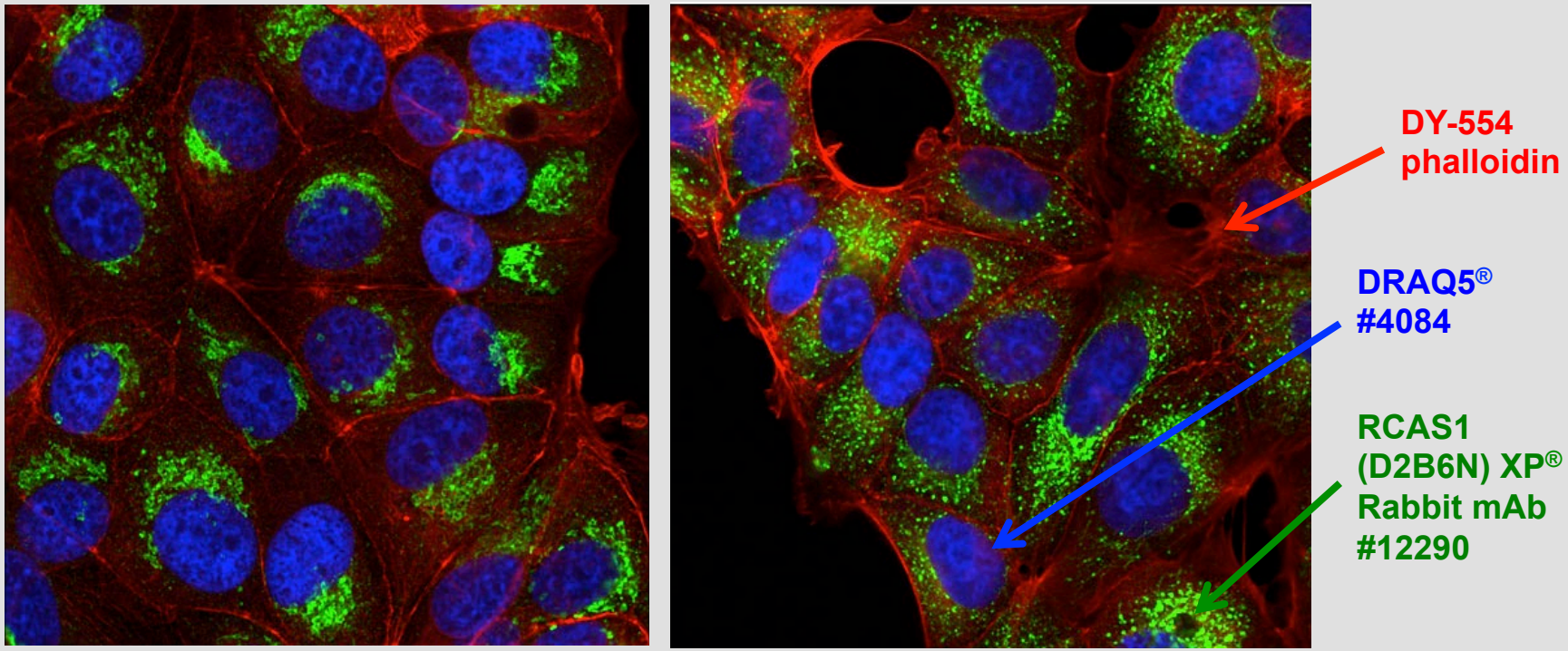
Antibodies validated for IF: Some examples

Untreated

Brefeldin A (#9972)



Analysis of vesicle transport

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Antibody Products for IF

Conjugated antibodies

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Fluorescent tags

Dye	Color	Excitation (nm)	Emission (nm)	Similar Color Dyes	Flow	IF	HCA	ICW
PacBlue	Blue	407 (UV)	421	AlexaFluor® 405, Marina Blue, Briliant Violet, Cascade Blue	✔	✔?	✔?	
Hoechst	Blue	345 (UV)	480	DAPI		DNA	DNA	
AlexaFluor® 488	Green	499 (blue)	520	FITC, Cy2	✔	✔	✔	
PE	Orange	480 (blue) or 565 (green)	575		✔			
AlexaFluor® 555	Red	533 (green)	568	TRITC, Rhodamine, Cy3	✔?	✔	✔	
AlexaFluor® 594	Red	591 (green)	618	Texas Red®	✔?	✔	✔	
Propidium Iodide	Red	536 (green)	617		DNA	DNA	DNA	
AlexaFluor® 647	Far Red	652 (red)	668	Cy5, APC	✔	✔	✔	
DRAQ5®	Far Red	647 (red)	681		DNA	DNA	DNA	DNA

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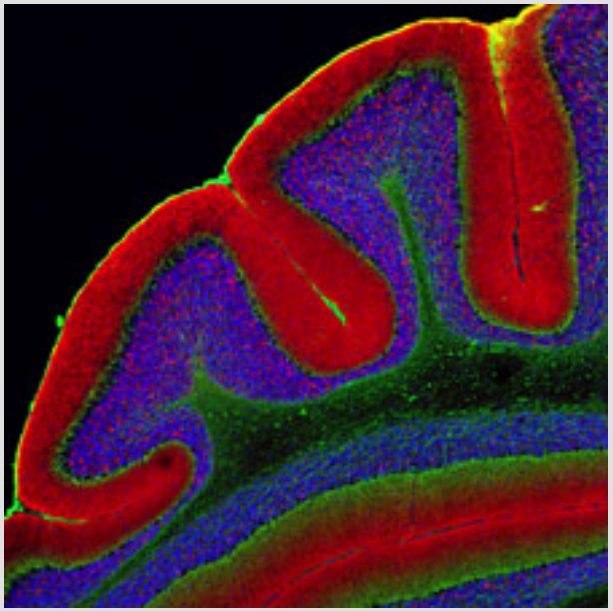
Conjugated antibodies at Cell Signaling Technology

Conjugated antibodies are crucial for multiplexing with IF

- CST™ antibodies plus:
  - AlexaFluor® 488, 555, 594, 647
  - Pacific blue
  - R-Phycoerythrin (PE)
- Anti-mouse, anti-rat and anti-rabbit secondary antibodies for multiplexing
- Optimized in-house conjugation for each antibody
- Tested and validated for fluorescent imaging and/or flow cytometry



Confocal analysis of mouse cerebellum using α-Synuclein Antibody #2628 detected with Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 555 Conjugate) #4413 (red) and Neurofilament-L (DA2) Mouse mAb #2835 detected with Anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) #4408 (green).

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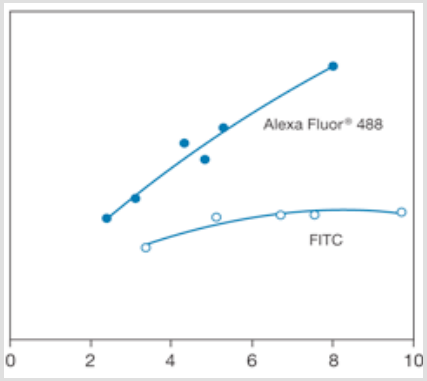
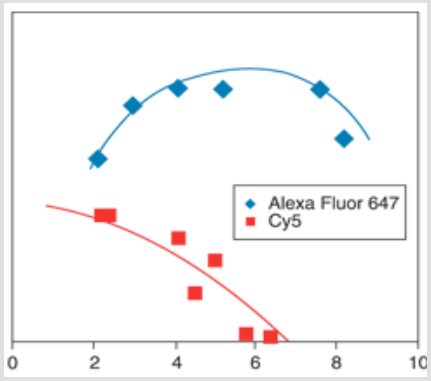
Benefits of IF imaging with AlexaFluor®

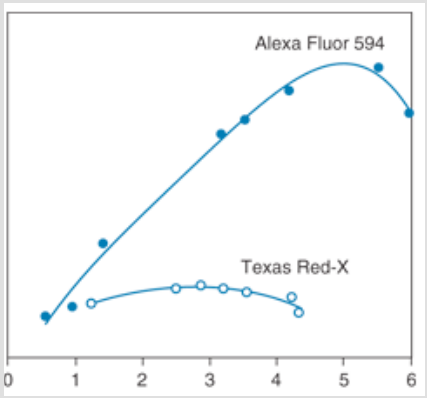
AlexaFluor® 488 vs. FITC



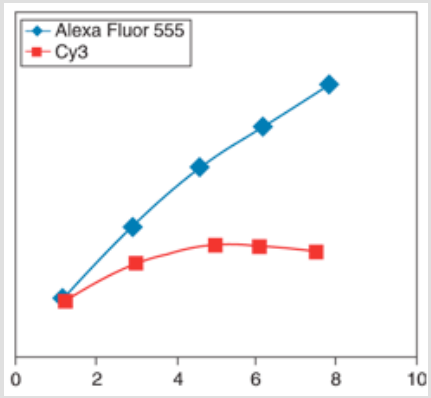
AlexaFluor® 647 vs. Cy5



AlexaFluor® 594 vs. Texas Red



AlexaFluor® 555 vs. Cy3



- Full spectrum coverage
- Brighter fluorescence output than similar fluorochromes
- Highly photo-stable
- Smaller: easier cell penetration

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Questions?

Let’s meet again in 10 mins!

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Obtaining informative IF images

CST Protocol optimization

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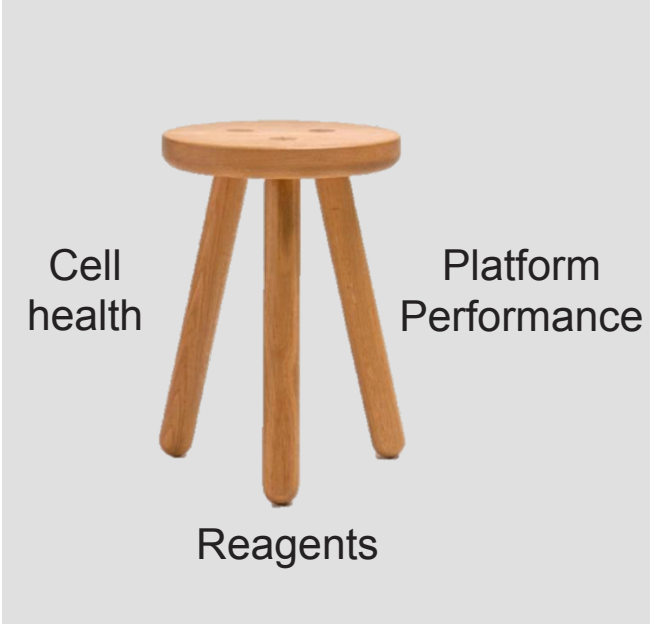
Important points to obtain correct IF images

The accuracy of IF images is dependent on the quality of all the reagents and protocols used

- Our scientists compare different reagents to understand what conditions give the best appropriate signal
- Reagents used by our scientists when validating CST primary antibodies
- Products and experience to support every step of your IF experiments
- Guaranteed performance



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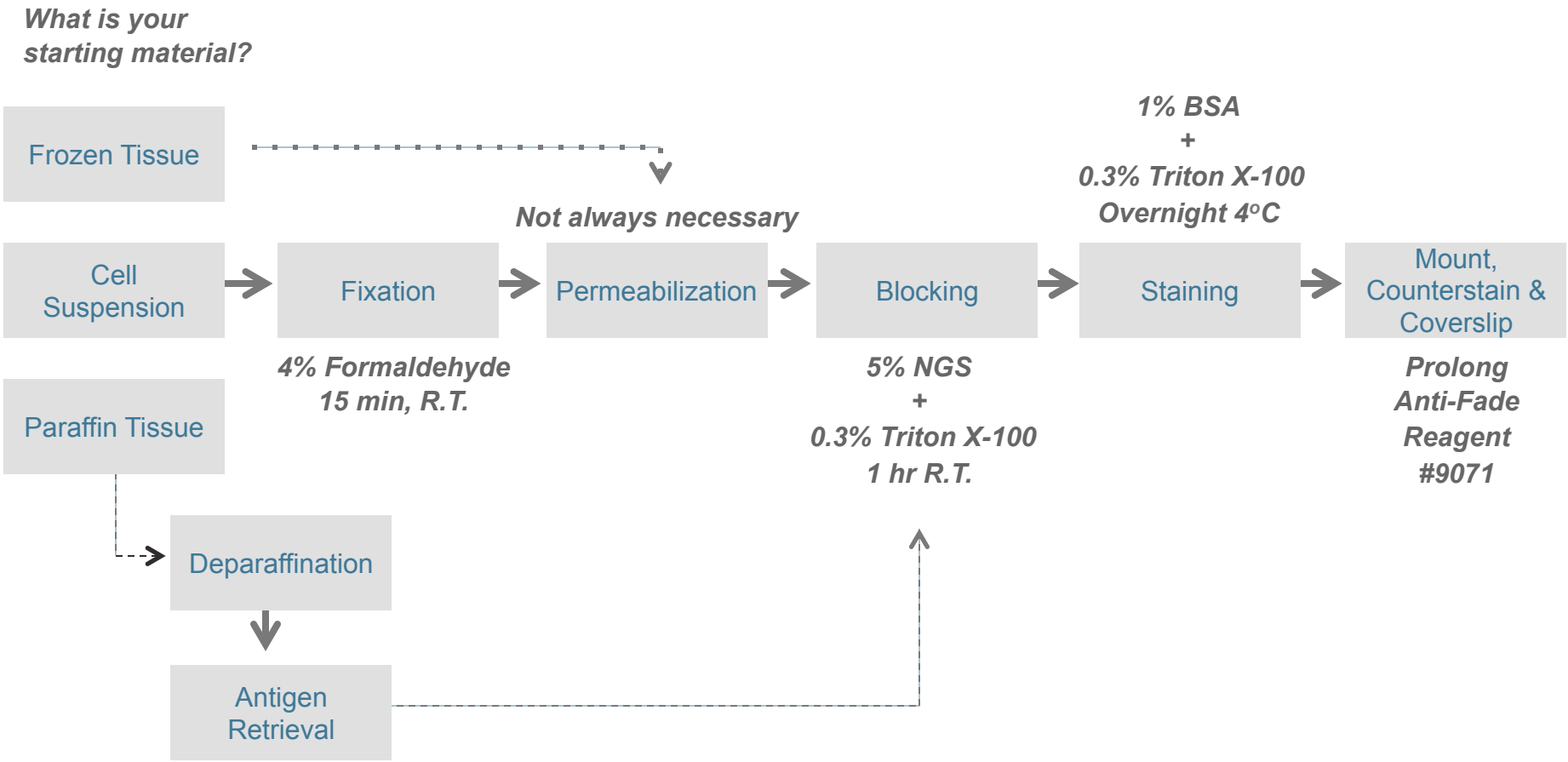
IF step by step



http://www.cellsignal.com/support/protocols

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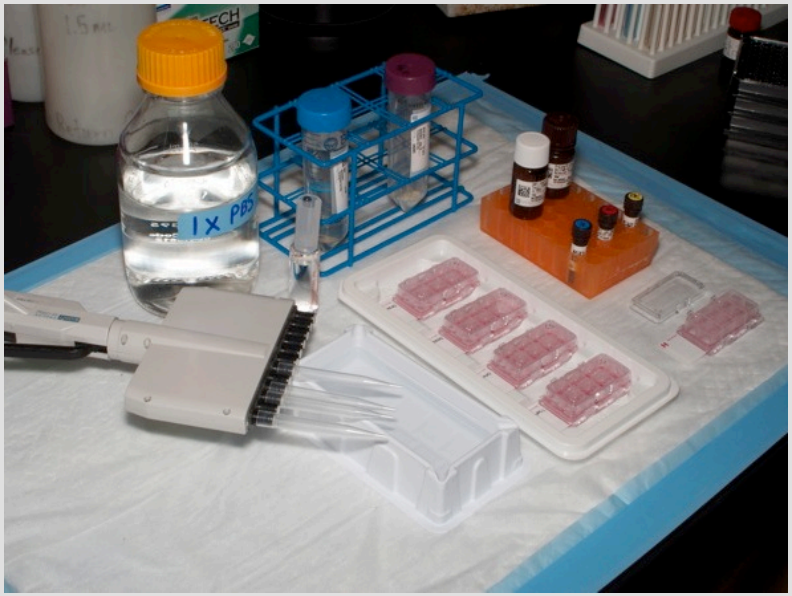
Preparing your samples: IF-IC

- Live cells should be healthy and of an appropriate confluence
- 50 –75 % confluence
- Cells should be grown, treated, fixed and stained directly in multi-well plates, chamber slides or coverslips
- Evenly distributed across the coverslip
- Expected morphology



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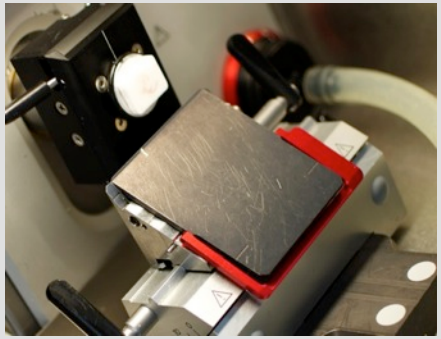
Preparing your samples: IF-F

- Frozen tissues offer good morphology with minimal negative impact on antigen preservation
- Samples are rapidly frozen in OCT medium and blocks cut with a Cryostat
- Sections are collected one-by-one at a thickness of 10-20µm and air-dried
- Trim away excess OCT, create a hydrophobic barrier, fix immediately
- For fixed frozen tissues proceed with immunostaining

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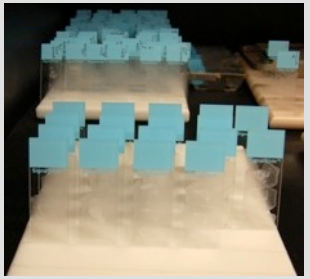
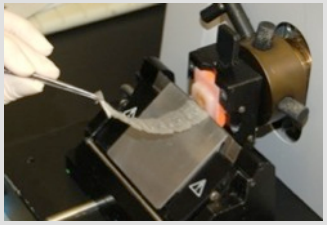
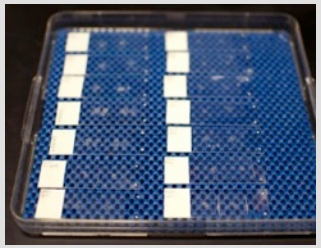
Preparing your samples: IF-P

- Water in tissue replaced by paraffin using an ethanol gradient and xylol
- Thin slices (4-6 µm) are cut on a microtome, floated in a water bath, mounted on slides, and dried overnight
- Deparaffinization – Removal using xylene
- Rehydration – Water gradually reintroduced using an ethanol gradient
- Antigen retrieval is crucial – Datasheet!
  - Citrate + microwave
  - EDTA + microwave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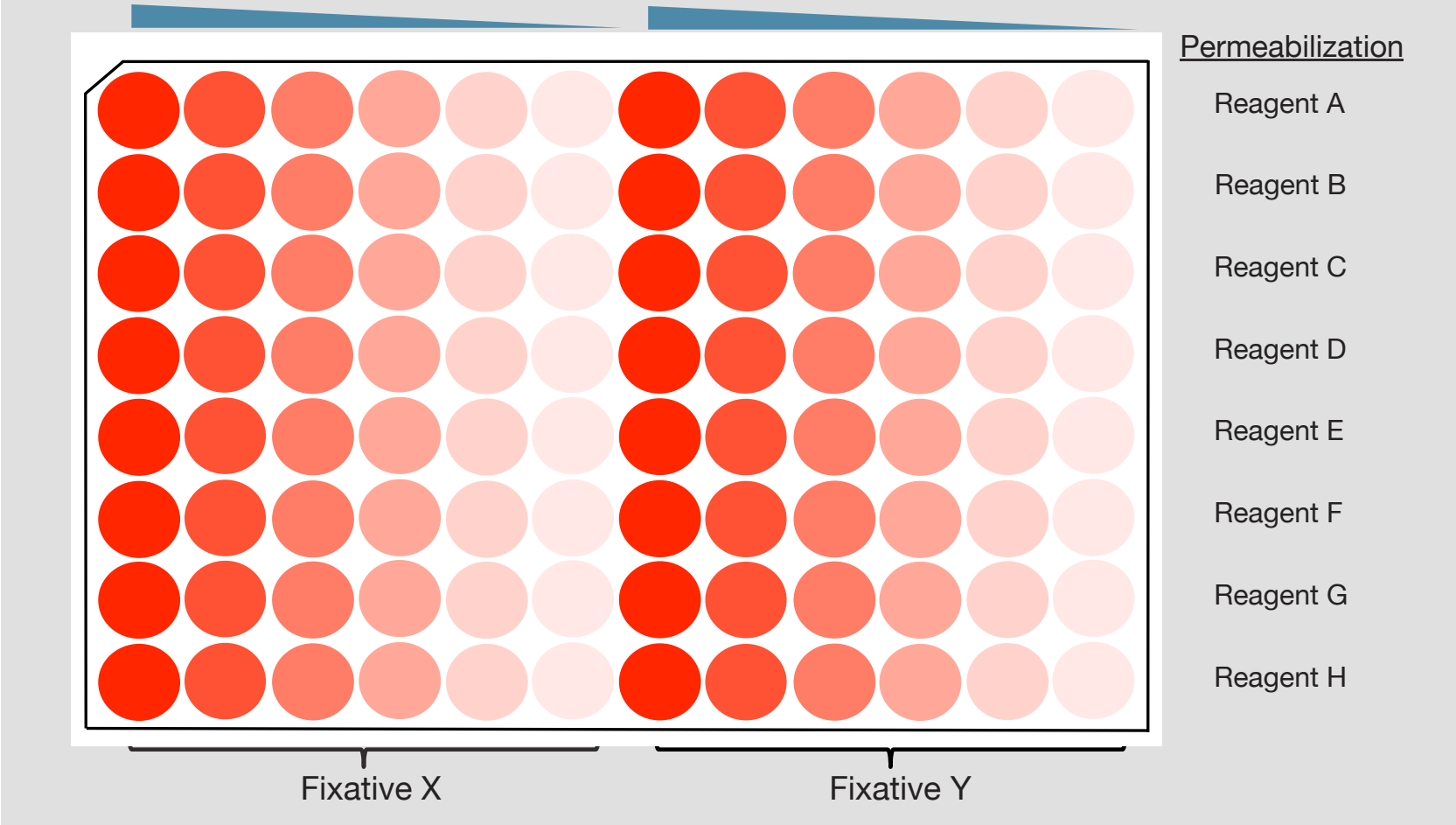
Key protocol step: Fixation and permeabilization



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PathScan® and StemLight® Kits for IF imaging

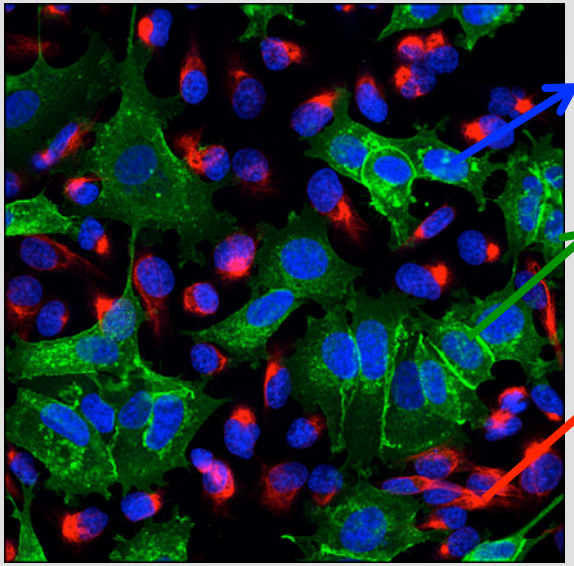
Kits to analyze cellular events using fluorescence microscopy or high content platforms

PathScan Multiplex Kits

- Primary Antibody cocktail + detection cocktail
- Apoptosis & proliferation #7851, EGF receptor activation #7967, EMT duplex #7771 and signaling nodes #8999

Multi-target HCA Kits
DNA damage #7101 and stress & apoptosis #7103 – 8 different 10x Abs

StemLight™ Pluripotency and iPS kits
(#9094, #9093, #9092 and #9656)



Confocal immunofluorescent analysis of paraffin-embedded human kidney using PathScan® EMT Duplex IF Kit

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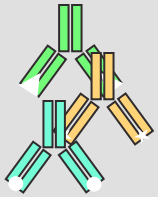
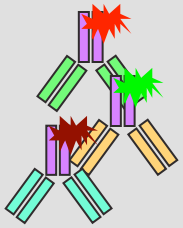
PathScan® multiplex IF kits

Day 1 - Component A



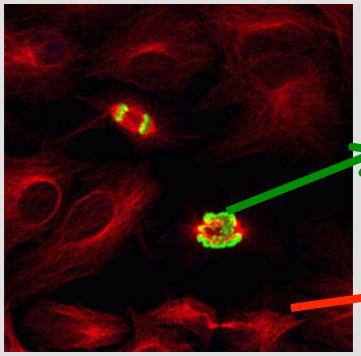
- P-Histone H3 Mouse IgG2a
- Cleaved-PARP Rabbit IgG
- Alpha-tubulin Mouse IgG2b

Day 2 - Component B

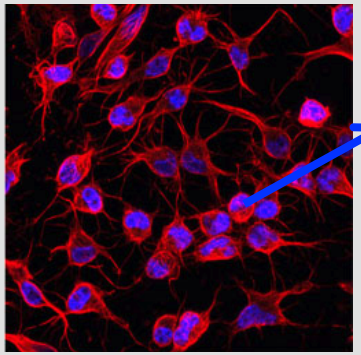


- a-Rabbit IgG Alexa Fluor® 647
- a-Mouse IgG2a Alexa Fluor® 488
- a-Mouse IgG2b Alexa Fluor® 555

Untreated



Staurosporine



PathScan® Apoptosis and Proliferation Multiplex IF Kit #7851

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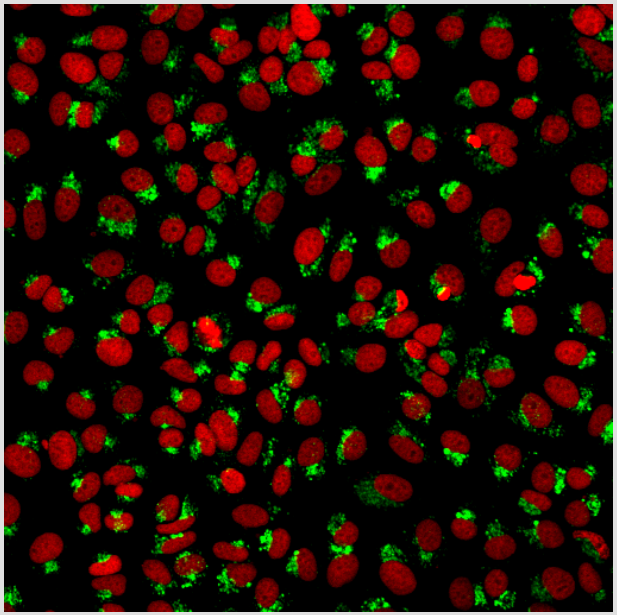
Key protocol steps: Fixation and permeabilization

Standard protocol

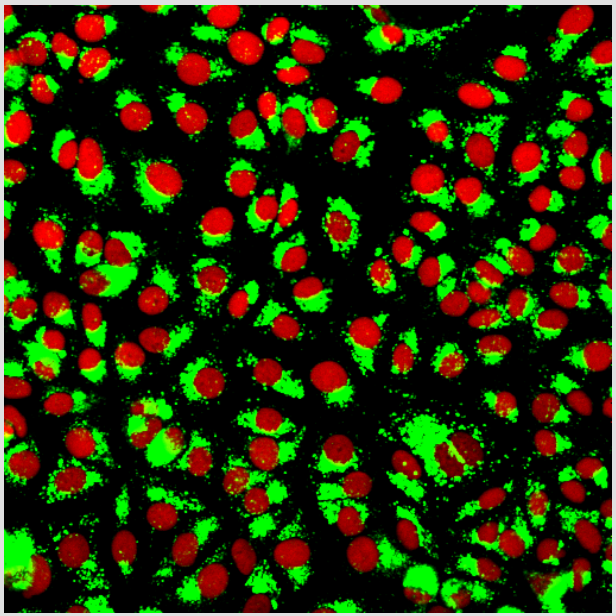
4% Formaldehyde fix
0.25% Triton-X100 perm



MFI=44.1, FI = 3.0

Optimized protocol

Methanol fix/perm
0.125% Triton-X100 perm



MFI=322, FI = 14.7

LC3B (D11) XP® Rabbit mAb #3868

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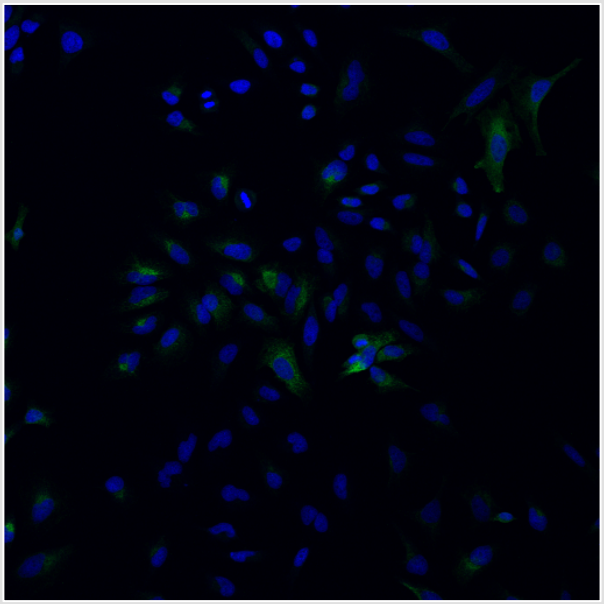
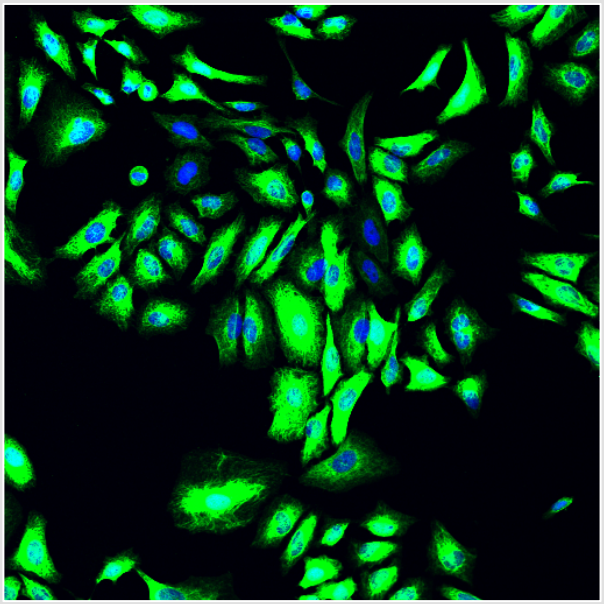
Fixation and permeabilization: re-validation of an antibody

Formaldehyde fixation



Methanol fixation



Keratin 17/19 (D32D9) XP® Rabbit mAb #3984

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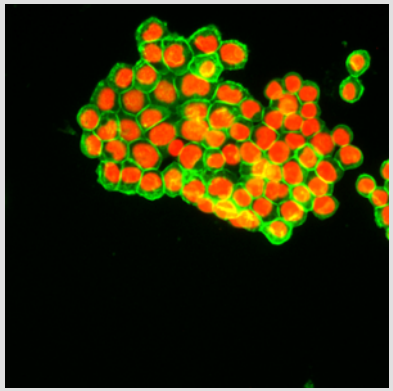
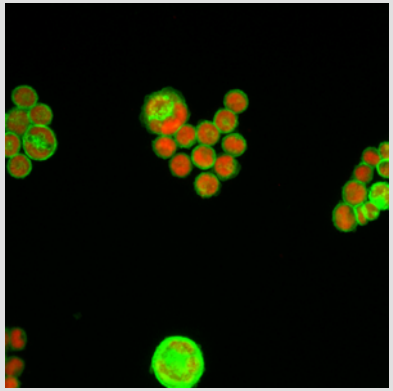
Key protocol step: Fixation

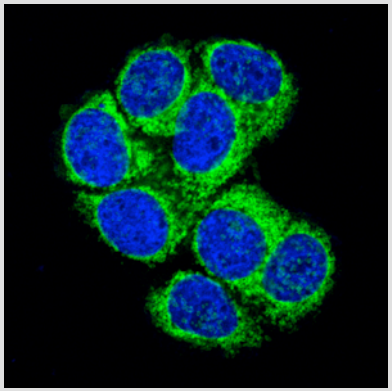


100% MeOH

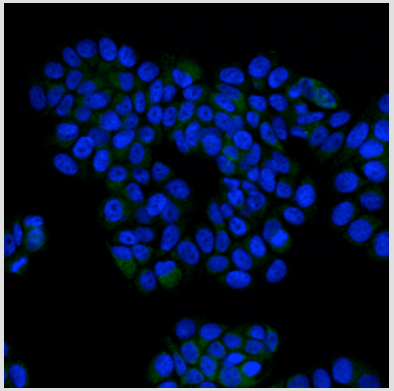


4% Formaldehyde

HER2/ErbB2 (D8F12) XP® Rabbit mAb #4290



2% Formaldehyde



4% Formaldehyde

Hexokinase I (C35C4) Rabbit mAb #2024

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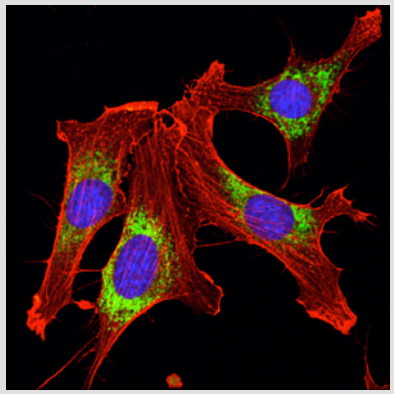
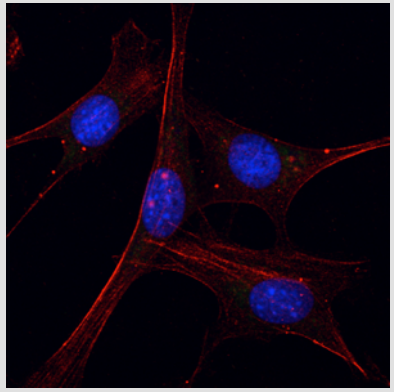
Key protocol steps: Permeabilization



Methanol



Triton-X100

PDI (C81H6) Rabbit mAb #3501

- Standard protocol: Permeabilization performed by using Triton X-100 while blocking and antibody incubation
- Permeabilization step is antibody dependent – Always check your antibody's datasheet for recommendations

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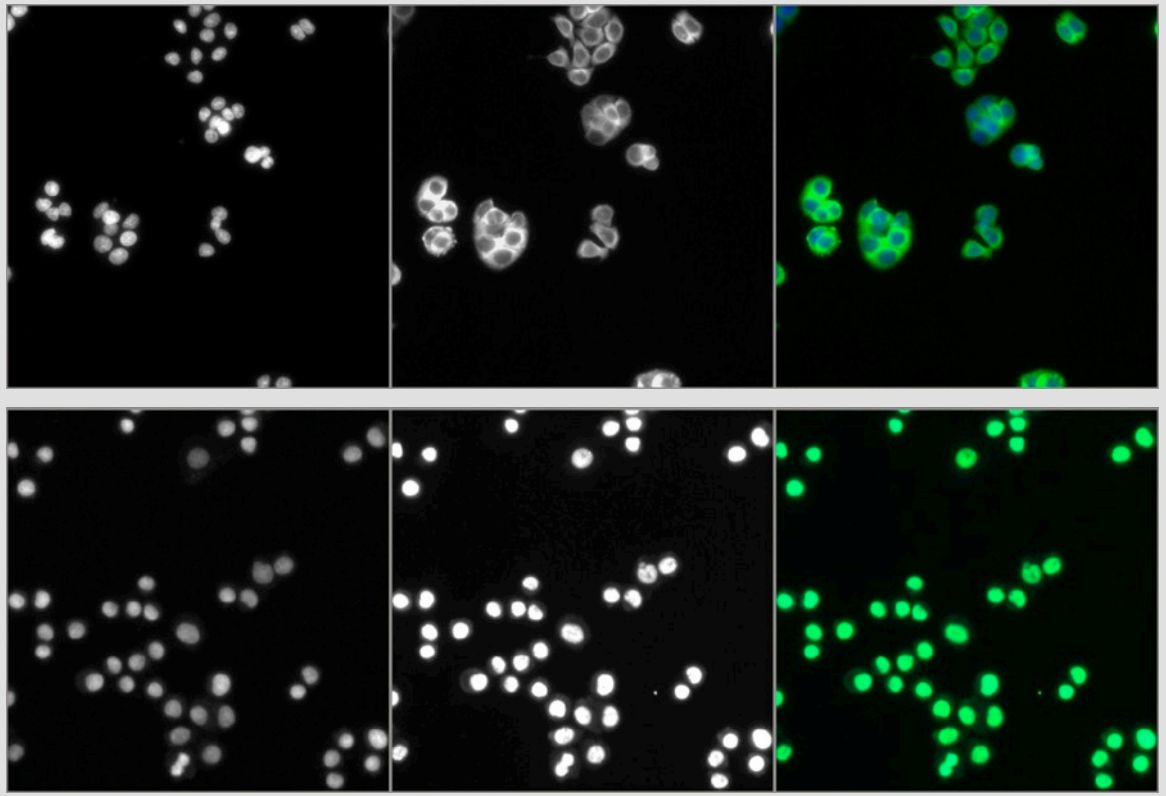
Fixation and permeabilization affect cellular localization



Di-Methyl-Histone H3 (Lys79) (D15E8) XP® Rabbit mAb #5427

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Key protocol steps: Blocking

- Standard protocol: Block with PBS/ 5% NGS/0.3% Triton X-100 – 1h, R.T.
- Blocking is antibody dependent – Always check your antibody's datasheet for recommendations
- Blocking time is critical and will impact your staining
- Do not wash between blocking and antibody dilution

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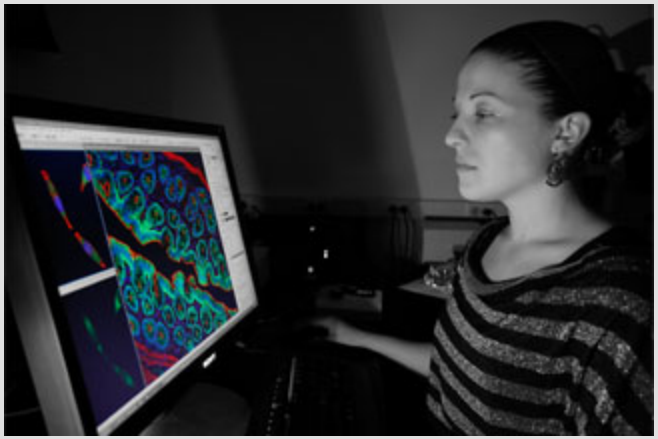
Key protocol step: Antibody incubation

- Antibody diluents: PBS, TBS, BSA or other carriers, detergents…
- Standard dilution buffer: PBS/1% BSA/0,3% Triton X-100
- Triton is a mild detergent that permeabilizes membranes
- **Choice of dilution buffer is antibody dependent**
- Incubate at 4°C overnight
- Ensure incubation is carried out in a humid light-tight box or covered dish/plate to prevent drying and fluorochrome fading



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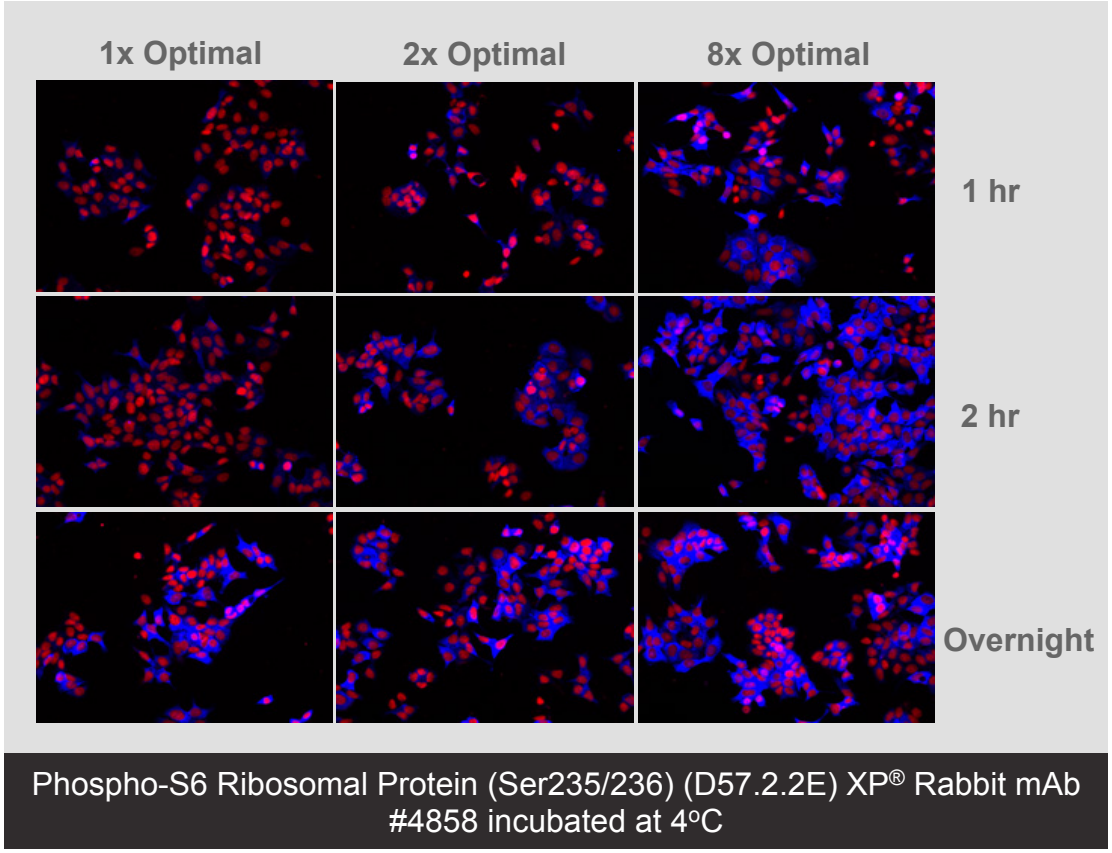
Can I perform a shorter primary antibody incubation?

- Able to achieve similar results by increasing antibody concentration
- **Pros:** Faster results
- **Cons:** Less assays per tube, increased background



Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb #4858 incubated at 4ºC

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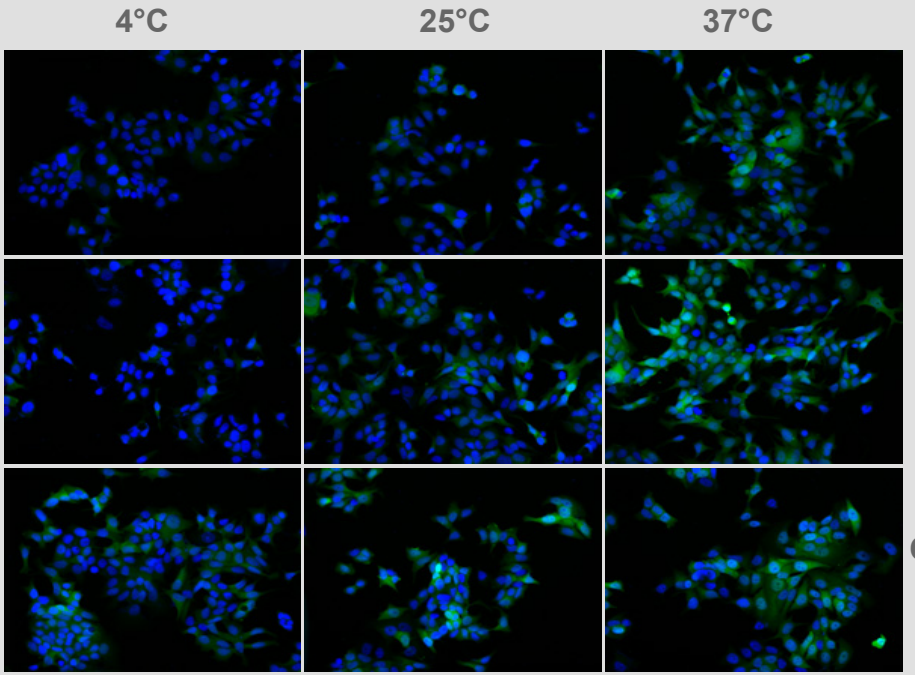
Do higher incubation temperatures accelerate antibody binding?

- With some antibodies comparable results are obtained with a shorter incubation at 37°C

	Positive MFI		
	4C	25C	37C
1h	834.43	1388.40	2206.37
2h	968.80	1465.58	**2381.96**
Overnight	1863.52	2339.23	3035.71

	Negative MFI		
	4C	25C	37C
1h	23.29	35.09	54.07
2h	27.68	35.09	**54.07**
Overnight	56.59	64.05	158.16



Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370, optimal dilution

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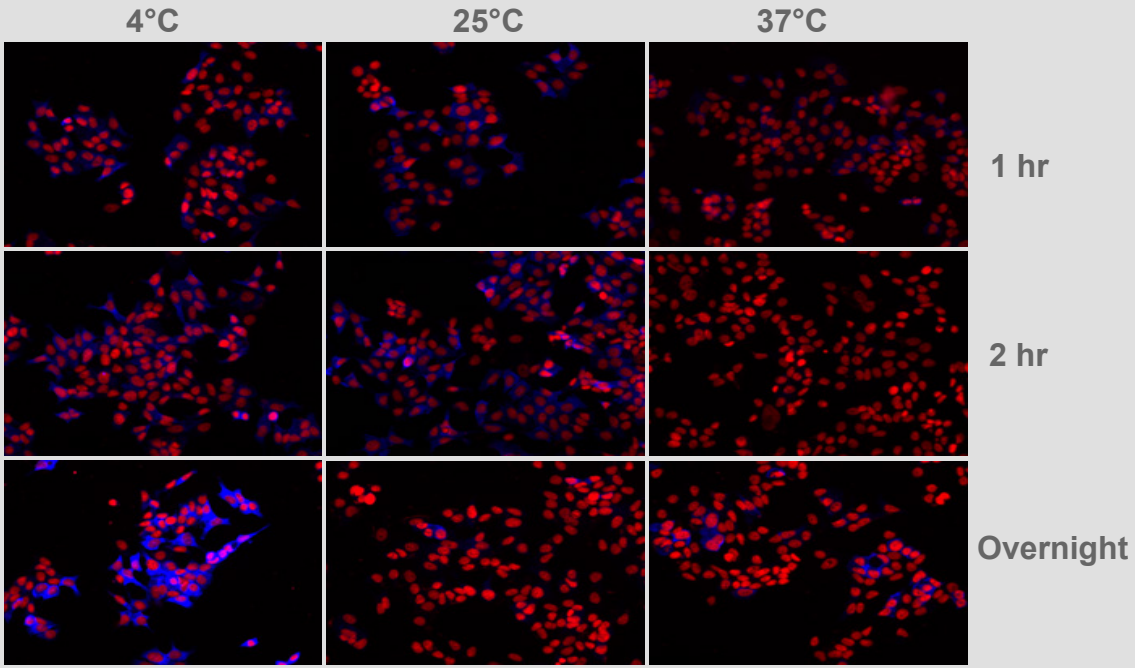
Do higher incubation temperatures accelerate antibody binding?

- However…Higher temperatures can negatively impact staining with some antibodies

Always follow product recommendations!



Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb #4858, optimal dilution

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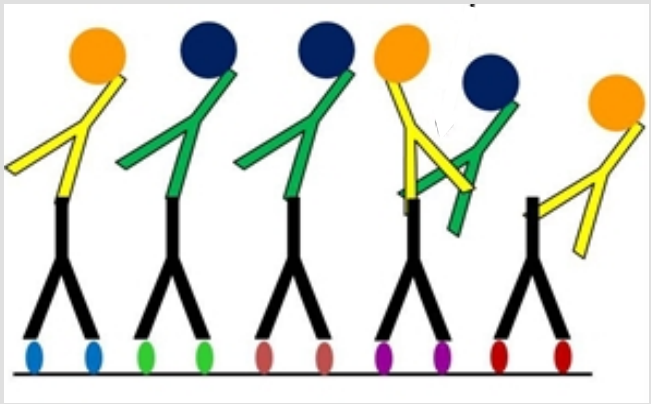
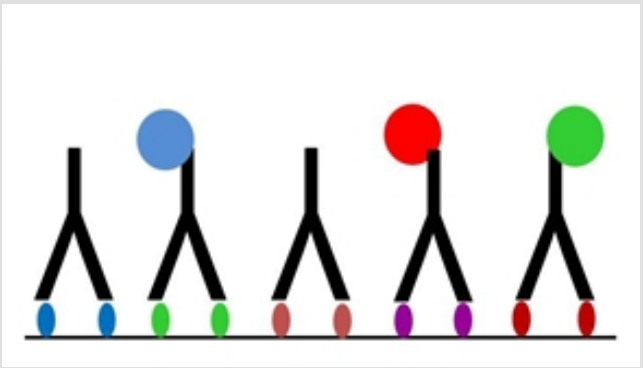
Indirect vs. direct antibody staining

Indirect staining



- Two-step process
- Chance of increased background
- Increase signal intensity

Direct staining



- Single-step process
- Easy to multiplex
- Decreased signal intensity

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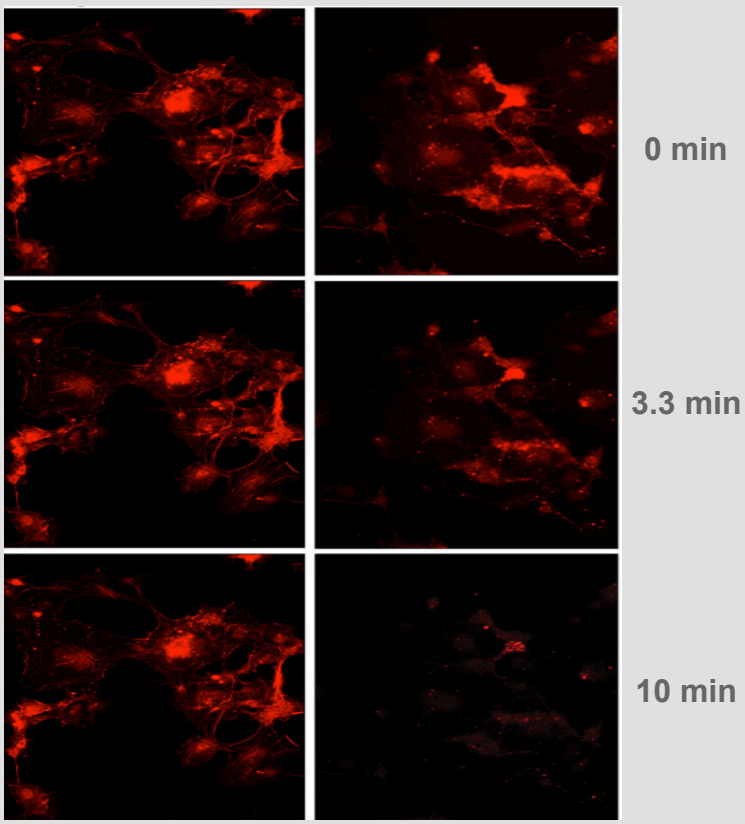
Final steps: Coverslip and counterstain



β-Actin Mouse mAb #3700 + Anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 555) #4409

Prolong® Anti-Fade (#9071 or #8961 with DAPI)

- Enhanced resistance to photobleaching
- Ready to use
- Little or no quenching of the fluorescent signal
- Hardens the coverslip and improves the lifetime of the slide
- Common Counterstains: DAPI (#4083), Hoechst (#4082), Propidium Iodide (#4087), DRAQ5® (#4084), Phalloidin (#8878, #8953, #8940)

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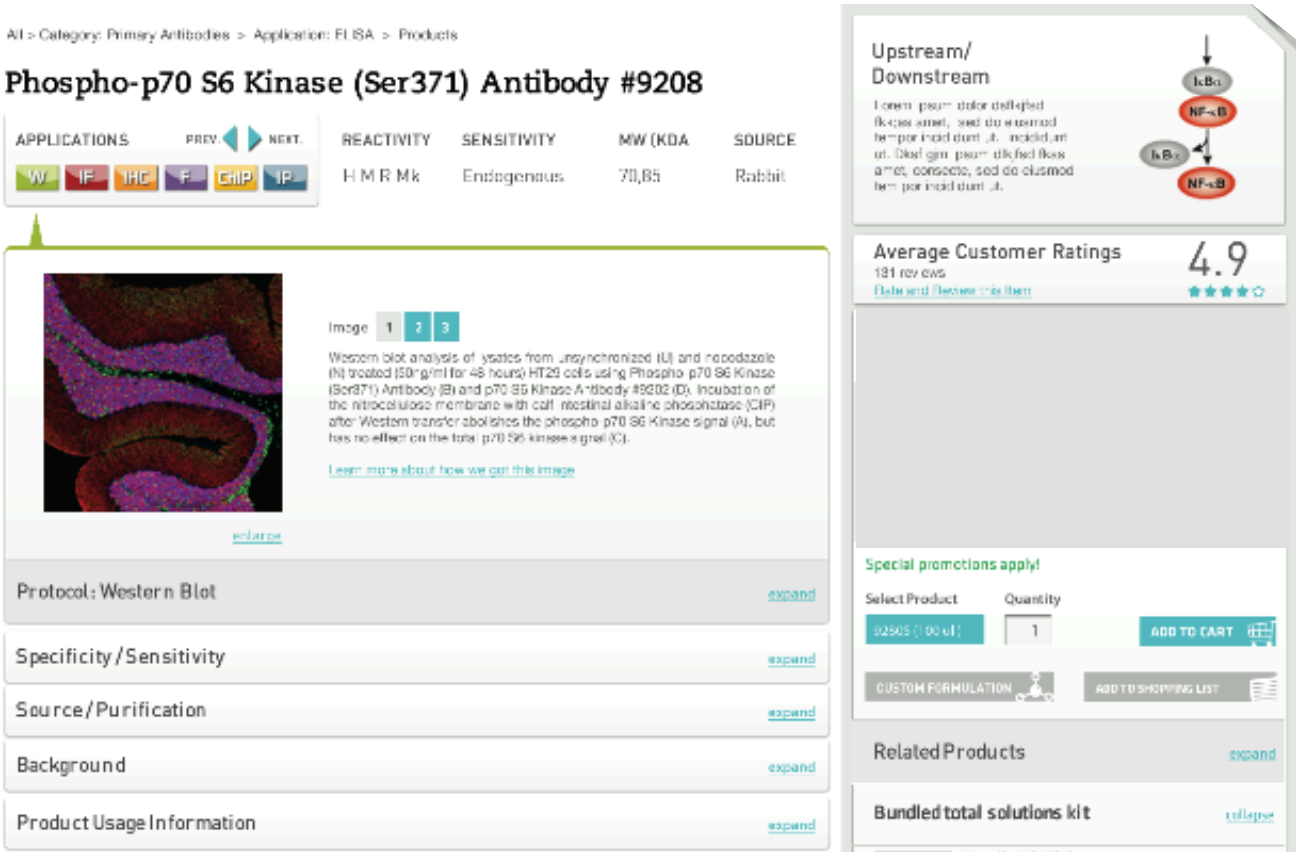
Because each antibody is different



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Technical support

Our help to you

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CST standard protocol for IF

1. Fix samples in methanol-free 4% formaldehyde in warm PBS for 15m, R.T.
2. Wash 3 x 5m in 1x PBS
3. Block samples for 1h at R.T. in 1x PBS + 5% NGS and 0.3% Triton X-100
4. Dilute primary antibody in 1x PBS + 1% BSA and 0.3% Triton X-100
5. Aspirate blocking solution, apply primary antibody, incubate overnight at 4°C
6. Wash 3 x 5m in 1x PBS
7. Dilute secondary antibody in 1x PBS + 1% BSA and 0.3% Triton X-100
8. Aspirate PBS, apply secondary antibody, incubate for 1h, R.T.
9. Wash 3x 5m in 1x PBS
10. Counterstain as necessary and mount in ProLong Gold anti-fade reagent

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But don’t forget…

- Antibody-antigen interactions are complex, and one standard protocol is not the answer for every product
- CST validation groups attempt to optimize high priority targets
- Unique protocols highlighted on both website and datasheet
- Deviation from these conditions is one of the most common themes in tech support

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Common problems with IF: Weak/ No signal

Possible cause	Solution
Target was not induced properly	• Optimal treatment conditions should be determined for each antibody • Use suitable controls
Inadequate fixation/ permeabilization	• Use the appropriate fixation and permeabilization protocol (product datasheet) • Fixative should be added immediately after treatment and at a high enough concentration to inhibit any phosphatase activity (4% formaldehyde) • Use methanol-free formaldehyde prepared fresh

- **Fixation**
  - aldehyde (cross-linking)
  - alcohol/acetone (precipitating)
  - glyoxal
  - Mirsky's
  - Streck's
- **Permeabilization (non-FFPE)**
  - Triton
  - Tween
  - SDS
  - Saponin
  - CHAPS
  - NP-40
  - alcohol

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Common problems with IF: Weak/ No signal

Possible cause	Solution
Too much washing	• Do not wash more than 3x5m
Incorrect use of secondary antibody	• Use recommended concentration • Ensure using correct host species
Lasers not compatible	• Ensure that the laser wavelength matches the excitation and emission wavelengths of the fluorochrome being used
Inadequate preparation of samples	• Antigen retrieval may be needed • Incomplete deparaffination • Inappropriate cell confluence

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Common problems with IF: Too much signal/ background

Possible cause	Solution
Autofluorescence	• Use unstained samples as controls • Wrong fixation reagent used – check product datasheet
Too much primary antibody used	• Used recommended antibody dilution • Ensure blocking step is performed appropriately • Ensure the antibody used is specific for the target
Insufficient washes	• Perform additional washes

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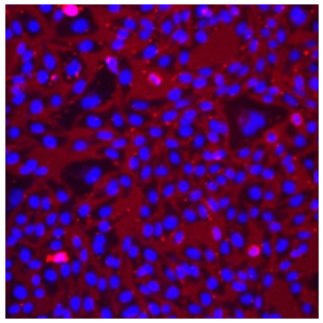
Common problems with IF: Too much signal/ background

Possible cause	Solution
Incorrect use of secondary antibody	• Ensure using correct host species • Use recommended antibody dilution
Incorrect fixation	• Use suitable fixation time • Ensure short times between sample preparation and fixation
Tissue sections too thick	• Paraffin sections: 4-6µm • Frozen sections: 10-20µm



- Using too much secondary antibody gives problems with background

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What fluorochromes do I use for intracellular targets?

- Fluorochromes compatible with your microscope
- Use the brightest fluorochrome for the lowest expressed protein – Stain index
- More stable and resistant to fixation fluorochrome for surface markers
- When multiplexing, check overlap and use different host species antibodies
- PE not recommended

Fluorochrome	Stain Index
PE-Cy5	353
PE	302
APC	278
Alexa Fluor® 647	214
PE-Cy7	139
PerCP-Cy5.5	107
Pacific Blue™	80
Alexa Fluor® 488	73
Alexa Fluor® 700	61
FITC	56
APC-Cy7	37
PerCP	37
AmCyan	25

Maecker & Trotter, 2008

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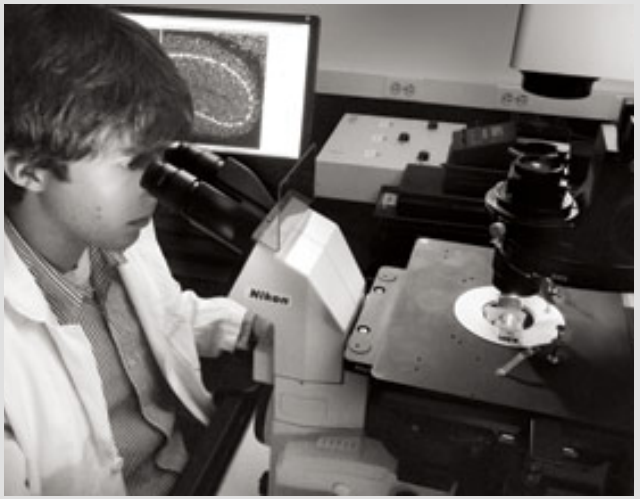
Technical support

CST scientists are available as technical resources to help you at any stage in your research

- Application support provided by the IF group
  - Protocol-fixation/perm/staining
  - Antibody concentrations
  - Has antibody been tested/validated for IF?
  - Recommendations for best antibody for your experiment when multiple options are available



Contact us at:
eusupport@cellsignal.eu

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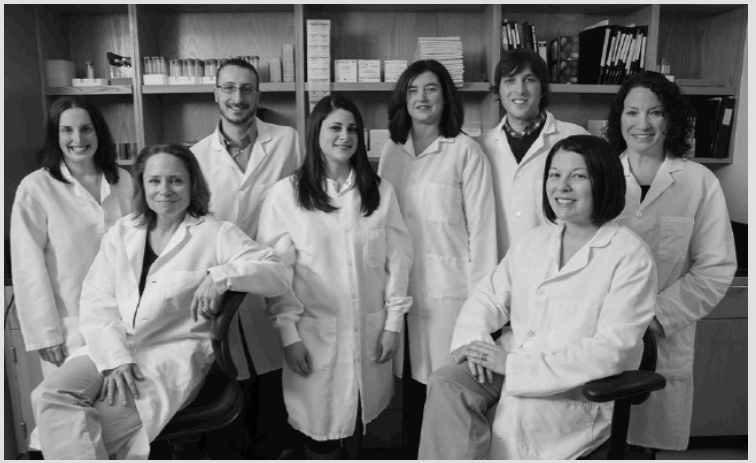
Take-home message

- Antibody-antigen interactions are complex - No protocol fits all
- Your assay is only as good as your reagents
- Don't assume any commercial antibody will work with your assay: Carefully review validation procedures
- CST validates each antibody and optimizes protocols for IF



When in doubt, ask us!

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Please Fill Out the Questionnaire!

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Thanks for listening!

Questions?

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About Us

Founded by research scientists in 1999, Cell Signaling Technology (CST) is a private, family-owned company with over 400 employees worldwide. Active in the field of applied systems biology research, particularly as it relates to cancer, CST understands the importance of using antibodies with high levels of specificity and lot-to-lot consistency. It's why we produce all of our antibodies in house, and perform painstaking validations for multiple applications. And the same CST scientists who produce our antibodies also provide technical support for customers, helping them design experiments, troubleshoot, and achieve reliable results. We do this because that's what we'd want if we were in the lab. Because, actually, we are.

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