

Intrinsic and Extrinsic Controls for Formalin Fixed, Paraffin Embedded Tissue

David L. Rimm M.D., Ph.D
Director, Yale Pathology Tissue Services
Professor, Dept. of Pathology
Yale University School of Medicine

Disclosure/Disclaimer

- I am a consultant to, stockholder in, and scientific co-founder of HistoRx Inc. the exclusive licensee of the AQUA[®] technology
- I am an author on the Yale held patent on the AQUA technology and receive royalties.
- *This project has been funded in whole or in part with the federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.*

The central problem:

Standardization of Protein Assessment of Formalin-Fixed Paraffin-Embedded tissue

- Definition of Extrinsic vs Intrinsic Control
- Solution of the Extrinsic control problem
- Progress toward an Intrinsic control or Tissue Quality Index (previously designated TIC for tissue immunocompetence index)

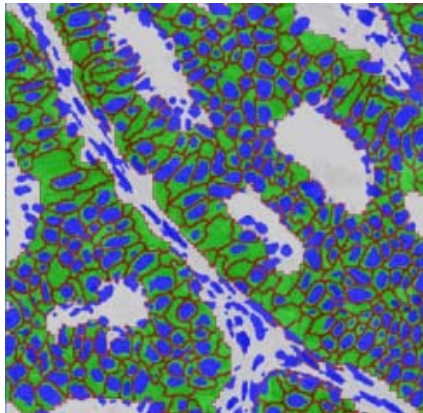
Extrinsic vs Intrinsic Controls

- Extrinsic controls; control for and standardize all the processes from the stainer through the analysis
- Intrinsic controls; control for and standardize all the processes from the patient to the stainer (pre-analytic variables)

Our solution to the Extrinsic Control Standardization Problem: The AQUA method of Quantitative Immunofluorescence

Other Software: **Think like a human**

Assign significance to morphologically defined entities and use feature extraction to emulate human assignments

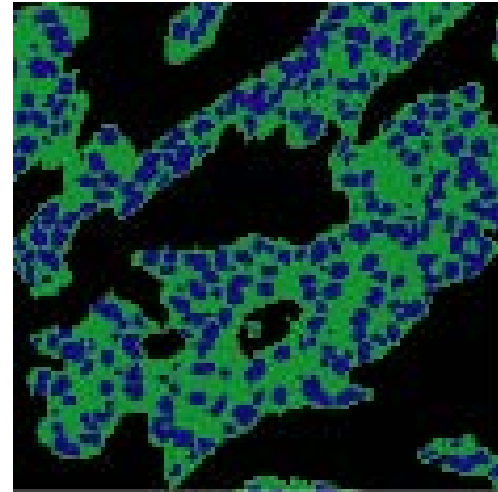


<http://www.tissuestudio.com/>

Example: a nuclear protein emulates the human definition of nucleus and finds round or roundish entities, then counts signal within the roundish entities

AQUA: **Think like a molecule**

Selection of regions only as a function of colocalization of molecular interactions

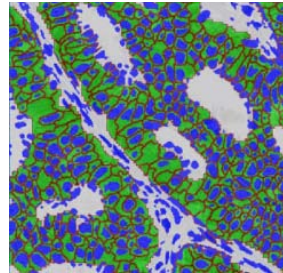


Example: a nuclear protein is measured by colocalization with DAPI in a cytokeratin positive region

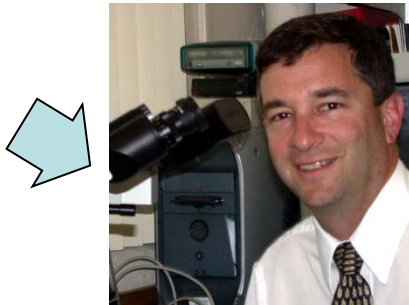
Understanding the Difference between AQUA and other tissue analysis software

Other Software: **Think like a human**
Assign significance to morphologically defined entities and use feature extraction to emulate human assignments

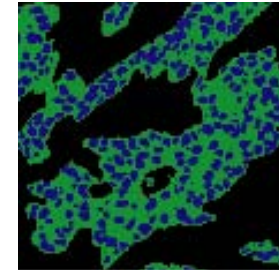
<http://www.tissuestudio.com/>



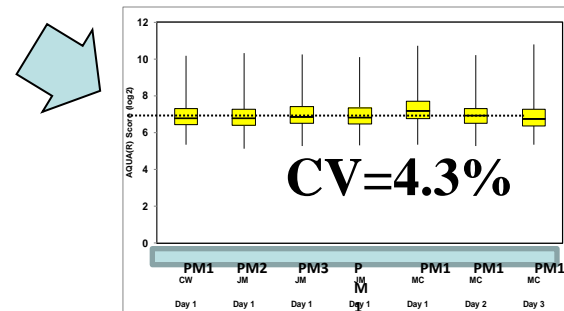
Problem: Feature extraction software does not “agree” with the pathologist since tumors (and pathologists) are very different



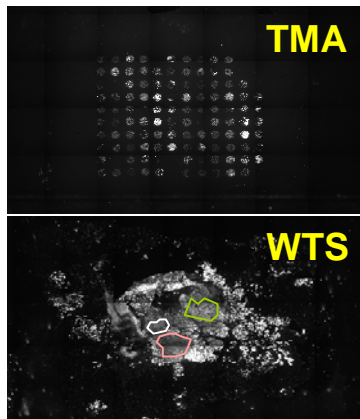
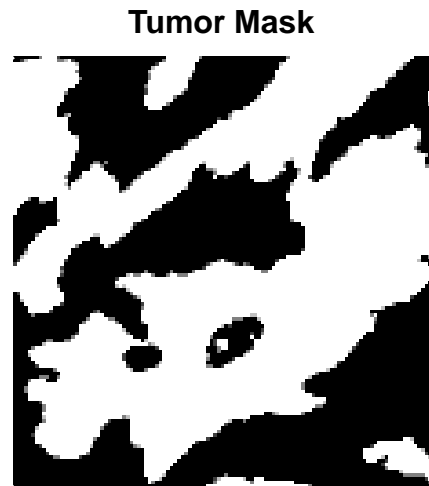
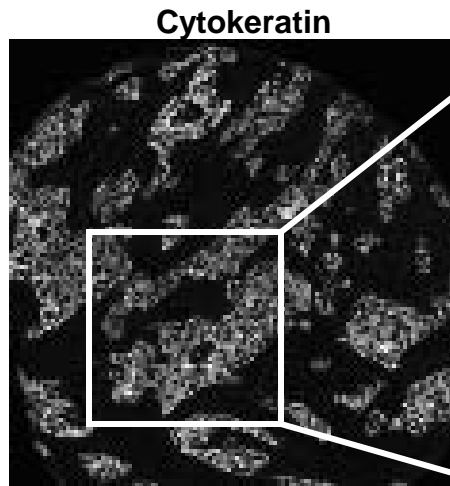
AQUA: **Think like a molecule**
Selection of regions only as a function of colocalization of molecular interactions



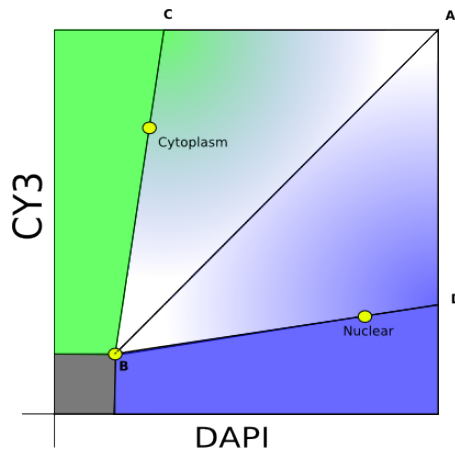
Solution: No pathologist to “agree” with since result is strictly derived from co-localization



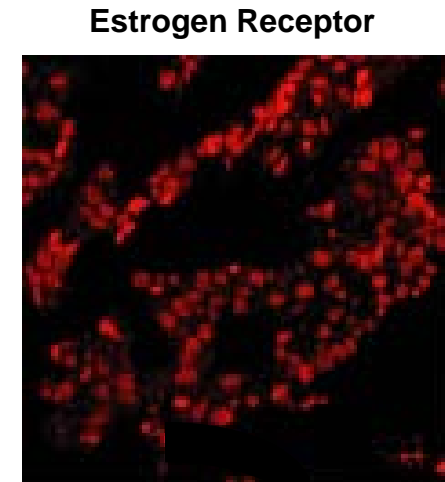
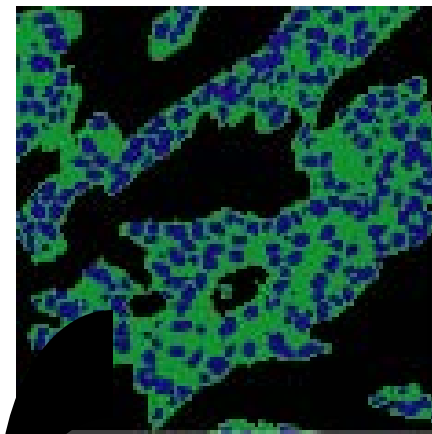
Generating the AQUA[®] score



TMA-Tissue Microarray
WTS-Whole Tissue Section



Combine DAPI image and cytokeatin image then cluster to assign each pixel to a subcellular compartment



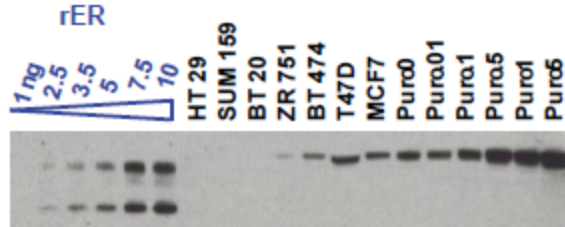
$$\frac{\Sigma \text{ target intensity in compartment pixels}}{\Sigma \text{ compartment pixel area}} = \text{AQUA score}$$

cell line panel
(known range of ER)

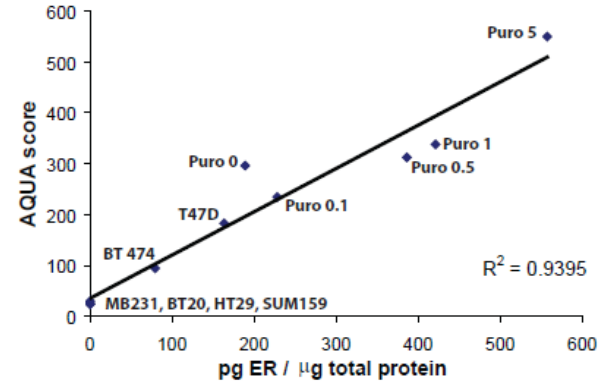
lysates

Western Blot
alongside
recombinant ER

calculate ER in pg/ μ g



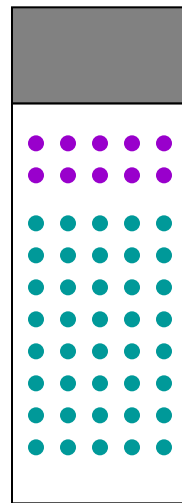
formalin-fixing &
paraffin-embedding



calculate ER as
AQUA scores

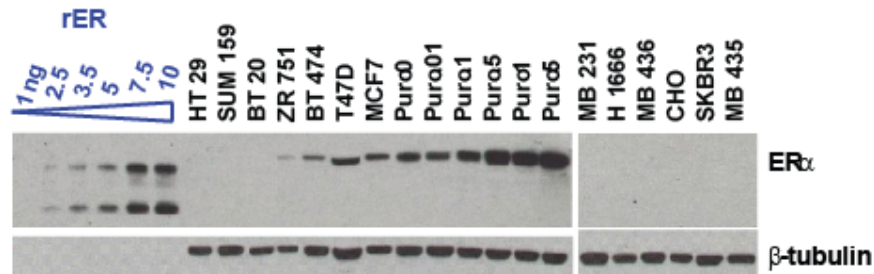
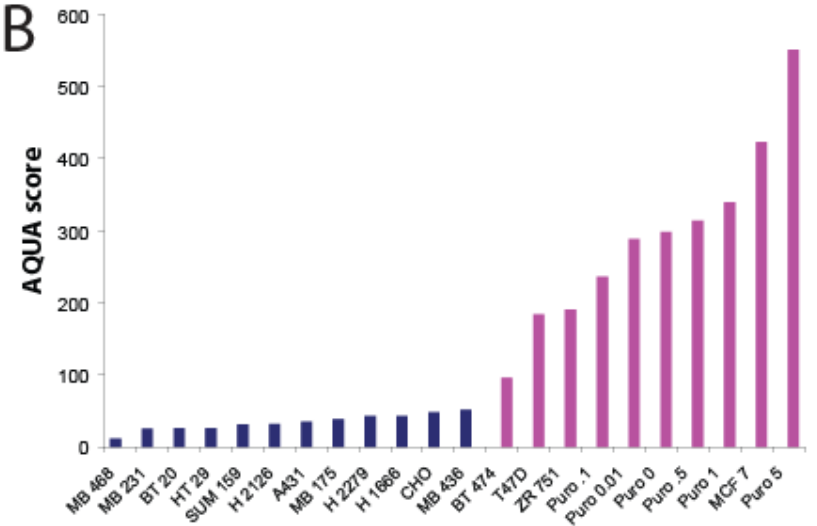
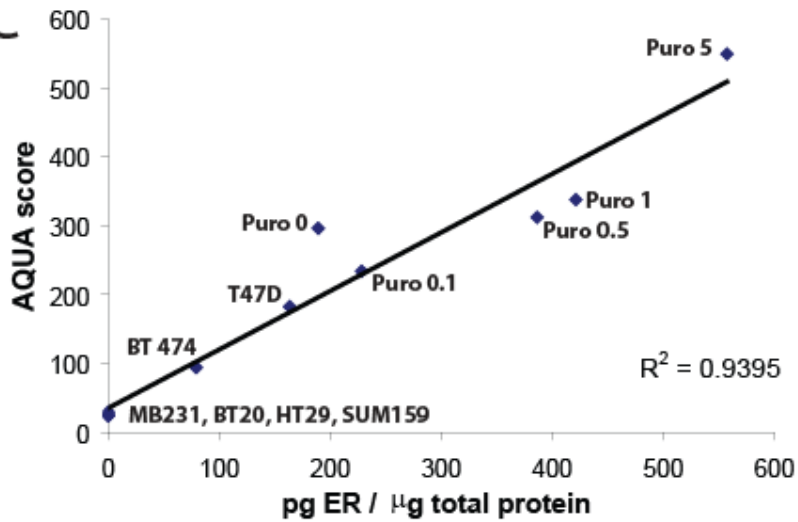
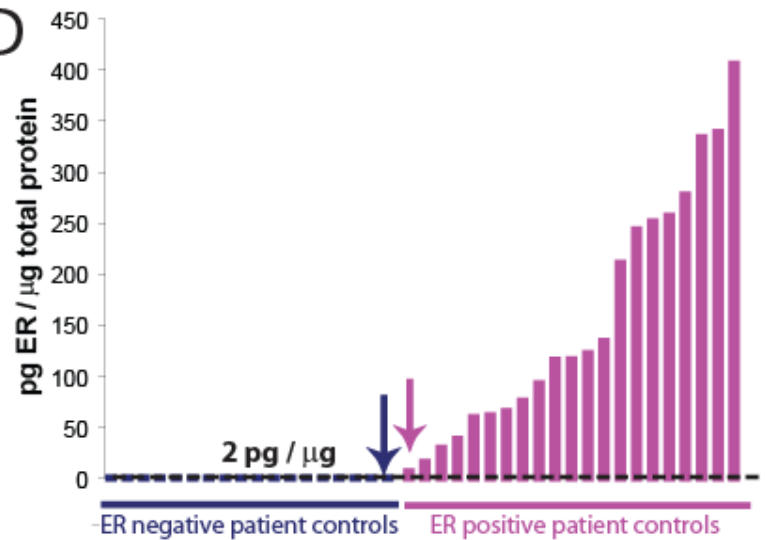


40 patient controls
(range of ER)



Control TMA

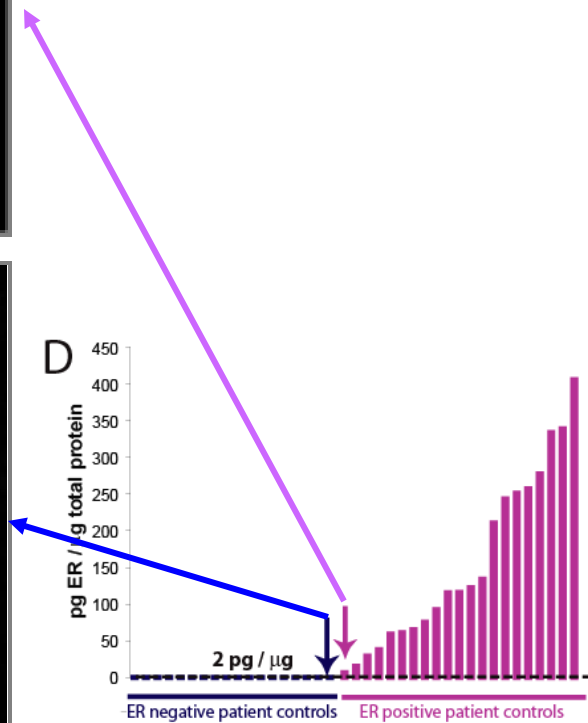
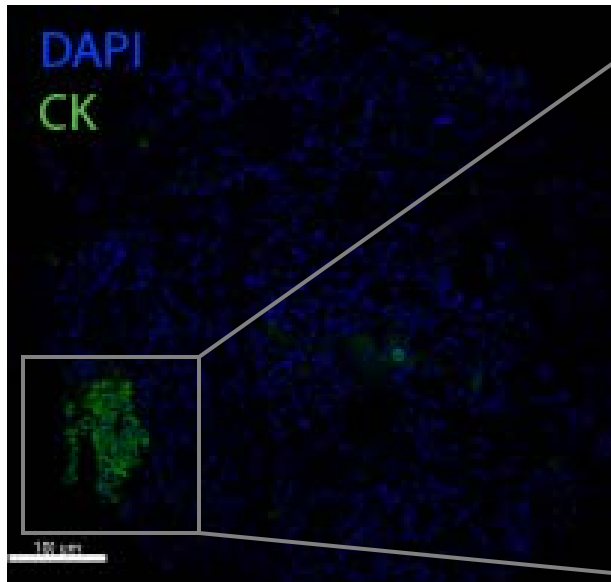
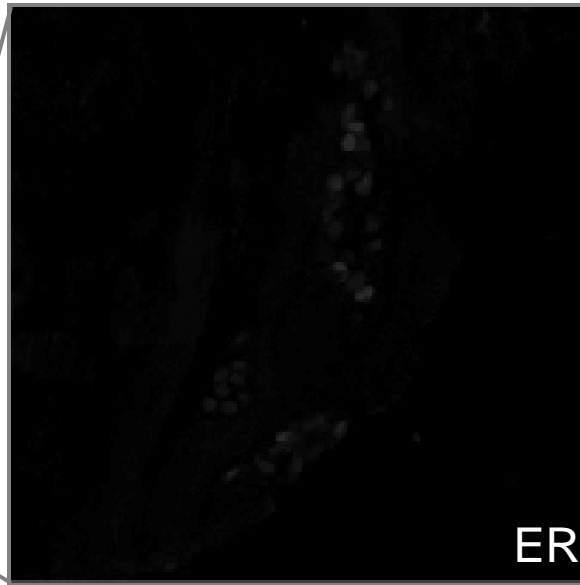
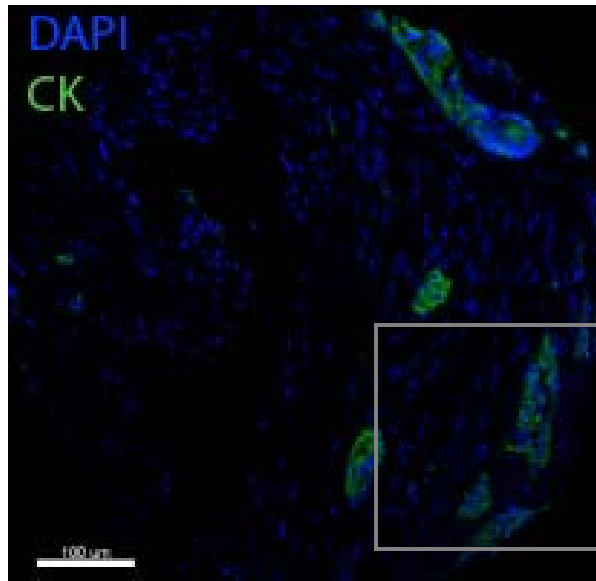
Convert AQUA scores to
pg/ μ g or other units

A**B****C****D**

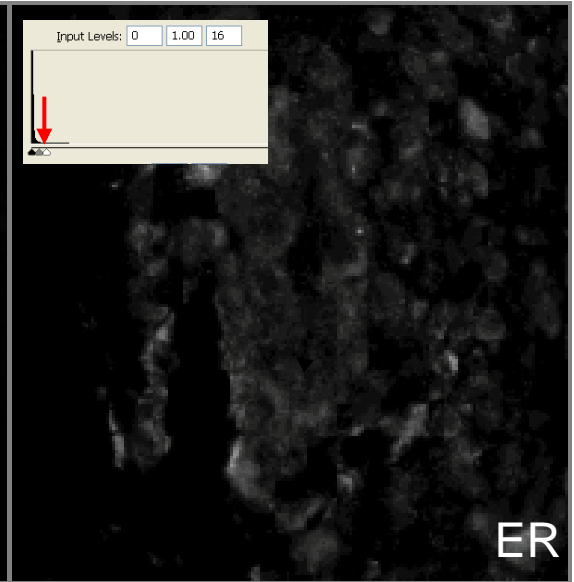
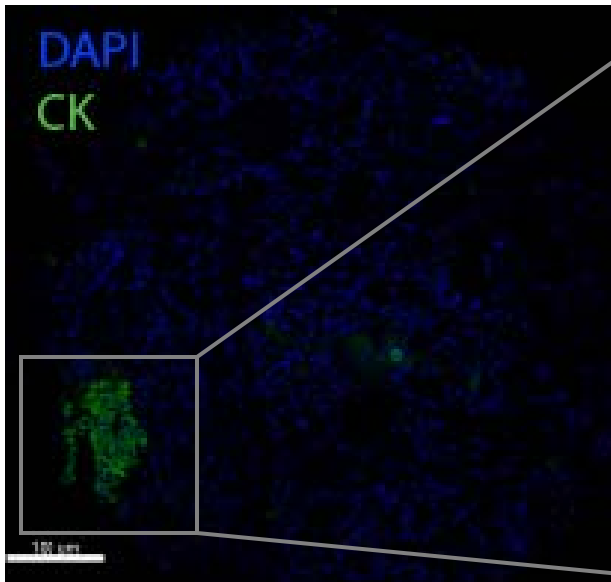
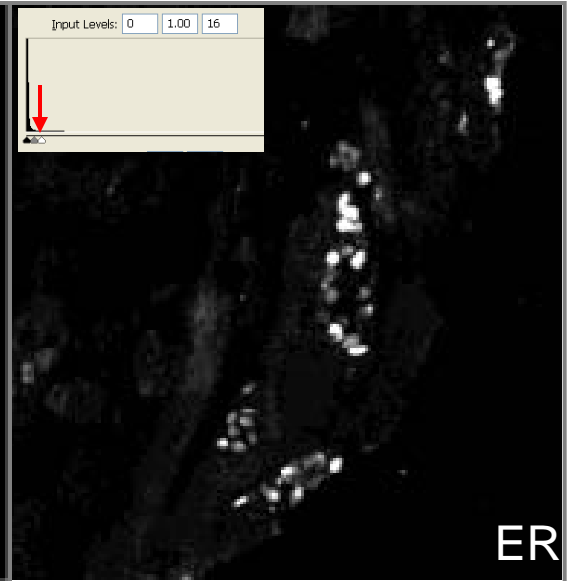
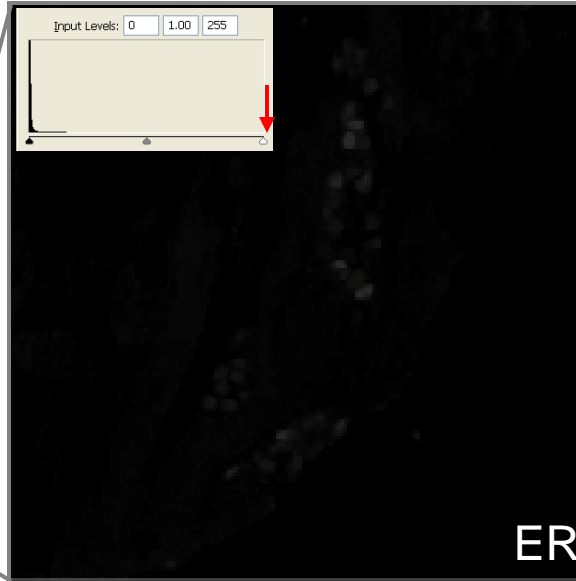
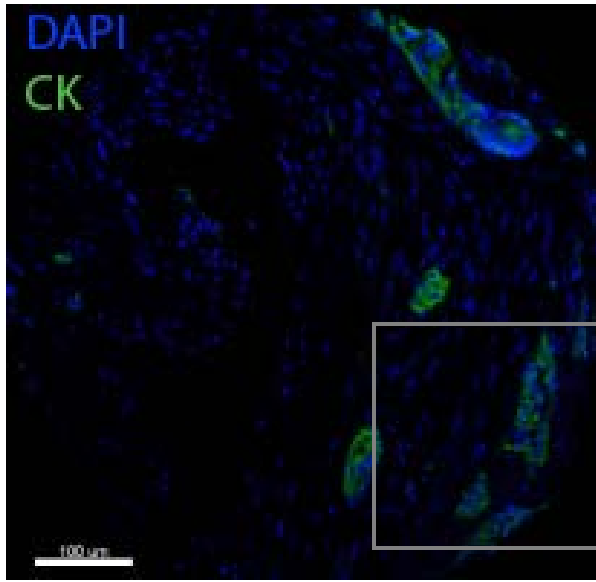
Standardized Index Array

ER antibody used is 1D5

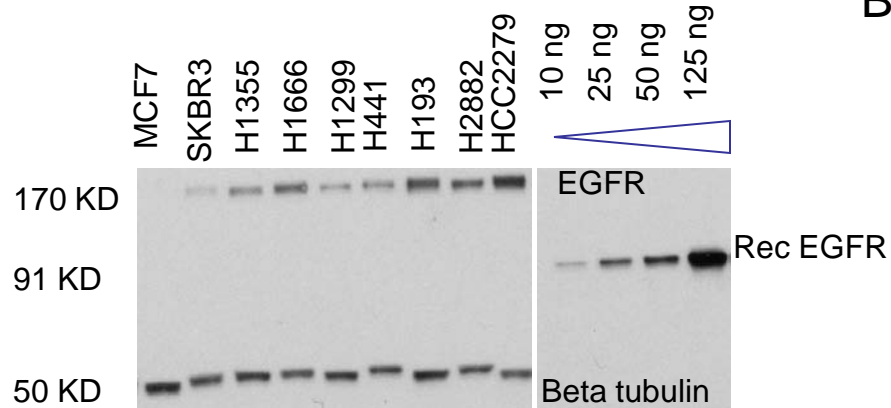
Lowest positive vs. highest negative



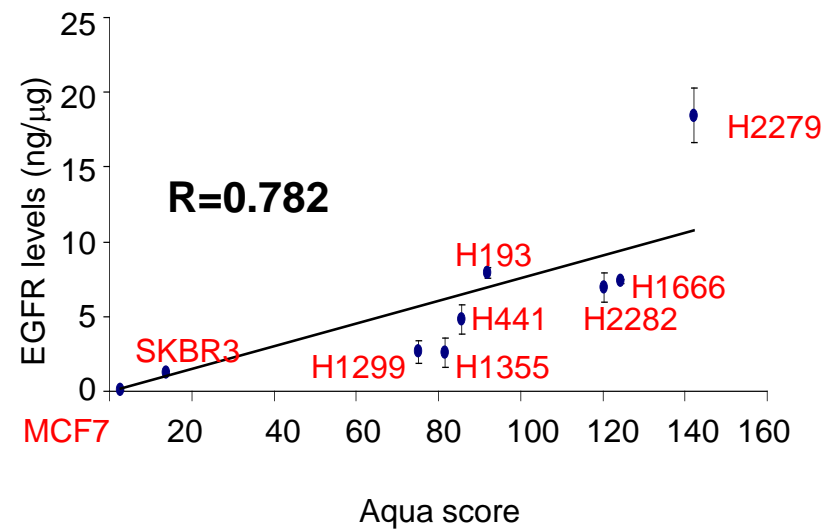
Alley Welsh



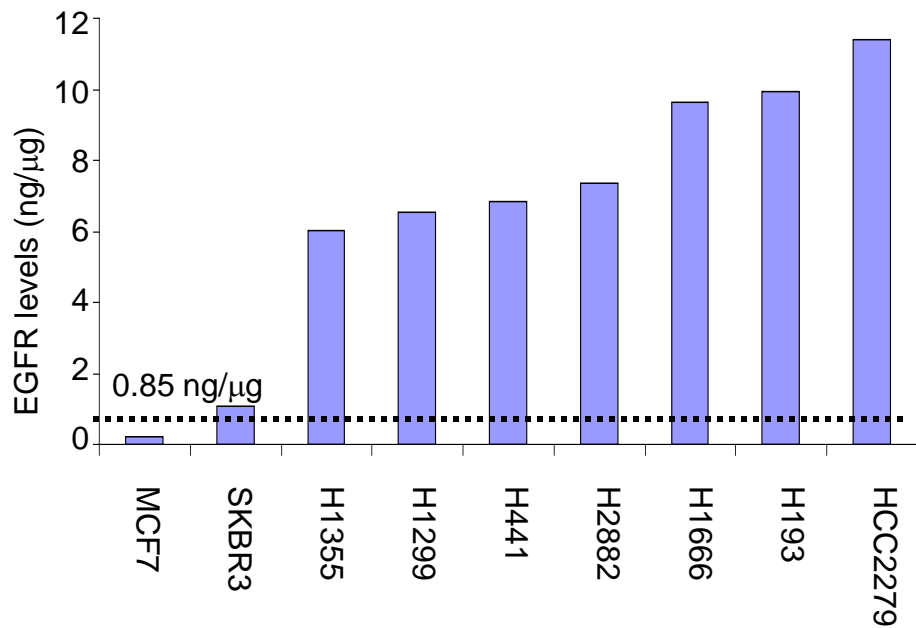
A.



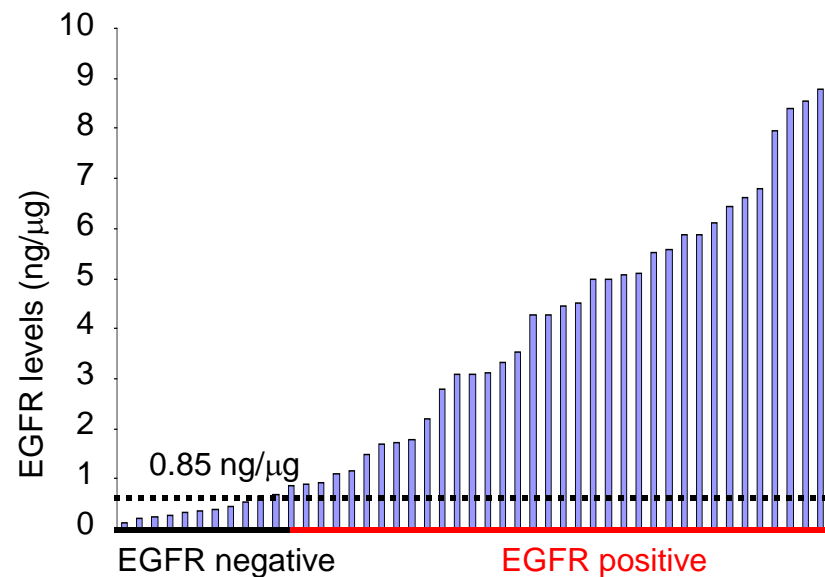
B.



C.

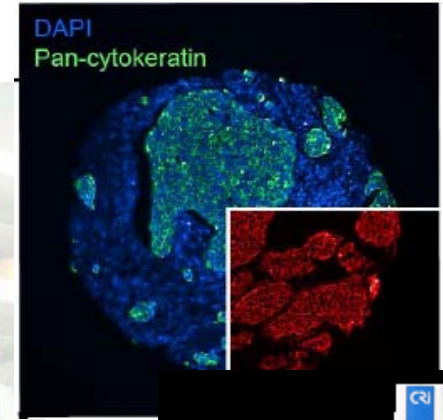
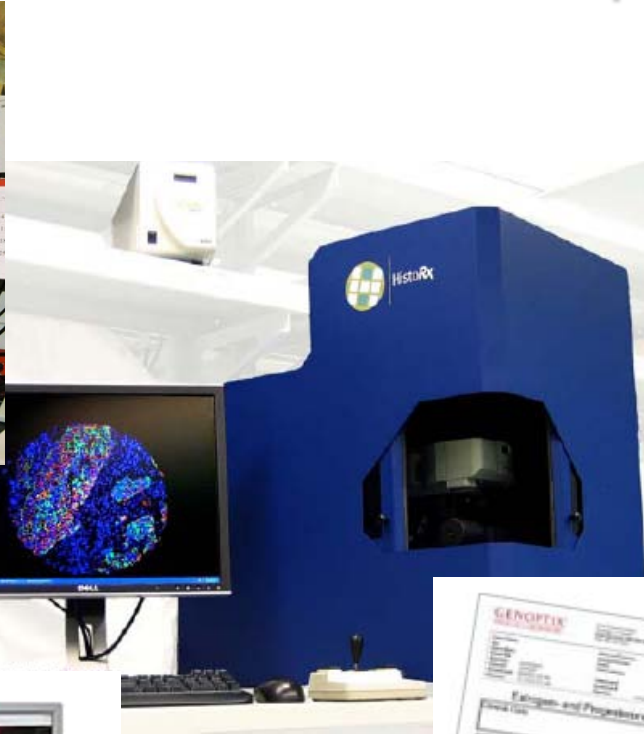


D.



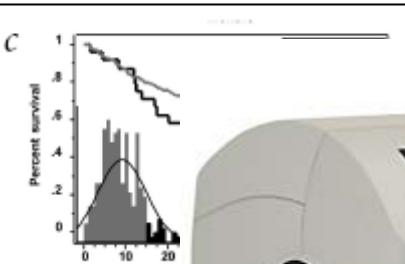
Tassos Dimou

Development and Commercialization Of a Quantitative Protein Measurement Technology (AQUA) from the lab to the patient



NATURE MEDICINE • VOLUME 8 • NUMBER 11 • NOVEMBER 2002

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Extrinsic vs Intrinsic Controls

- Extrinsic controls control for and standardize all the processes from the stainer through the analysis
- Intrinsic controls control for and standardize all the processes from the patient to the stainer (Pre-analytic variables)

Goals of our Project

- Development of a Tissue Quality Index (TQI):
- obtained by developing a quantitative intrinsic control that can measure the degree of degradation of any FFPE sample.

Pre-analytical Variables (incomplete list)

- Variable warm ischemic time
- Variable cold ischemic time
- Variable manipulation during gross cutting and prepping
- Variable temperature during fixation
- Variable total fixation time
- Variable thickness of tissue blocks
- Variable half life of fixative
- Variable types/brands/ components of fixatives
- Variable types of tissue processors
- Variable solutions in the processor
- Variable temperatures of different processor components
- Variable types of embedding paraffin
- Variable slide drying times
- Variable slide oven temperatures

Pre-Analytic Variables; Can we treat them as a black box?

If we cannot control pre-analytical variables can we quantify the damage or tissue degradation caused by them?

Can we disqualify specimens for companion dx testing?



Intrinsic Controls for FFPE tissue

Goal 1: To generate two “discovery” tissue sets to assess “pre-analytical” variability.

Goal 2: Assessment of markers of cold ischemia (“housekeeping markers”) on discovery cohorts

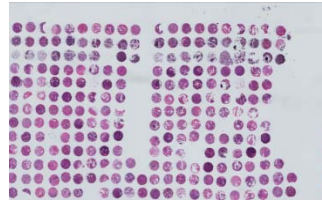
Goal 3: Assessment of markers of hypoxia on discovery cohorts

Goal 4: Generation of a Multiplexed “Tissue Immunologic Competence” (TIC) Model (now Tissue Quality Index (TQI)) for normalization of tissue handling that measures tissue integrity for immunological assessment

Goal 5: Validation testing of the TIC Model in two core vs. resection specimen studies

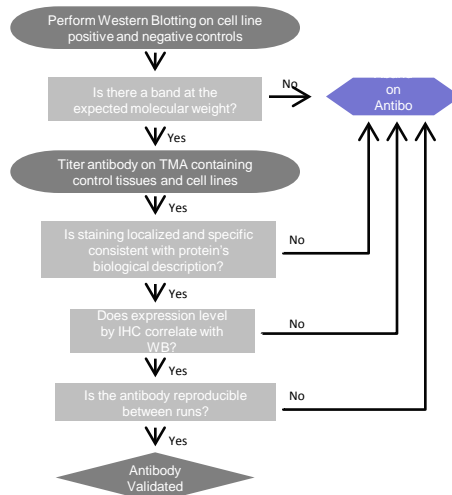
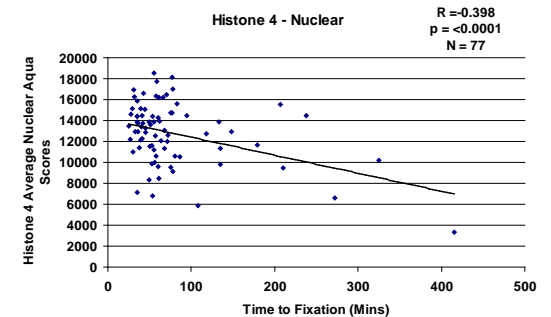
Approach

Generate
Intrinsic Control
cohorts



Select and validate
potential
antibodies/reagents

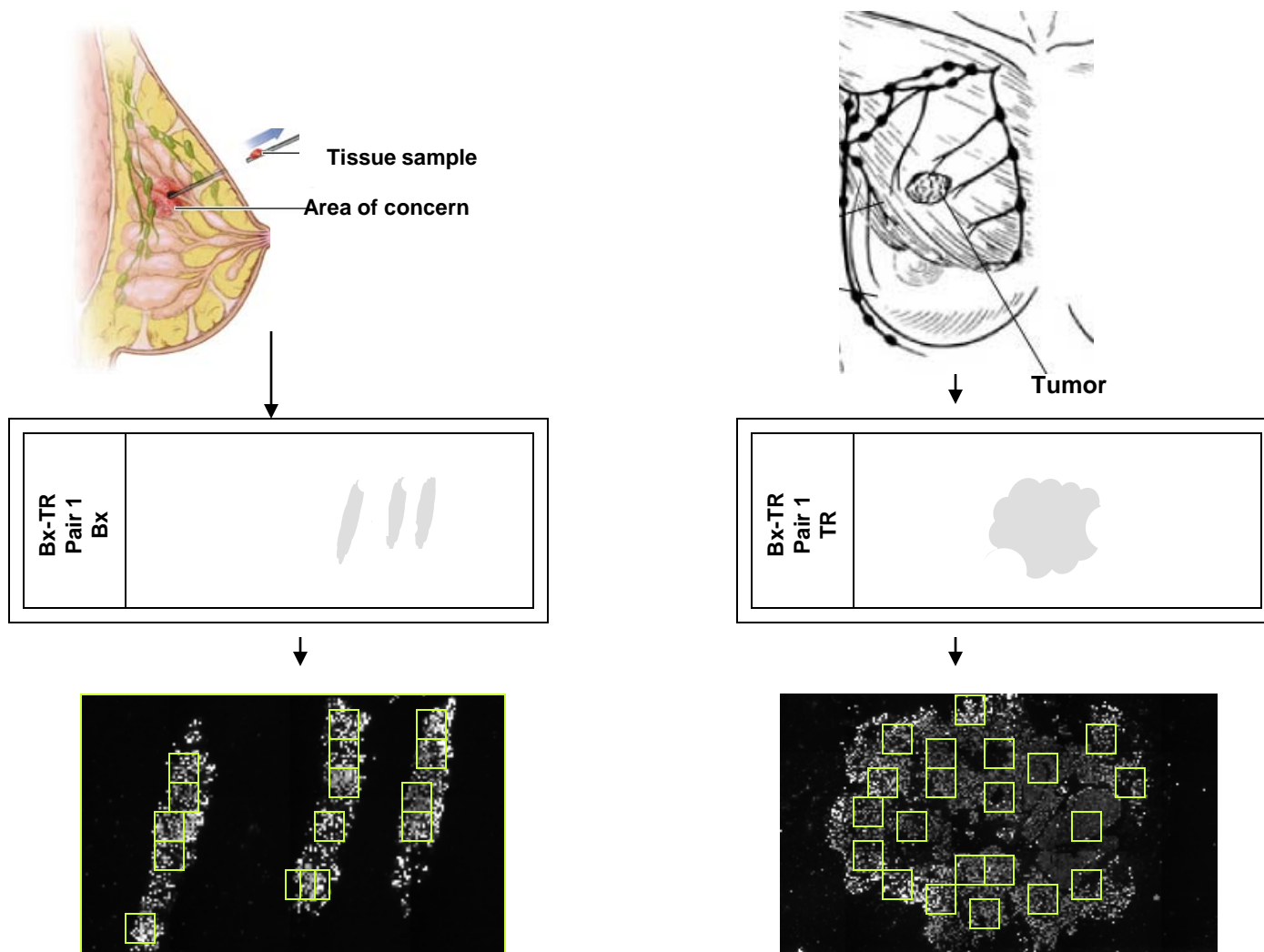
Test each reagent
individually on
Intrinsic Control
Cohorts




Generate simplest
Multi-variable model
that can assess
tissue quality (TQI)

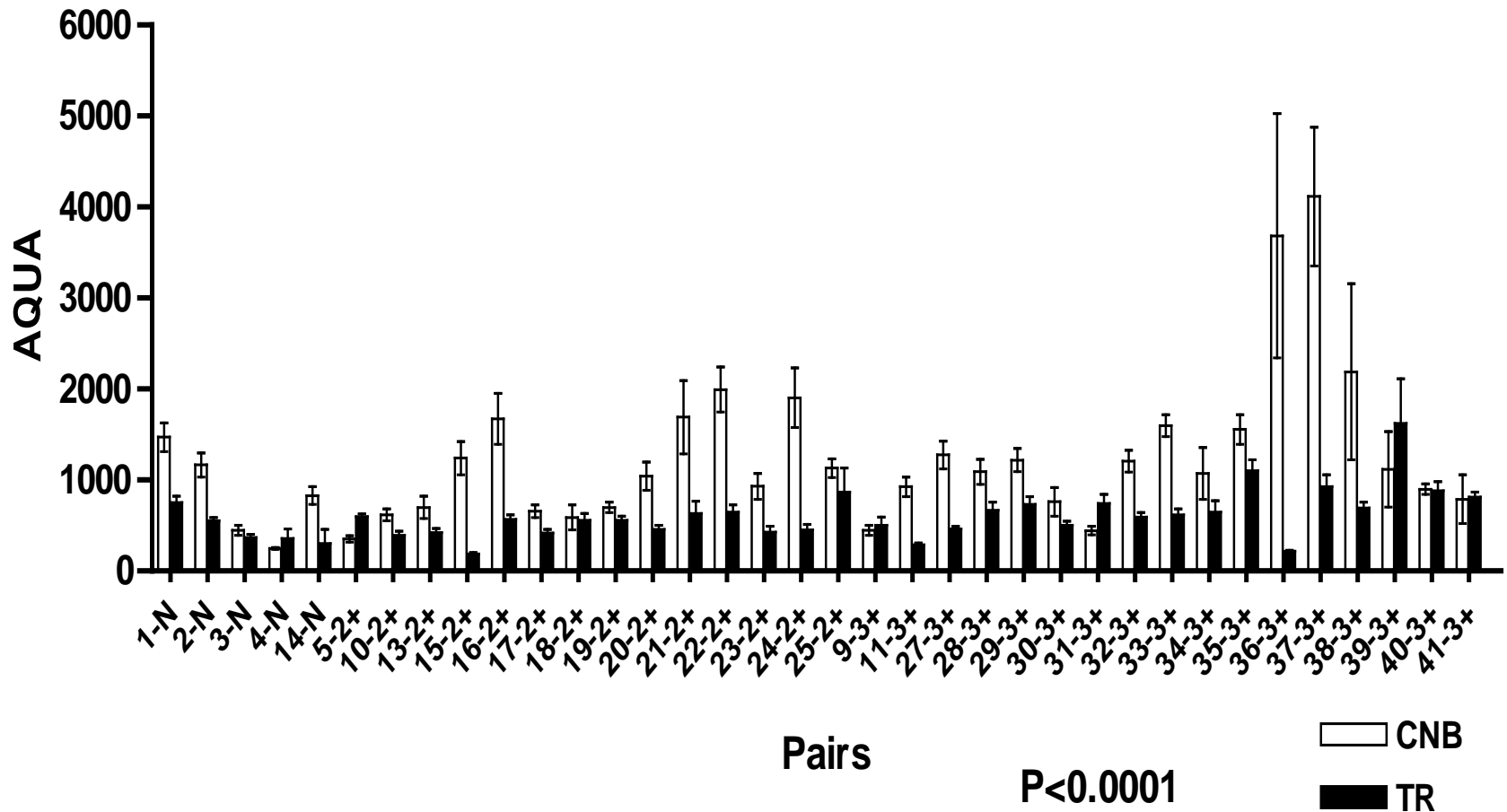
Validate TQI

Core Bx – Resection Pair Cohorts

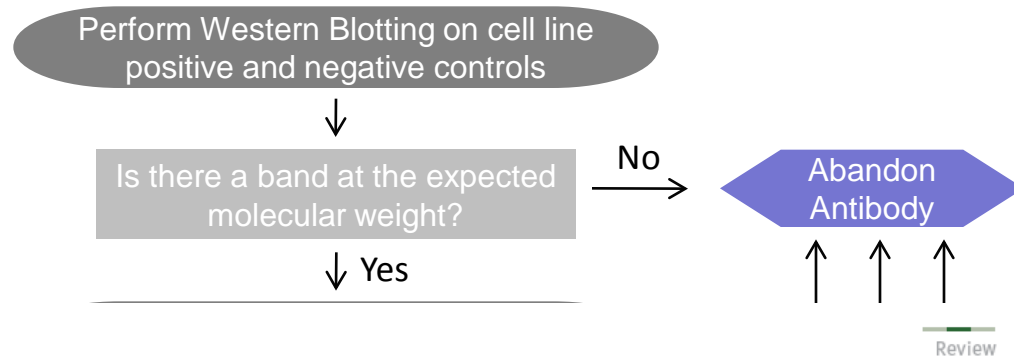


 Field of View (FOV)

Example of CNB – Resection Cohort (from other studies)



Antibody Validation (Overview)



Review

Antibody validation

Jennifer Bordeaux, Allison W. Welsh, Seema Agarwal, Elizabeth Killiam, Maria T. Baquero, Jason A. Hanna, Valsamo K. Anagnostou, and David L. Rimm

Department of Pathology, Yale University School of Medicine, New Haven, CT, USA

BioTechniques 48:197-209 (March 2010) doi 10.2144/000113382

Keywords: antibody; validation; immunohistochemistry; immunofluorescence

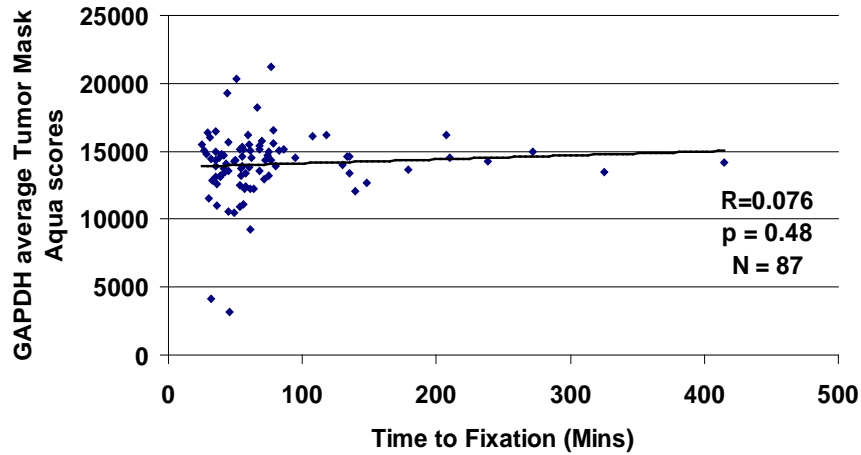
Antibodies are among the most frequently used tools in basic science research and in clinical assays, but there are no universally accepted guidelines or standardized methods for determining the validity of these reagents. Furthermore, for commercially available antibodies, it is clear that what is on the label does not necessarily correspond to what is in the tube. To validate an antibody, it must be shown to be specific, selective, and reproducible in the context for which it is to be used. In this review, we highlight the common pitfalls when working with antibodies, common practices for validating antibodies, and levels of commercial antibody validation for seven vendors. Finally, we share our algorithm for antibody validation for immunohistochemistry and quantitative immunofluorescence.

Symbol	Description	Origin	Antibody Clone/Isotype	Catalog #	Validated	Supplier
Markers of Cold Ischaemia						
ACTB	Beta-Actin	Rabbit	13E5/IgG	4970	yes	Cell Signaling Technology
TUBB	Beta-Tubulin	Rabbit	pF3/IgG	2128	yes	Cell Signaling Technology
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Rabbit	14C10/IgG	2118	yes	Cell Signaling Technology
HIST4	Histone 4	Mouse	L64C1	2935	yes	Cell Signaling Technology
HIST3	Histone 3	Mouse	96C10/IgG1, kappa	3680	yes	Cell Signaling Technology
HIST2A	Histone 2A	Mouse	L88A6/IgG1	3636	yes	Cell Signaling Technology
RPL19	Ribosomal Protein 19	Mouse		sc-100830	no	Santa Cruz Biotechnology
RPL9	Ribosomal Protein 9	Mouse		sc-100828	no	Santa Cruz Biotechnology
RPS16	Ribosomal Protein 16	Rabbit	polyclonal	sc-102087	no	Santa Cruz Biotechnology
LMNA/C	Lamin A/C	Rabbit		2032	yes	Cell Signaling Technology
LDH	Lactat Dehydrogenase	Rabbit			yes	Cell Signaling Technology
Markers of Hypoxia						
VEGF	Vascular Endothelial Growth Factor	Mouse	VG1/IgG1, kappa		no	DAKO
CCND1	Cyclin D1	Rabbit	IgG		yes	Thermo Fisher Fremont
Caspase	Cleaved Caspase 3 (Asp175)	Rabbit		9661	yes	Cell Signaling Technology
HIF1	Hypoxia Inducible Factor 1	Rabbit		NB 100-449	yes	Novus Biological
AKAP13	A-kinase anchoring protein13	Mouse		sc-81902	yes	Santa Cruz Biotechnology
CDC42		Mouse		sc-8401	yes	Santa Cruz Biotechnology
CCNB1	Cyclin B1	Mouse	GNS-11/IgG2	554178	yes	BD Biosciences
UBE2Q2	Ubiquitin conjugated enzyme E2 Q2	Mouse	IgG2a	sc-100625	no	Santa Cruz Biotechnology
HIF-2alpha	Hypoxia inducible factor - 2alpha	Mouse	ep190b/IgG1	ab8365	yes	abcam
HIF-3A	Hypoxia inducible factor - 3A	Rabbit	polyclonal(aa581-592)	LS-B714	in progress	Lifespan Biosciences
CA9	Carbonic Anhydrase IX	Rabbit	polyclonal(aa581-592)LS-B273		no	Lifespan Biosciences
Eosin	Shandon EosinY aqueous			6766009	yes	Thermo Electron Corporation
Markers of phosphorylated proteins						
pAKT 473	phospho-Akt (ser473)	Rabbit	D9E/IgG	4060	in progress	Cell Signaling Technology
pAKT 308	Phospho-Akt (Thr308)	Rabbit	C31E5E/IgG	2965	in progress	Cell Signaling Technology
pMAPK	Phospho-p44/43MAPK (Erk1/2) (Thr292/Tyr204)	Rabbit	IgG	4370	yes	Cell Signaling Technology
pER	Phospho-Estrogen Receptor alpha (Ser118)	Mouse	16J4/IgG2b	2511	yes	Cell Signaling Technology
4G10	Anti-Phosphotyrosine	Mouse	IgG2b	05-1050	yes	Millipore

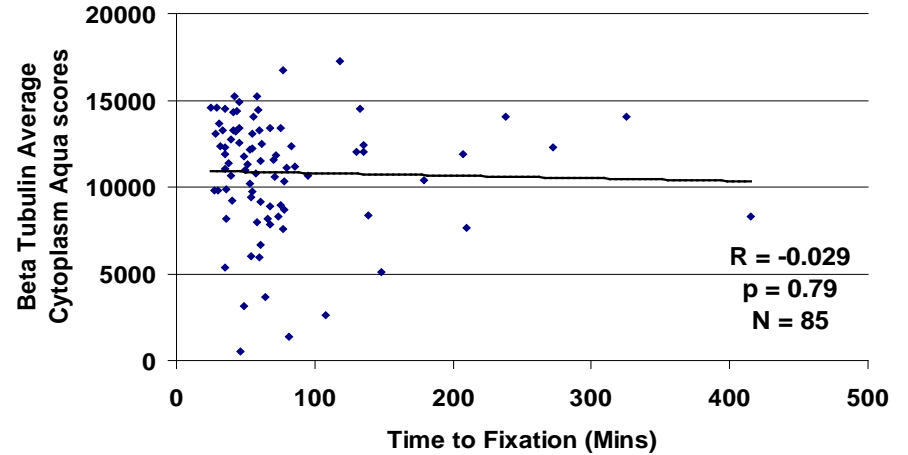
These markers show either an increase or a decrease of expression with time to fixation on the time to fixation TMA

Correlation between markers of cold ischemia and hypoxia with time to fixation

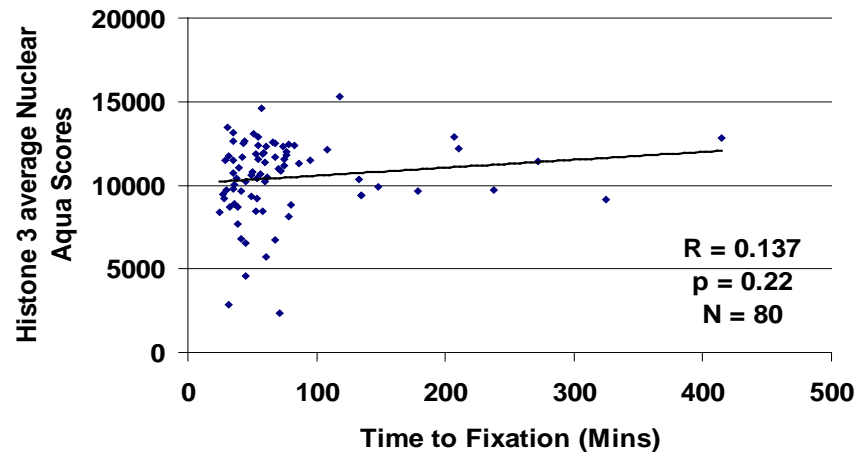
GAPDH - Tumor Mask



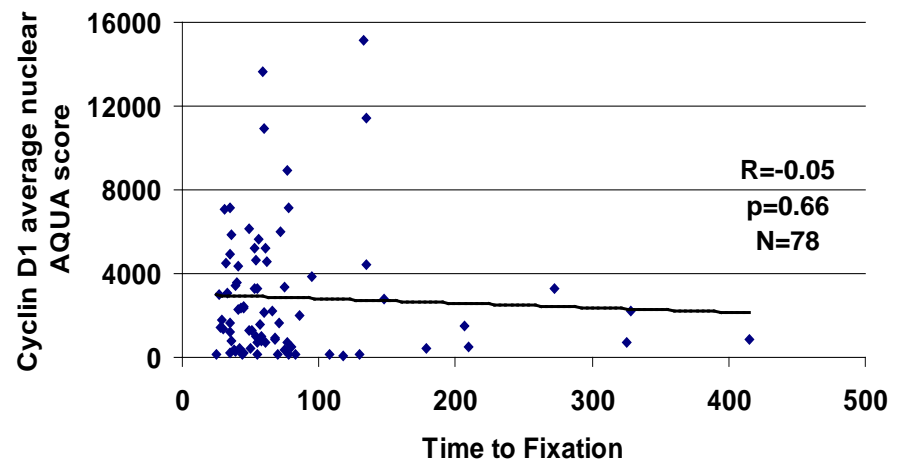
Beta Tubulin - Cytoplasm

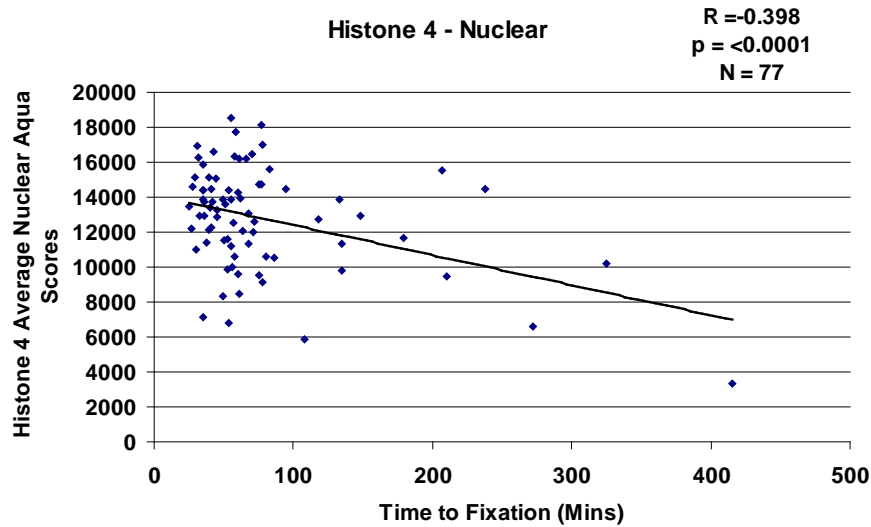


Histone 3 - Nuclear

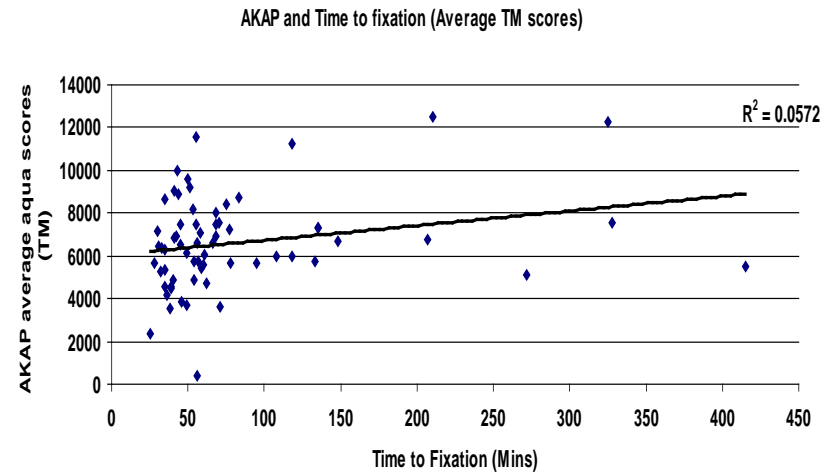
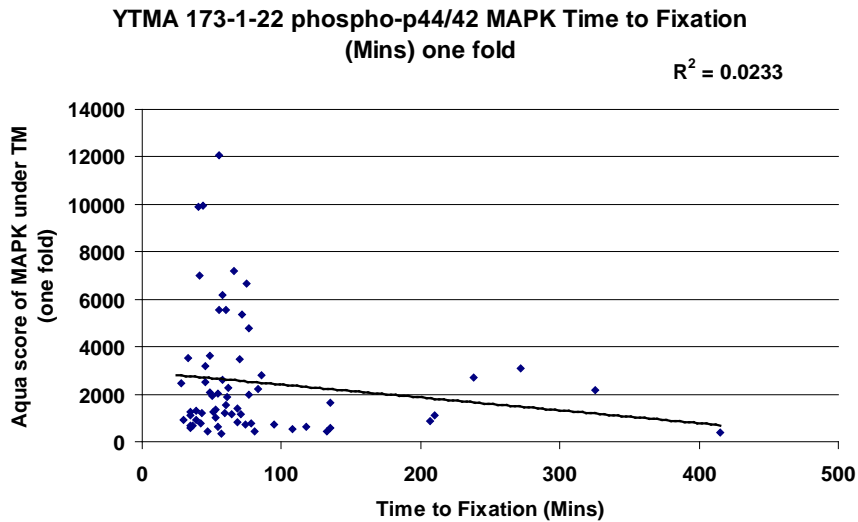


Cyclin D1-Nuclear

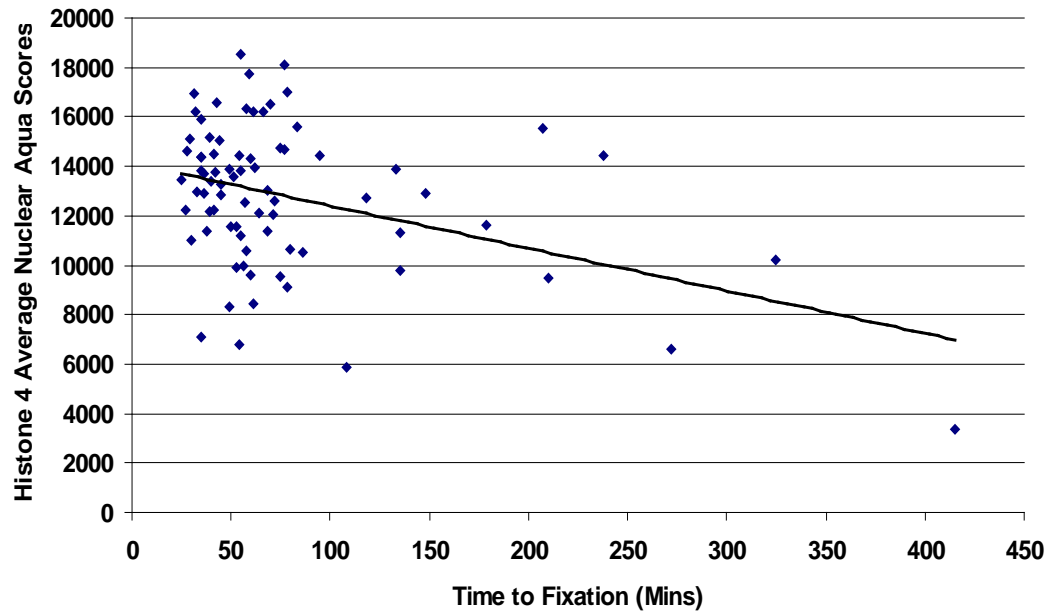




While some show a downward or upward trend, heterogeneity is a concern



Histone 4 on Time to Fixation Array

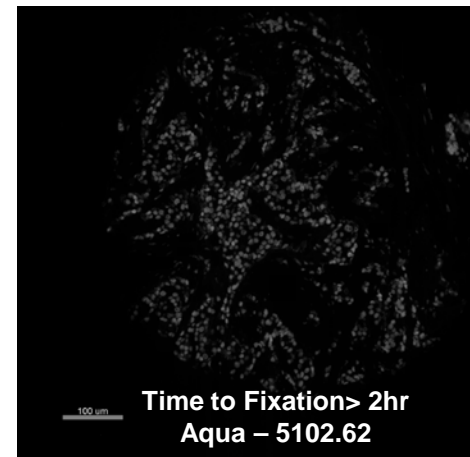
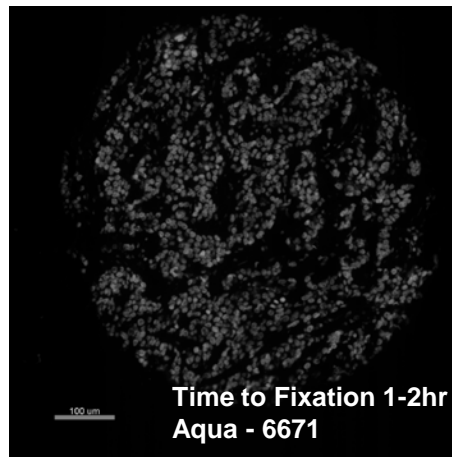
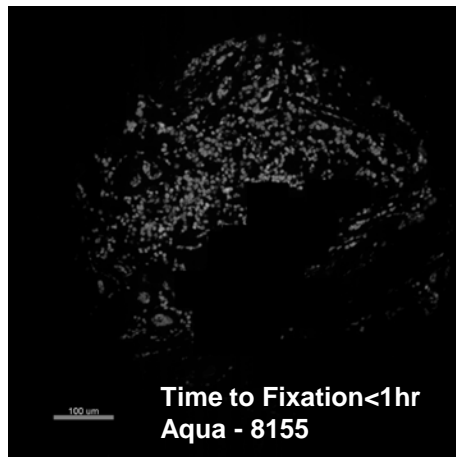


Spearman Rank Correlation:

$P < 0.0001$

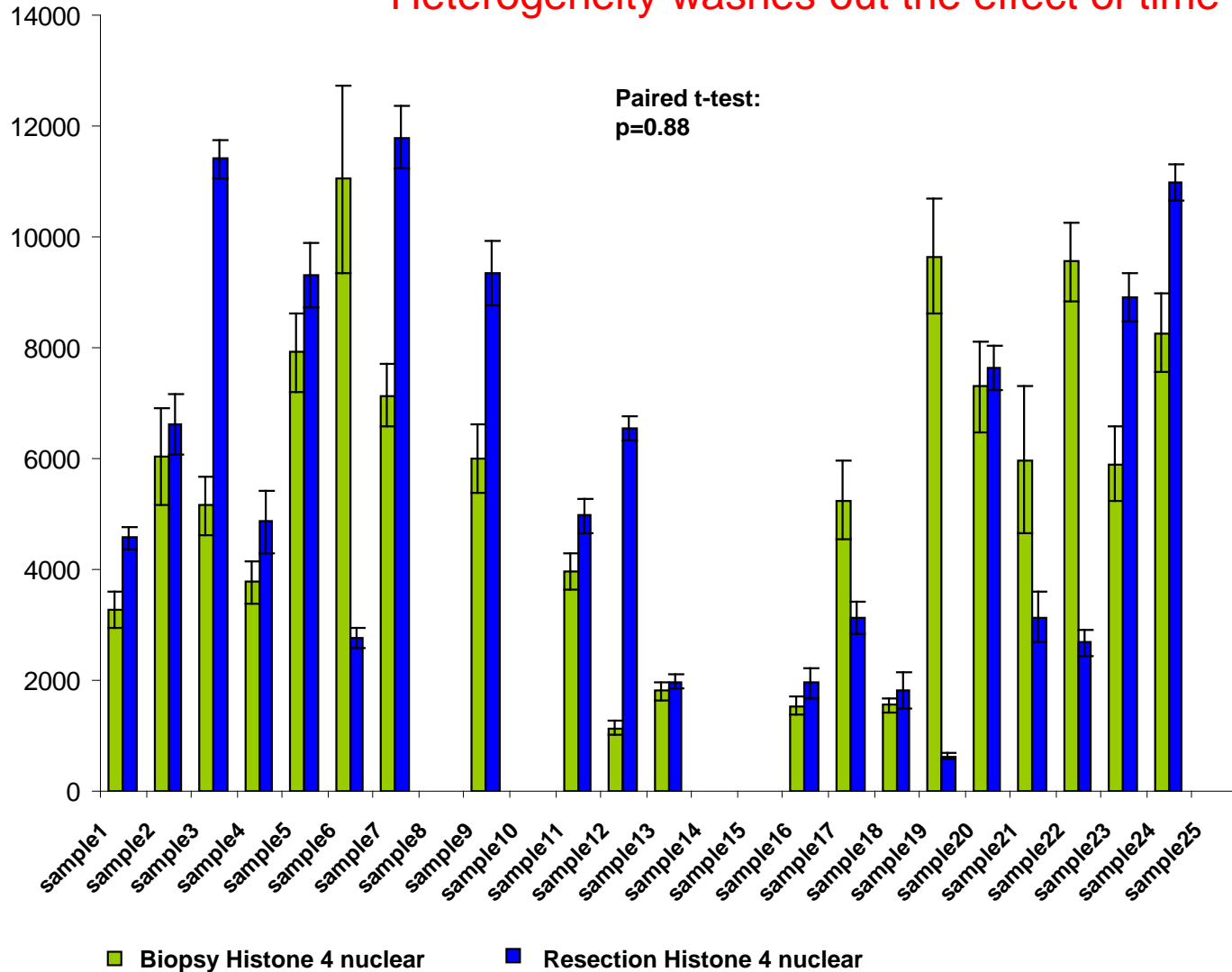
$R = -0.398$

$N = 77$



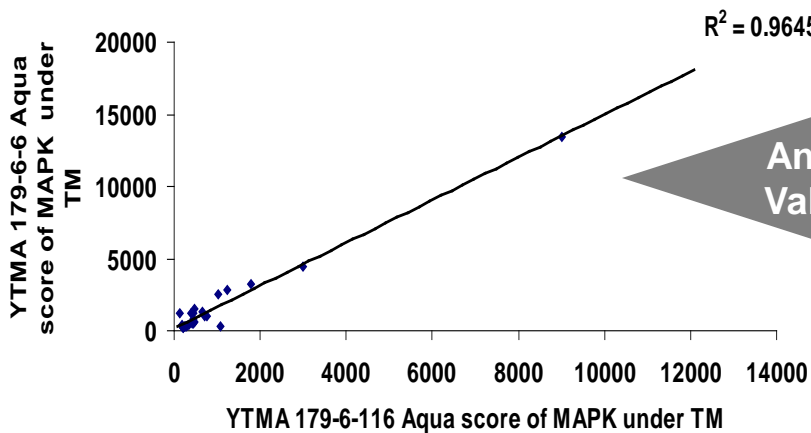
Histone 4 on 25 matched pairs of biopsies and resections

Heterogeneity washes out the effect of time to fixation



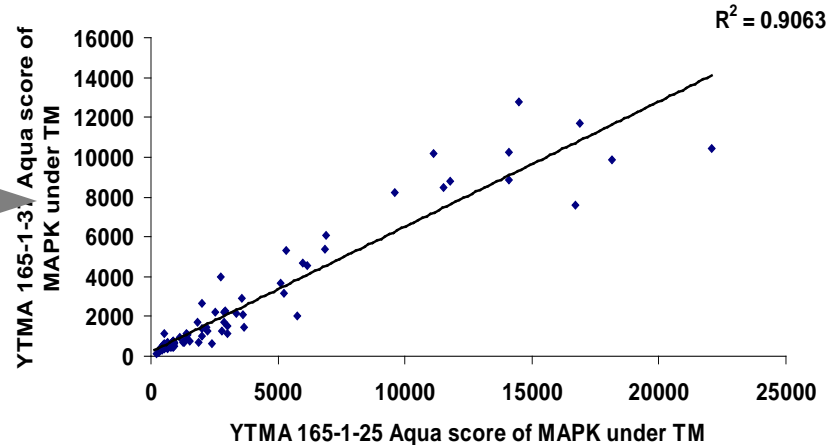
Assay Reproducibility

phospho-MAPK test to test reproducibility

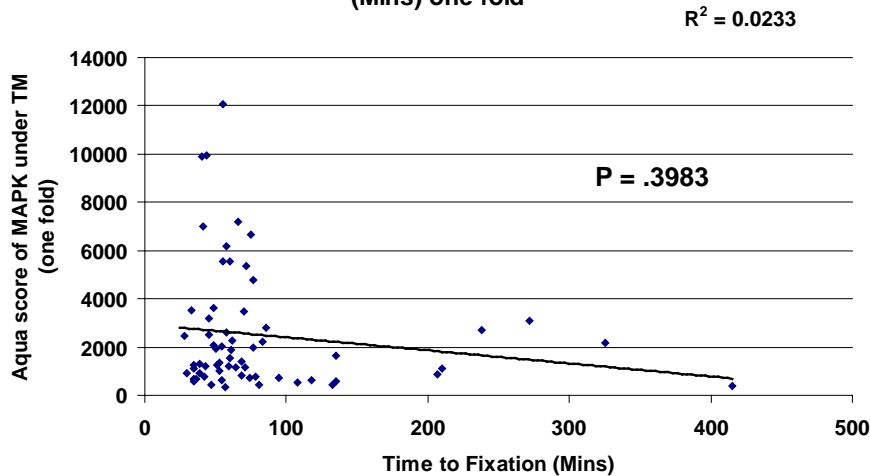


Antibody Validated

YTMA 165- Tic Reproducibility

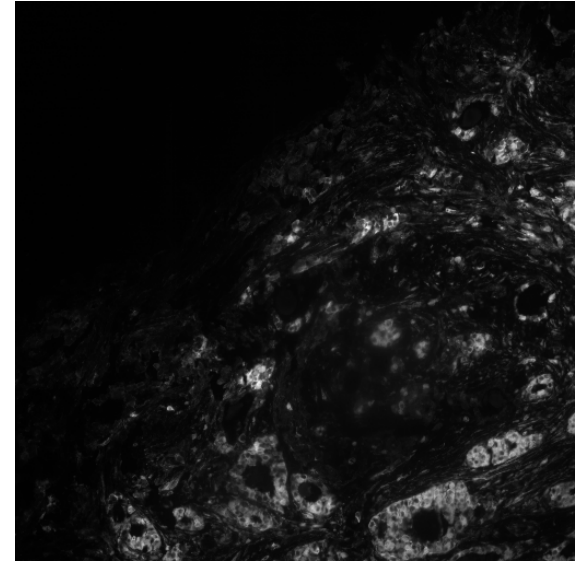
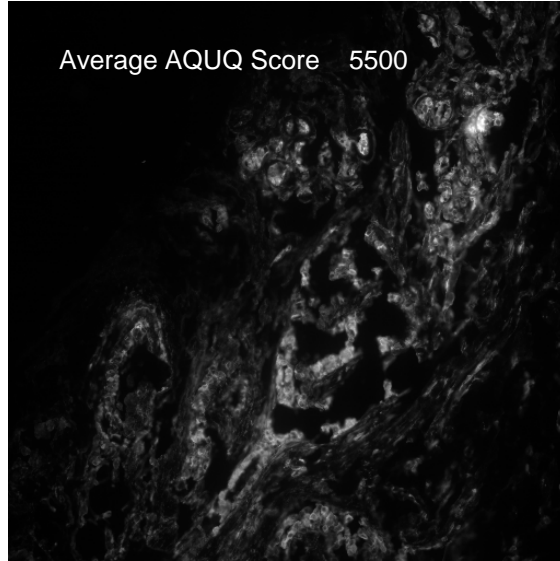
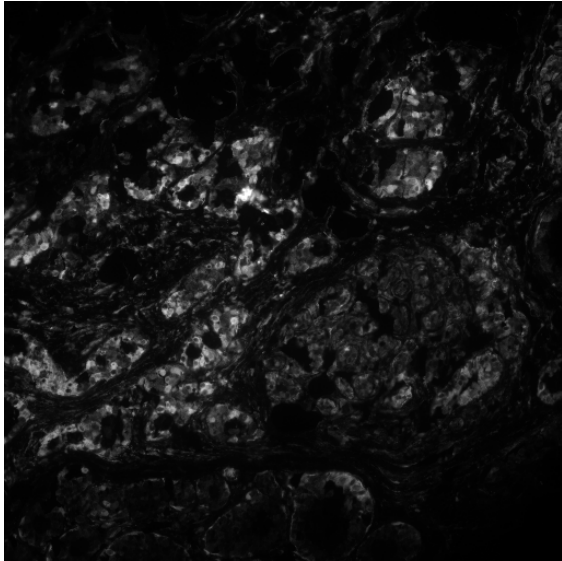


YTMA 173-1-22 phospho-p44/42 MAPK Time to Fixation (Mins) one fold

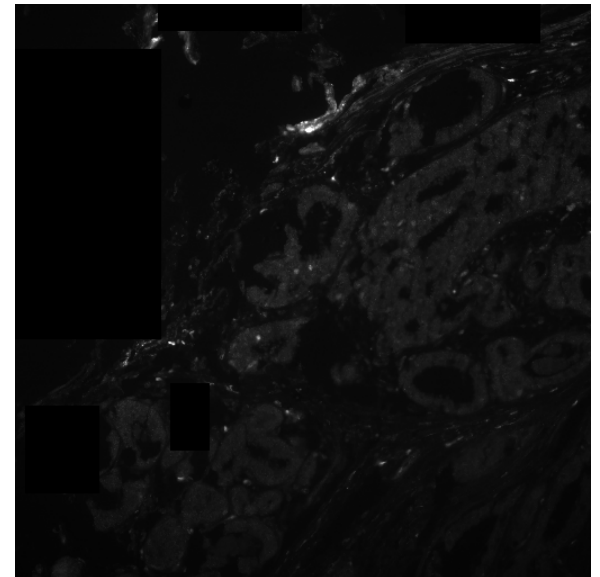
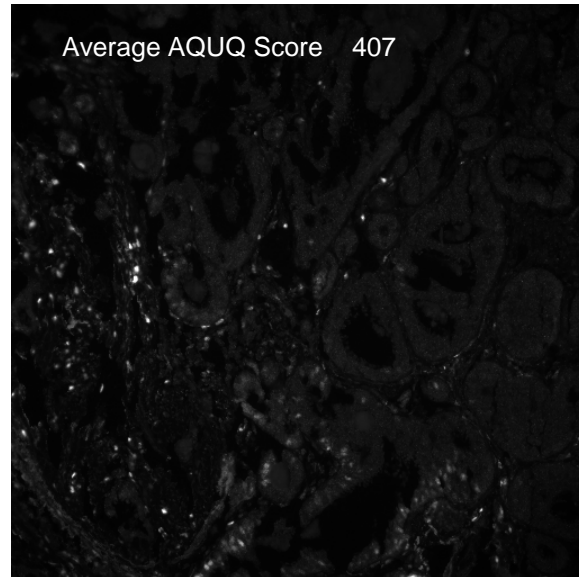
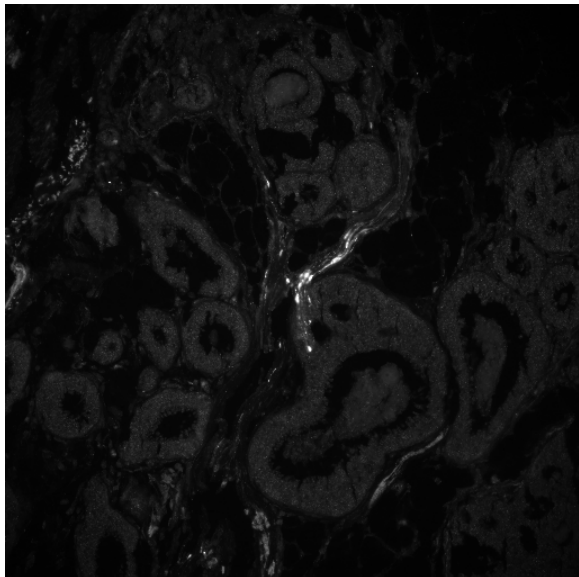


The Spearman Rank Correlation shows a trend (p-value not significant) towards negative correlation.

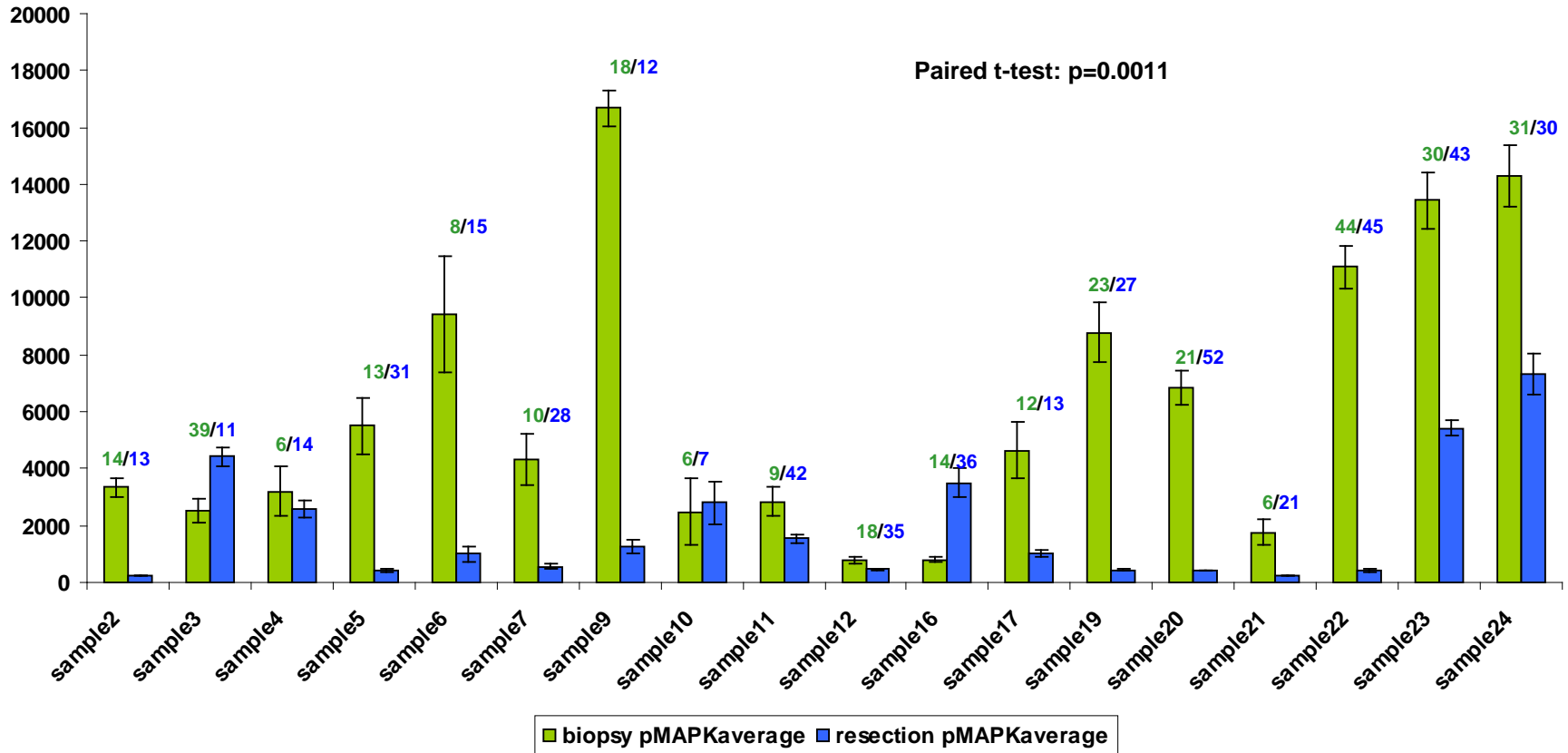
Biopsy patient 5 pMAPK Immunohistochemistry



Resection patient 5 pMAPK Immunohistochemistry



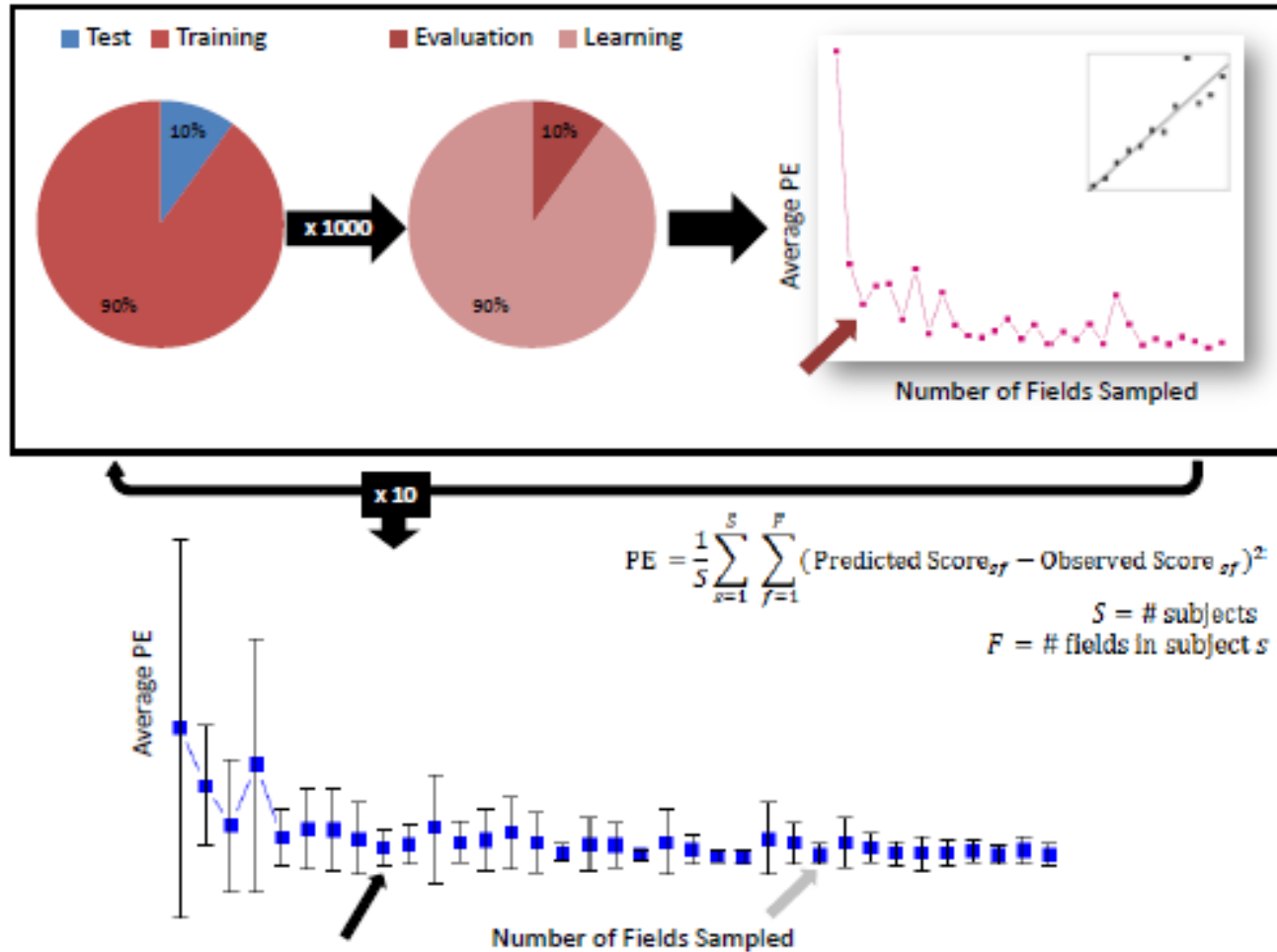
Assessment of pMAPK expression on 25 matched pairs of biopsies and resections



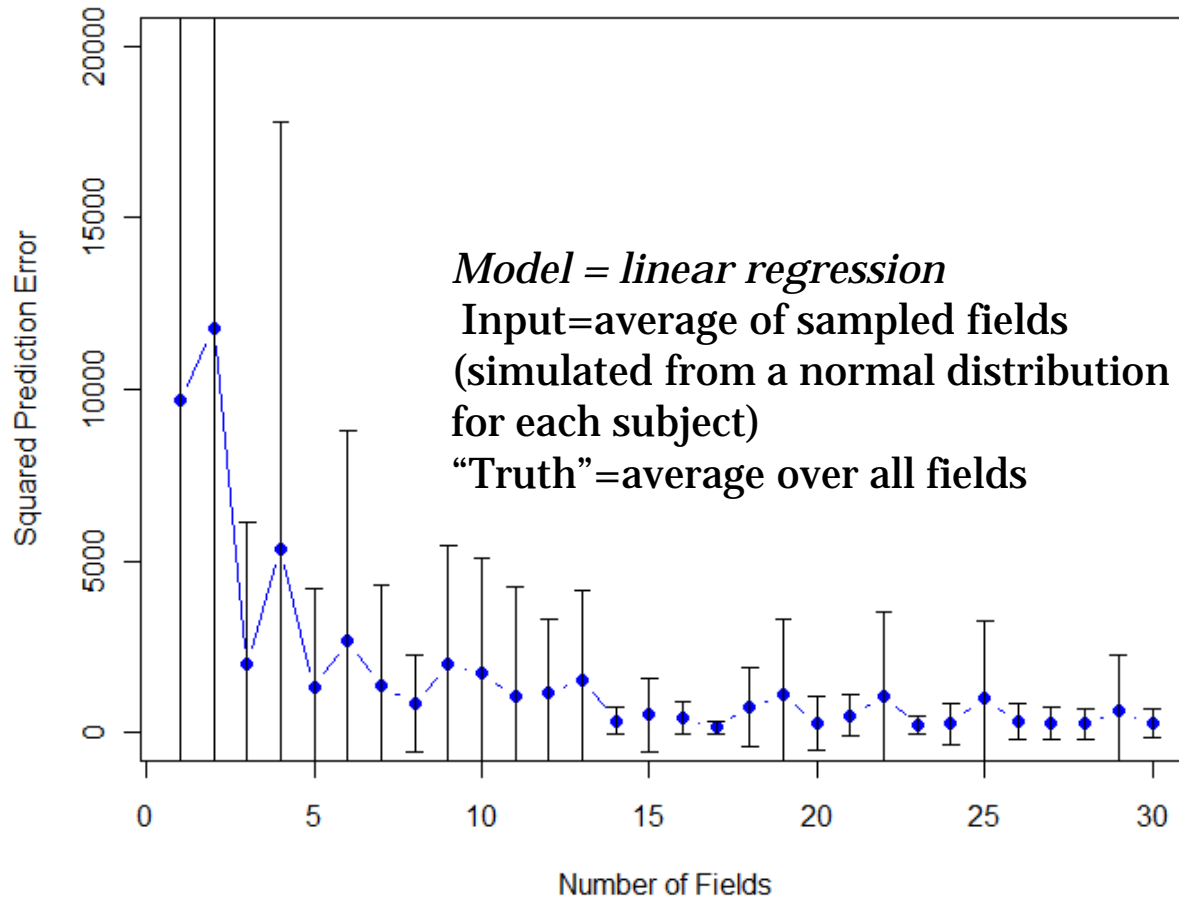
Samples missing: 1,8,13,14,15,18,25

Results for 18 pairs

Mixed Effects Model for Estimating Number of Fields Required for Immunostaining



Estrogen Receptor: Estimated Prediction Error Criterion



The number of FOVs required are a function of the protein examined

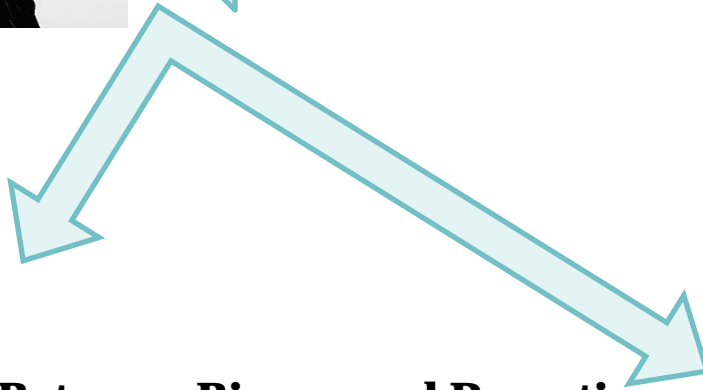
Marker	Optimal Number of 20X FOVs	SE of Optimal Number (FOVs)
ER	8	3.4
HER-2	5	3.0
AKT	4	1.5
ERK	6	2.5
S6K1	6	3.4
GAPDH	12	4.1
Cytokeratin	3	4.3
MAP-Tau	14	4.2
MAP-Tau (direct sampling)	14	4.2

Number of 20X fields of view (FOVs) to find stable minimum in mixed effects modeling

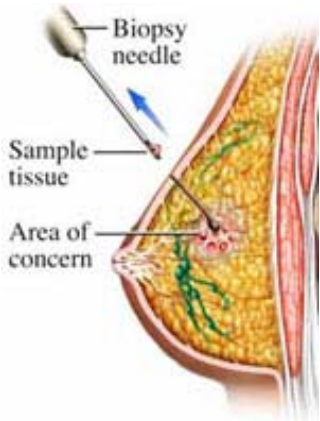
Measurement of Variability and Heterogeneity of Estrogen Receptor



Between Patients
Mean difference: **149 pts** (CI: 92-241)



Between Biopsy and Resection
Mean decrease: **134 pts** (CI: 62-204)



Between Regions within a Sample
Mean difference: **39 pts** (CI: 29-53)



Problem!! Need to find markers that are both highly homogeneous and highly sensitive to pre-analytic variables

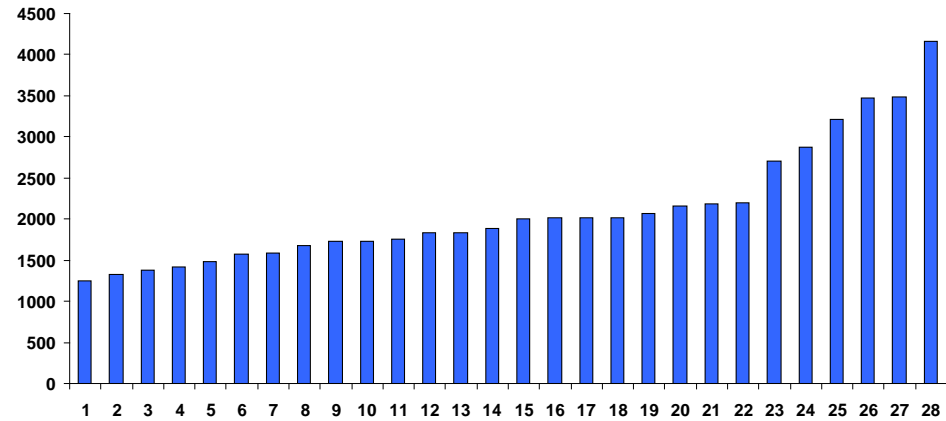
- Go beyond antibodies? Eosin
- Phospho-modification? pTyr antibodies
- Your suggestions here_____

Staining with Eosin at 1 to 50

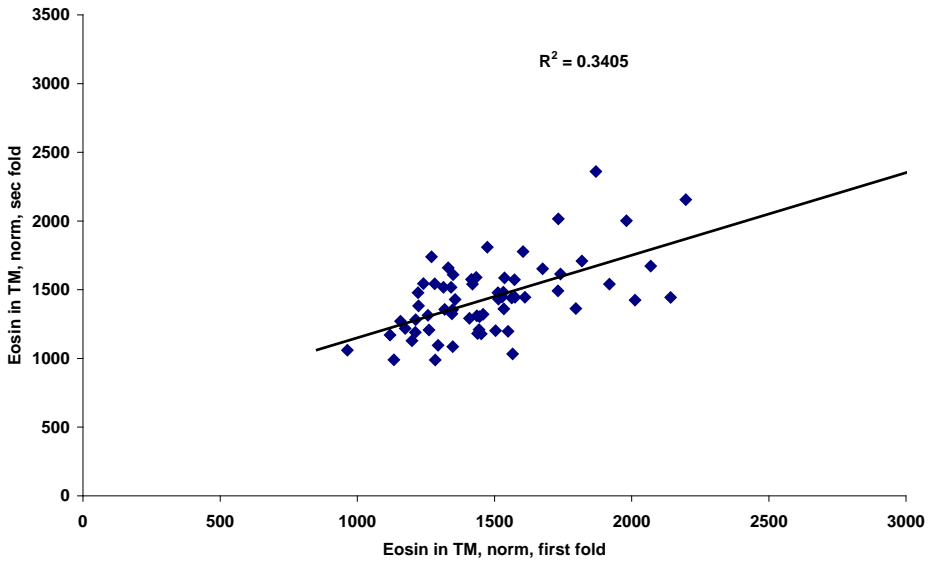
-to measure the amount of free amines and protein degradation

-Predict increase free amine with increased degradation

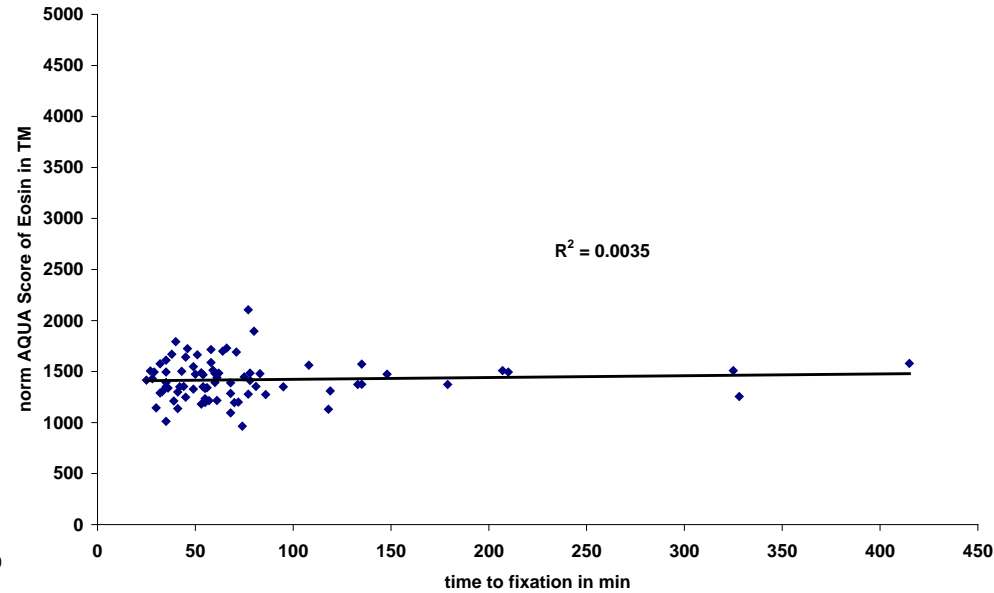
Distribution of norm AQUA Scores in TM for Eosin at 1 to 50, 5 min at RT



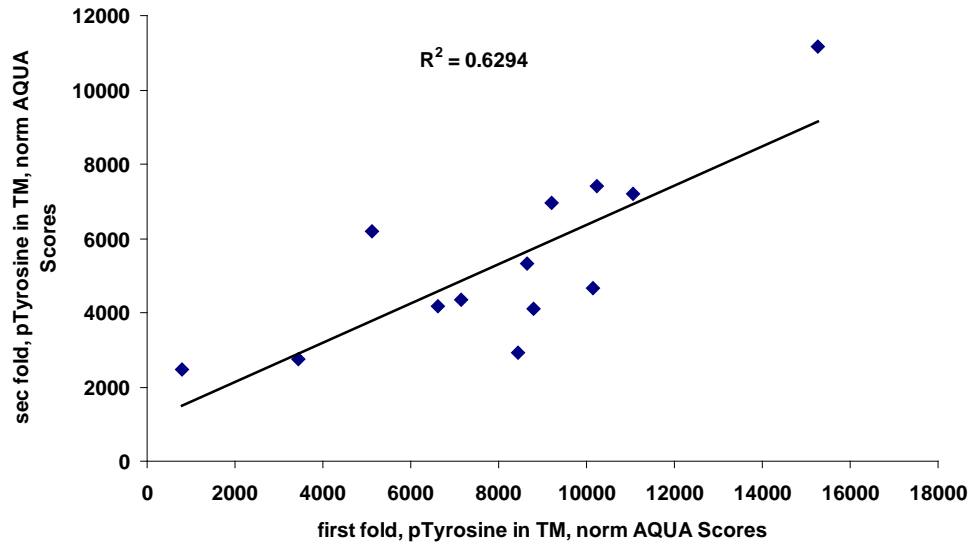
Intra Array Reproducibility of Eosin on YTMA173-2-8, march2011



Average Eosin Expression in TM on YTMA173-2-8, March2011

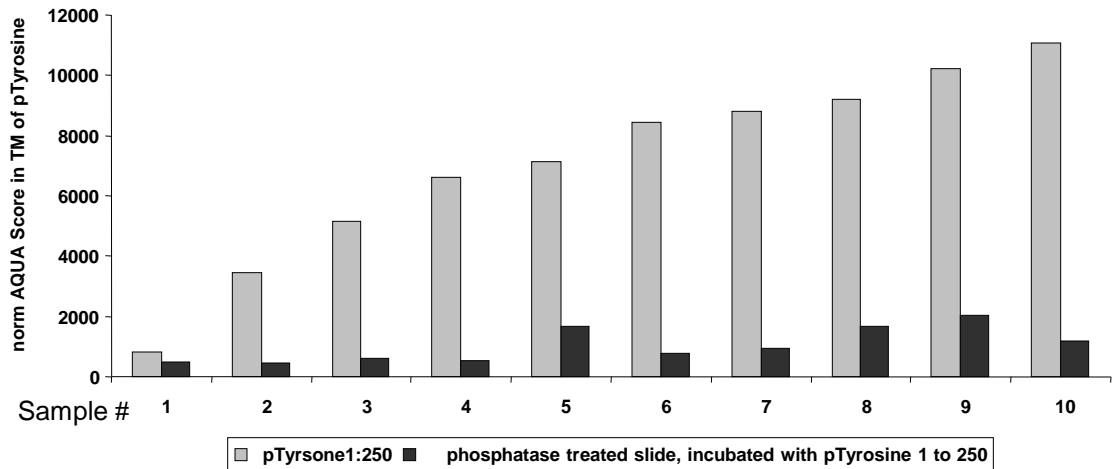


Reproducibility for pTyrosine on breast tests

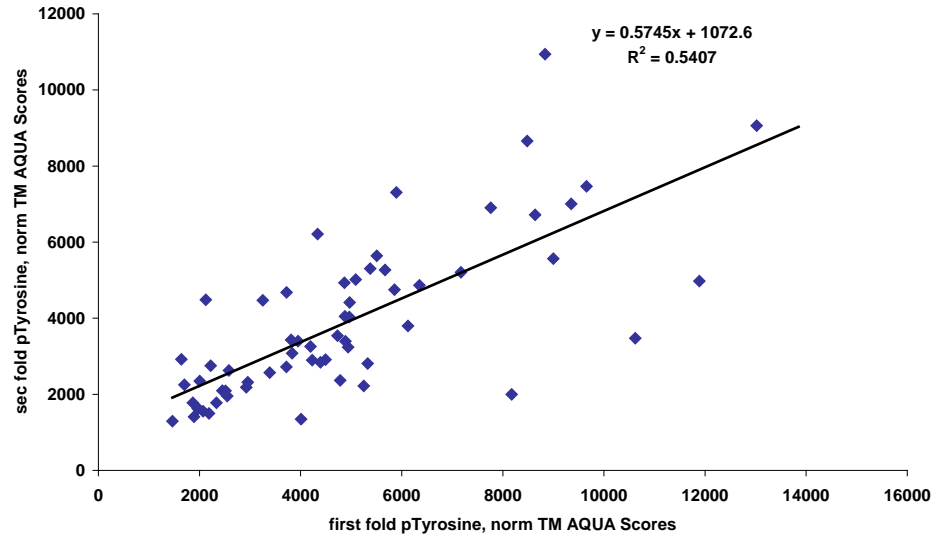


Antibody Validated

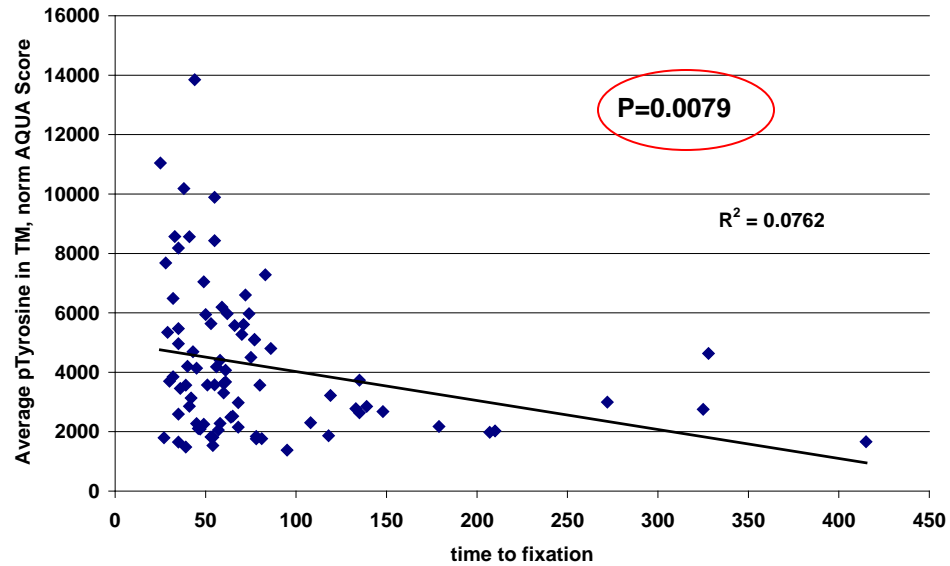
Treatment of breast test with Lambda Phosphatase for 2 hours at 37degree celsius



Intra Array Reproducibility for pTyrosine 4G10
on YTMA 173-2 at 1 to 250, March 2011

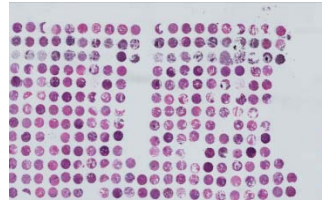


Average pTyrosine Expression on YTMA173-2 and Time to Fixation



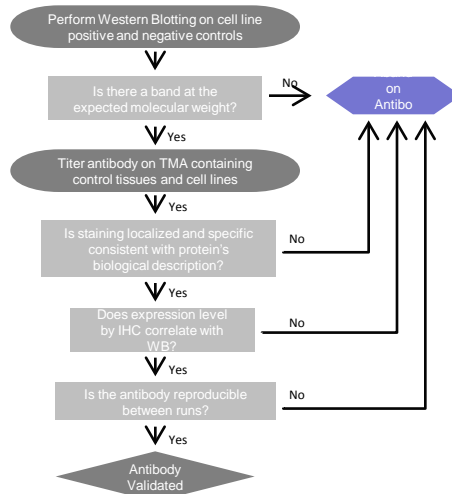
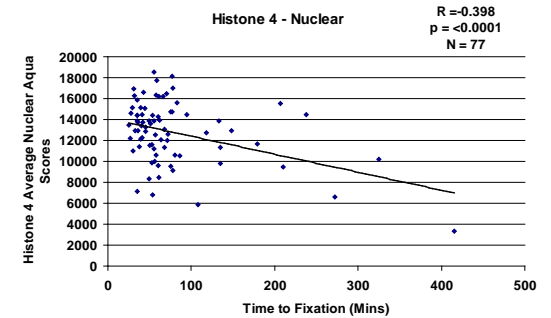
Summary

Generate
Intrinsic Control
cohorts



Select and validate
potential
antibodies/reagents

Test each reagent
individually on
Intrinsic Control
Cohorts



Generate simplest
Multi-variable model
that can assess
tissue quality (TQI)

Validate TQI

Yale Pathology Tissue Services

Thanks to:

Lori Charette

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Maria Baquero

Alley Welsh

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Hollis Viray

Yalai Bai

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Yale Collaborators

Annette Molinaro

Karen Lostrito

Juliana Tolles

Harriet Kluger

Ruth Halaban

Steve Ariyan

Daniel Boffa

Catherine Sullivan

Frank Detterbeck

Lynn Tanoue

Lyndsay Harris



Outside Yale Collaborators

Konstantinos Syrigos (Athens)

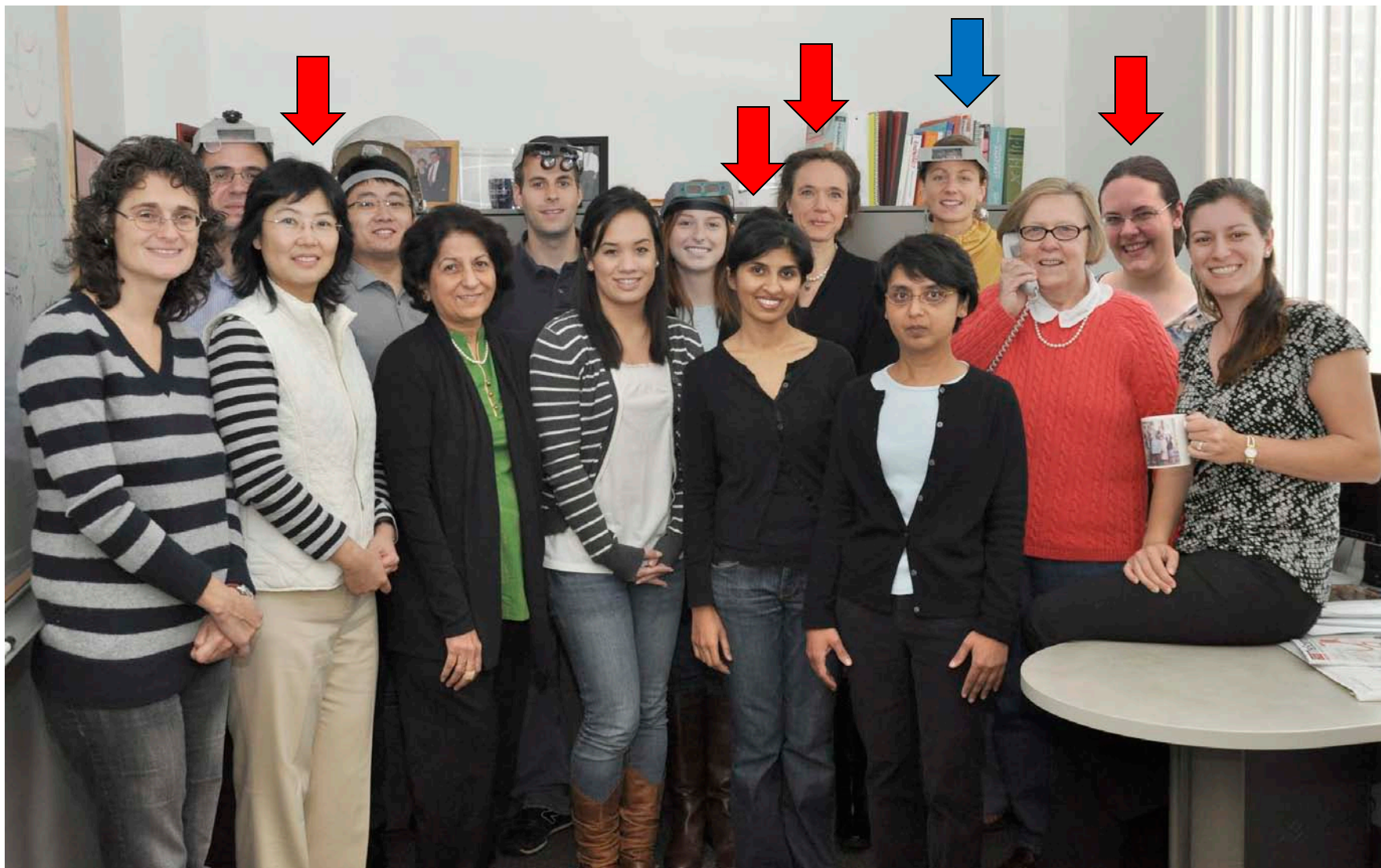
Gerold Bepler (Moffitt-KCI)

Daniel Hayes and SWOG

Elaine Alarid (UW)

Bruce Haffty (CINJ)

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Rimm Lab 2010

www.tissuearray.org

Why we measure protein



Same Genome - Different Proteome

Why we measure protein *in situ*

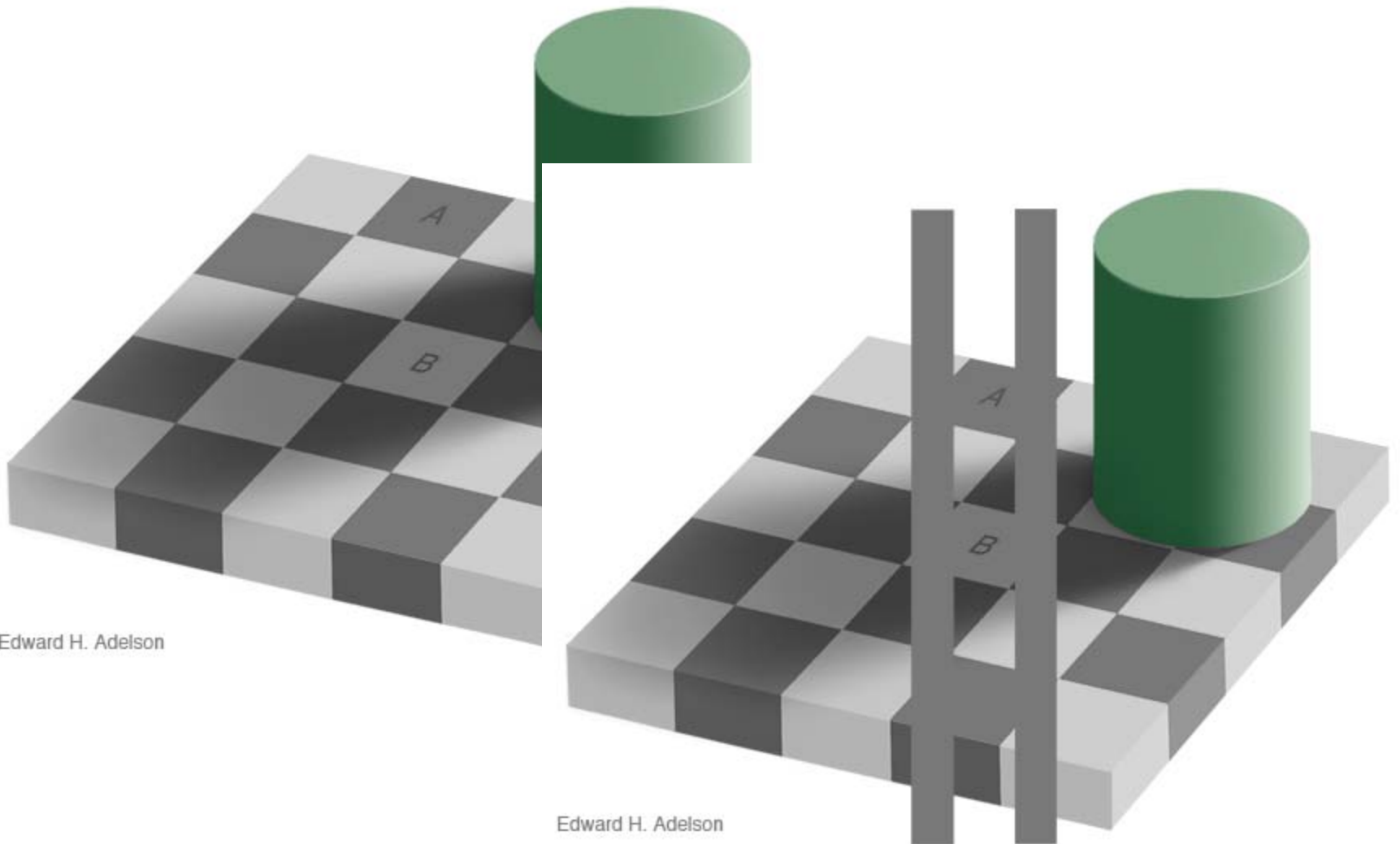


Auguste Renoir :
The Luncheon of the Boating Party
C.1881



Claude Monet:
The Stroll, Camille Monet and Her
Son Jean (Woman with a Parasol)
C. 1875

Why we measure protein with a machine



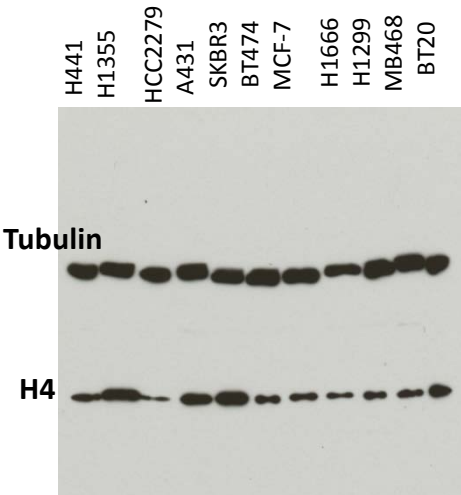
Edward H. Adelson

Edward H. Adelson

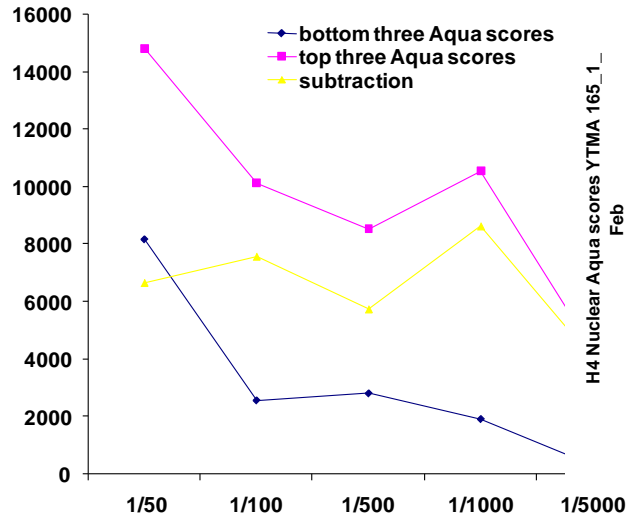
Marker of cold ischemia: Histone H4

Antibody Validated

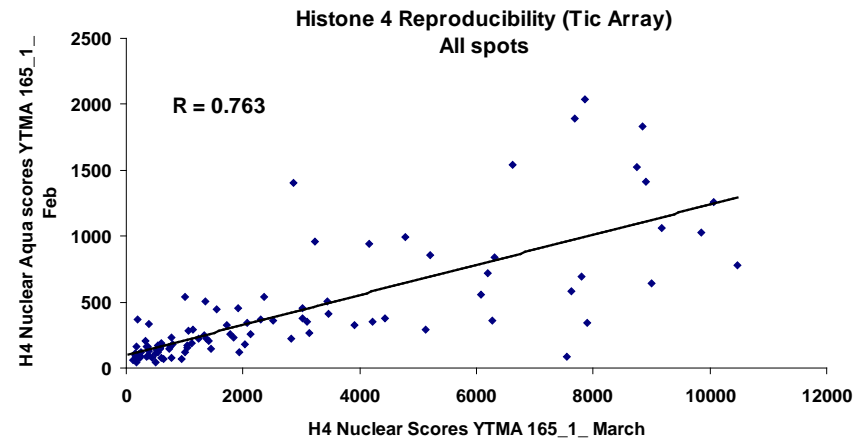
Western Blotting



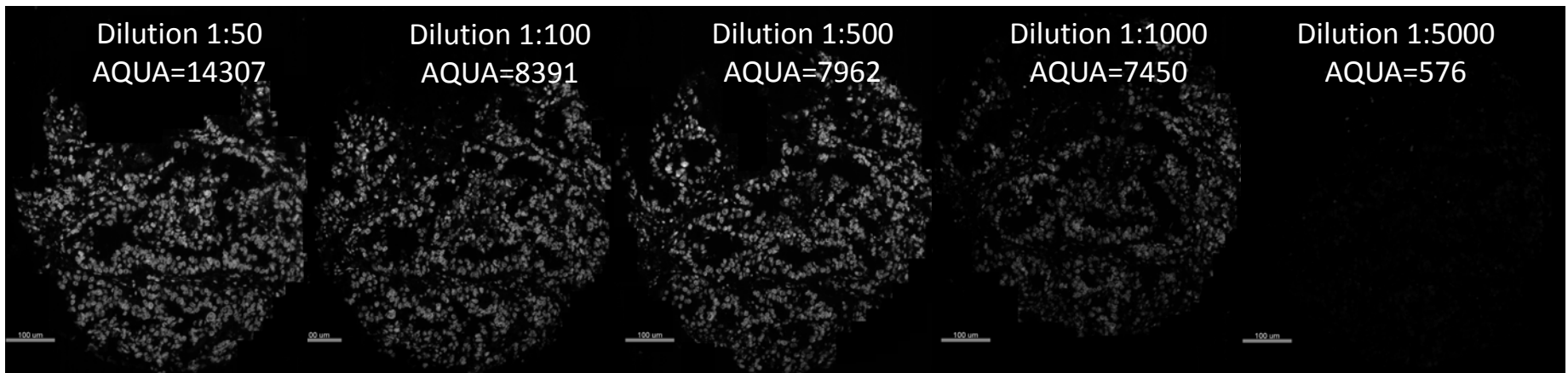
Expression Range Graph



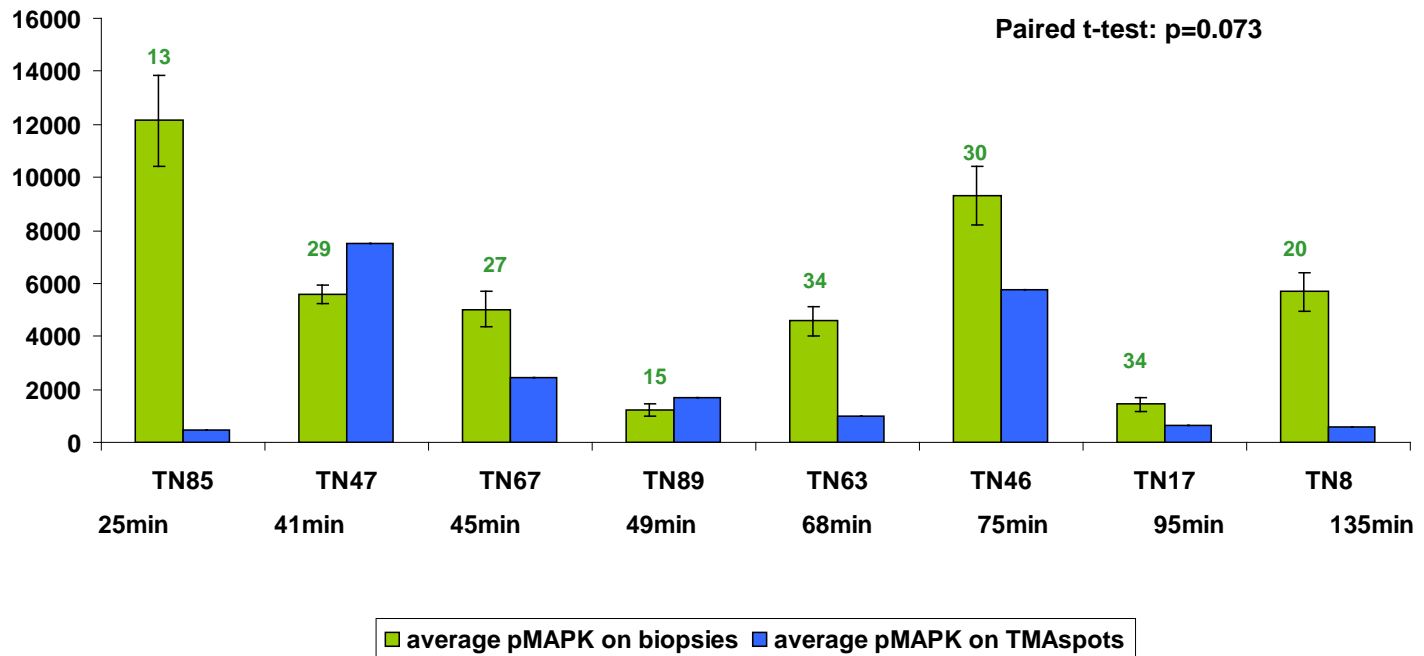
Assay Reproducibility



Quantitative Immunofluorescence



Assessment of pMAPK Expression on Biopsies and matched TMA Spots on Time to Fixation Array

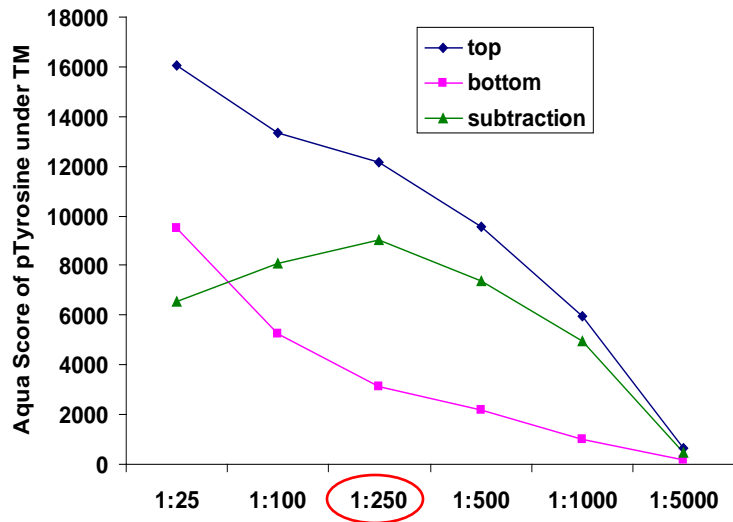


4G10 Platinum, Anti-Phosphotyrosine

Mouse monoclonal Antibody cocktail IgG2b

Millipore, Cat. # 05-1050

pTyrosine Expression Range Graph



pTyrosine Expression Range Graph

