



# **Intrinsic Controls for Formalin Fixed, Paraffin Embedded Tissue**

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# Disclosure

- I am a consultant, stockholder and scientific founder of HistoRx
- I am an author on the Yale held patent on the AQUA technology.

# Pre-analytical Variables (incomplete list)

- Variable warm ischemic time
- Variable cold ischemic time
- Variable manipulation during 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 drying times
- Variable 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?



# Toward Intrinsic Controls for FFPE tissue

- **How do we quantify protein expression on slides (AQUA)**
- Using AQUA to quantify the effects of pre-analytic variables
- Progress toward an intrinsic control

# AQUA<sup>®</sup>: objective analyte measurement on a tissue slide based on co-localization

**Step 1:** Mask (define region of interest, exclude stroma, blank space, etc) = colocalization with Cytokeratin for carcinoma

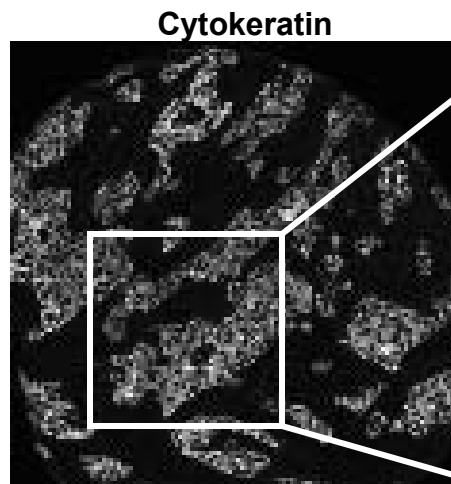
**Step 2:** Define the numerator (target) and denominator (compartment)

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

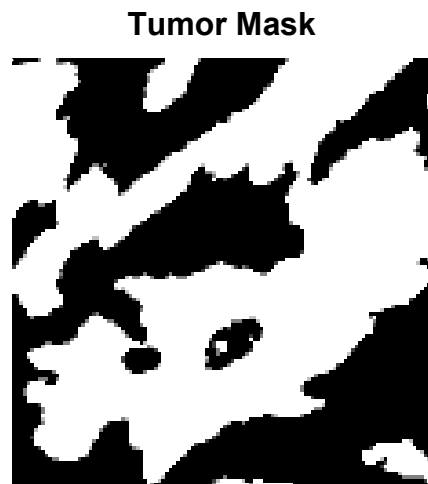
**Step 3:** Calculate the AQUA score

**Step 4:** Convert to absolute concentration or normalize to set of uniform standards

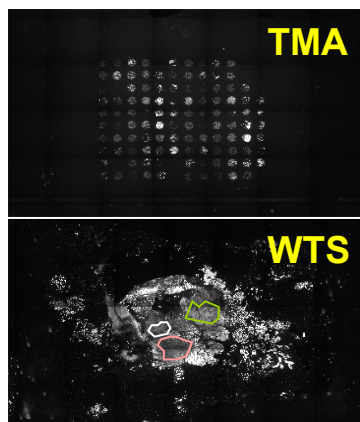
# Generating the AQUA<sup>®</sup> score



Cytokeratin



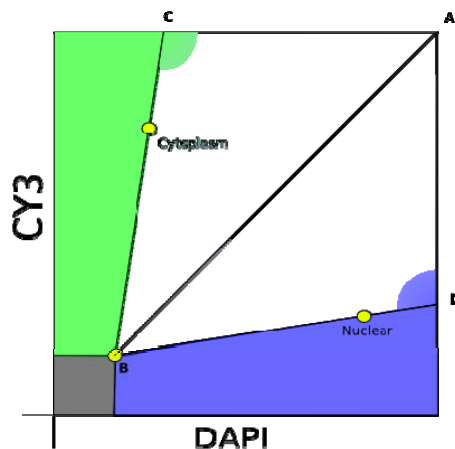
Tumor Mask



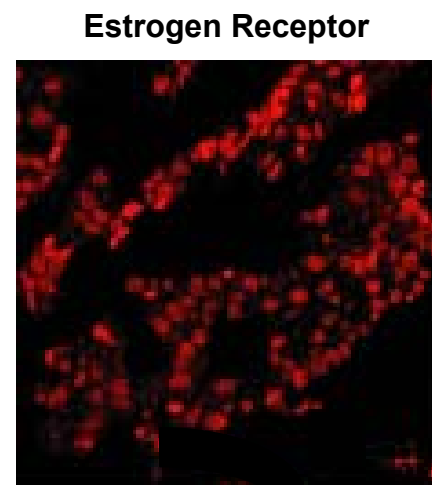
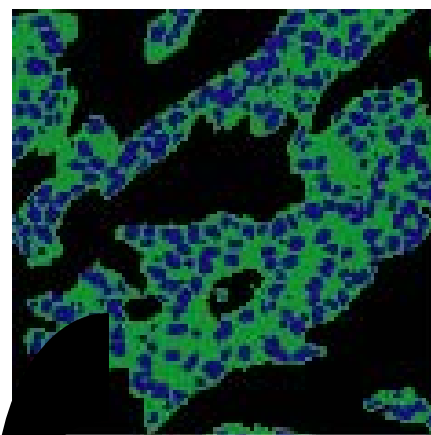
TMA

WTS

TMA-Tissue Microarray  
WTS-Whole Tissue Section



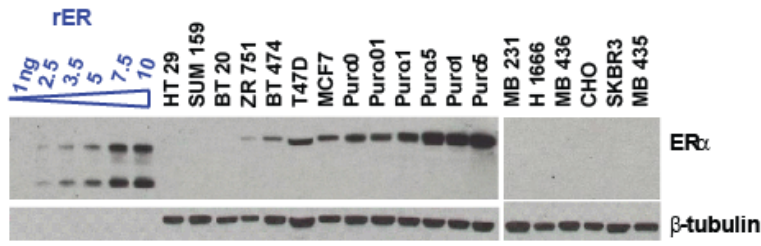
Combine DAPI image and cytokeatin image then cluster to assign each pixel to a subcellular compartment



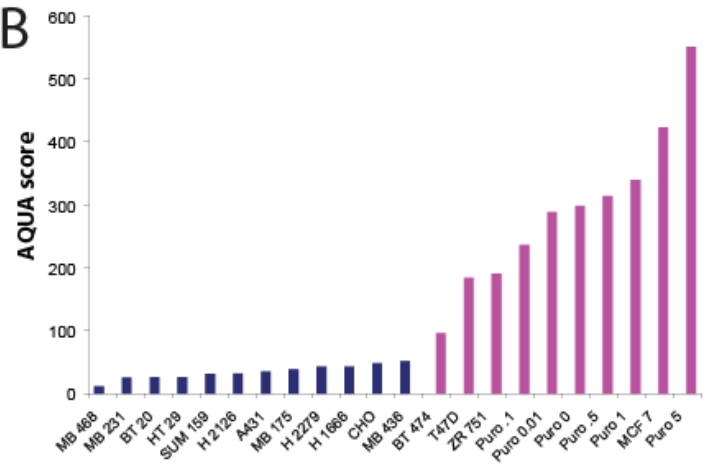
Estrogen Receptor

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

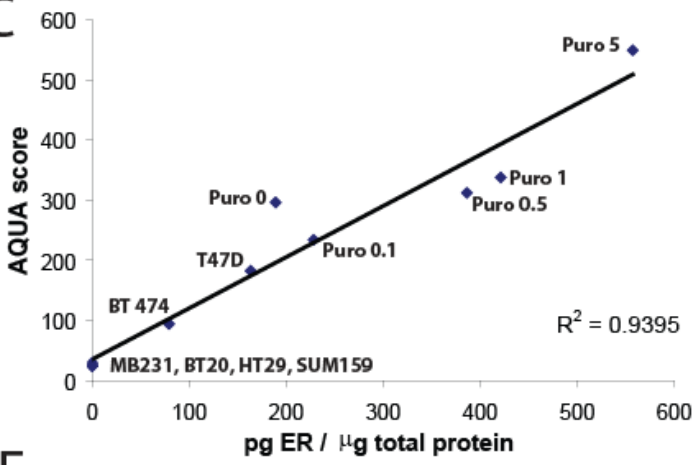
**A**



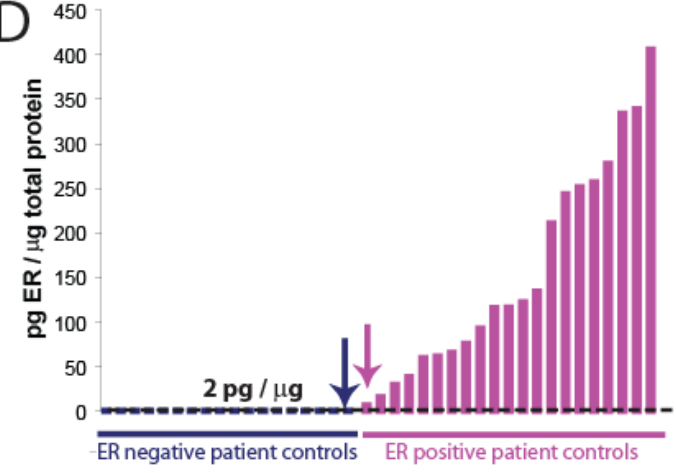
**B**



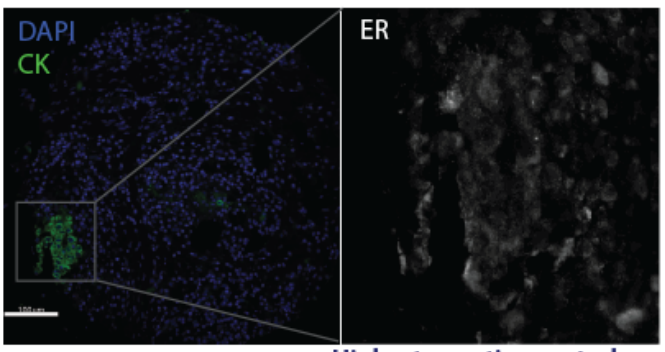
**C**



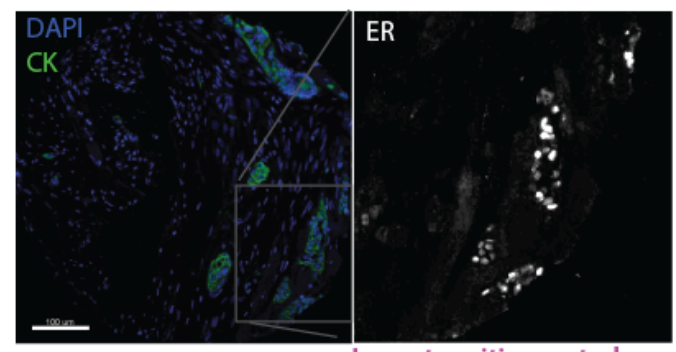
**D**



**E**



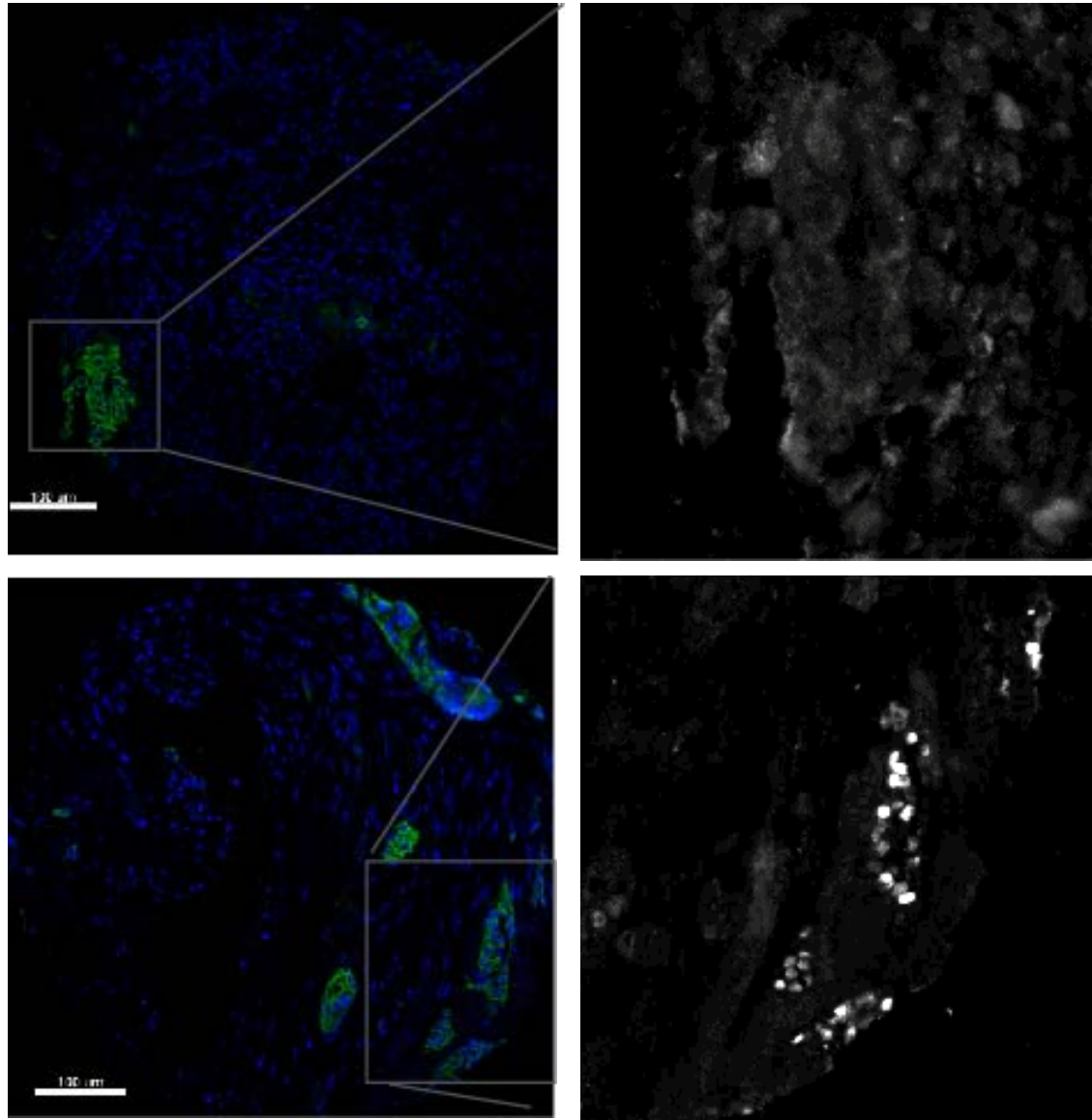
Highest negative control case

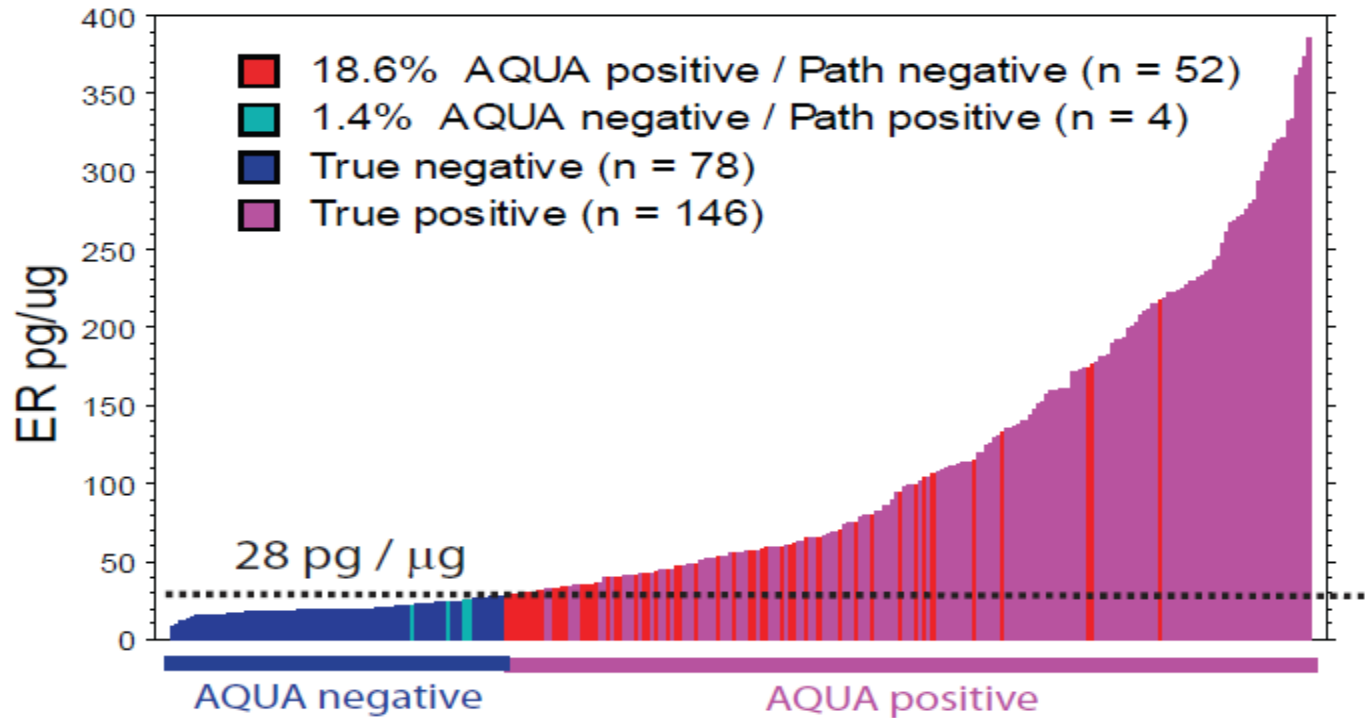


Lowest positive control case

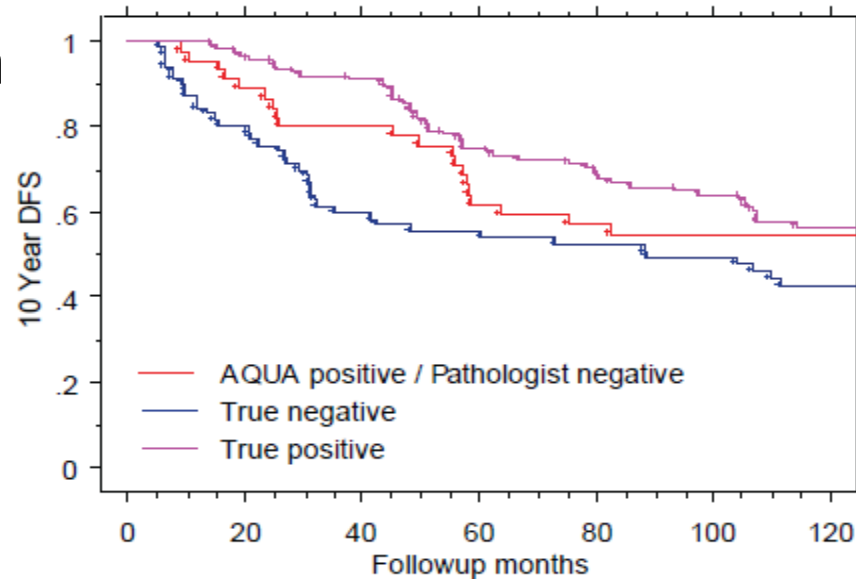


# Expanded “levels” to visualize threshold

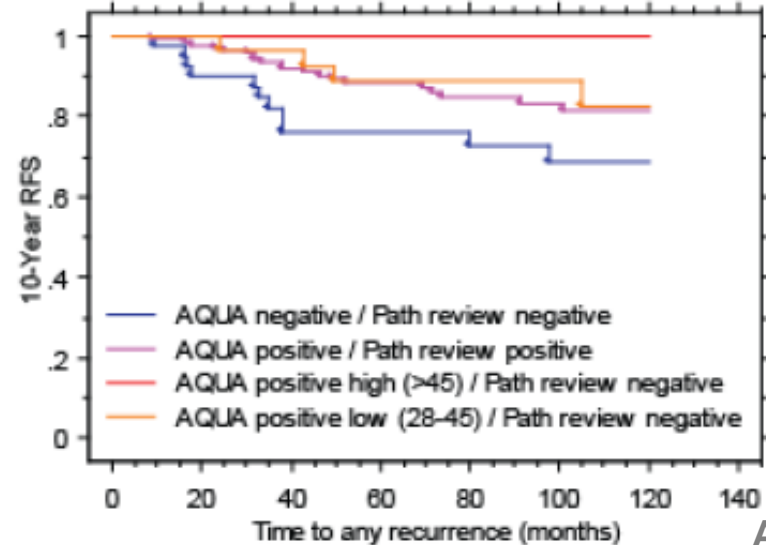
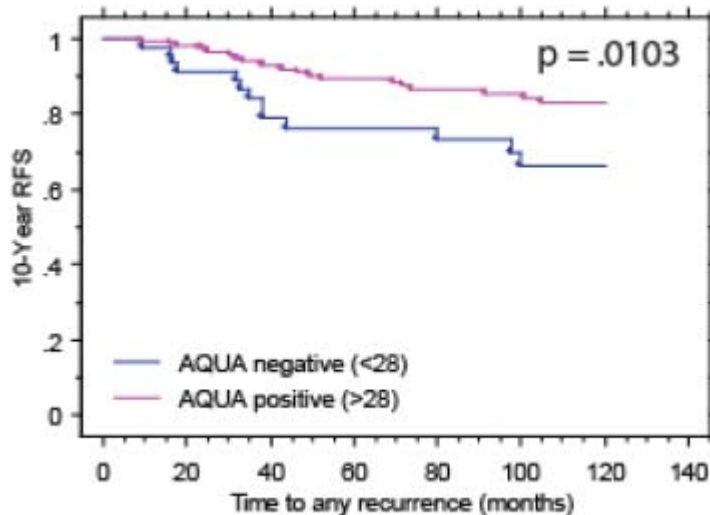
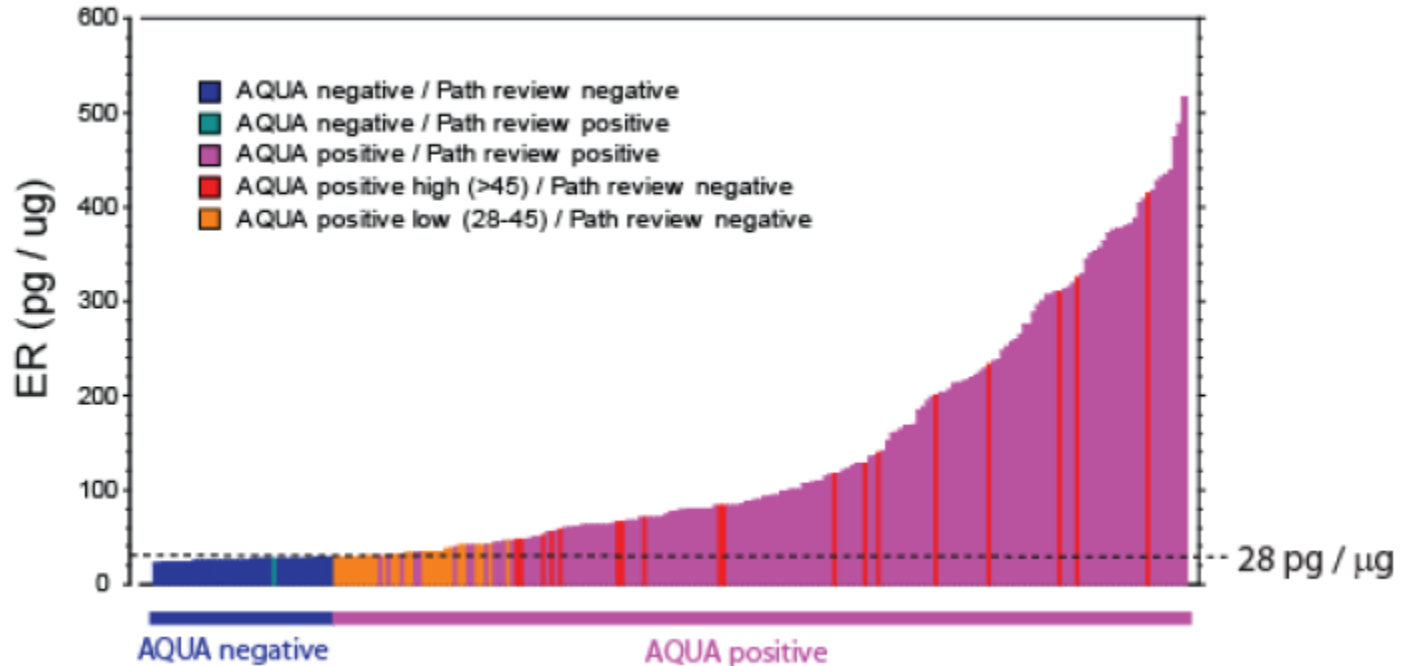




Misclassification  
analysis of  
YTMA-49  
1962-1982  
(n=280)

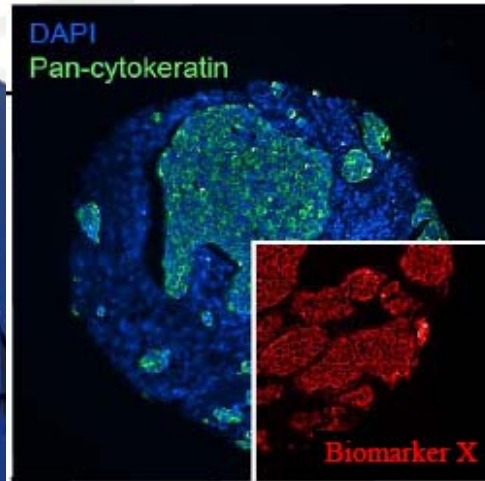
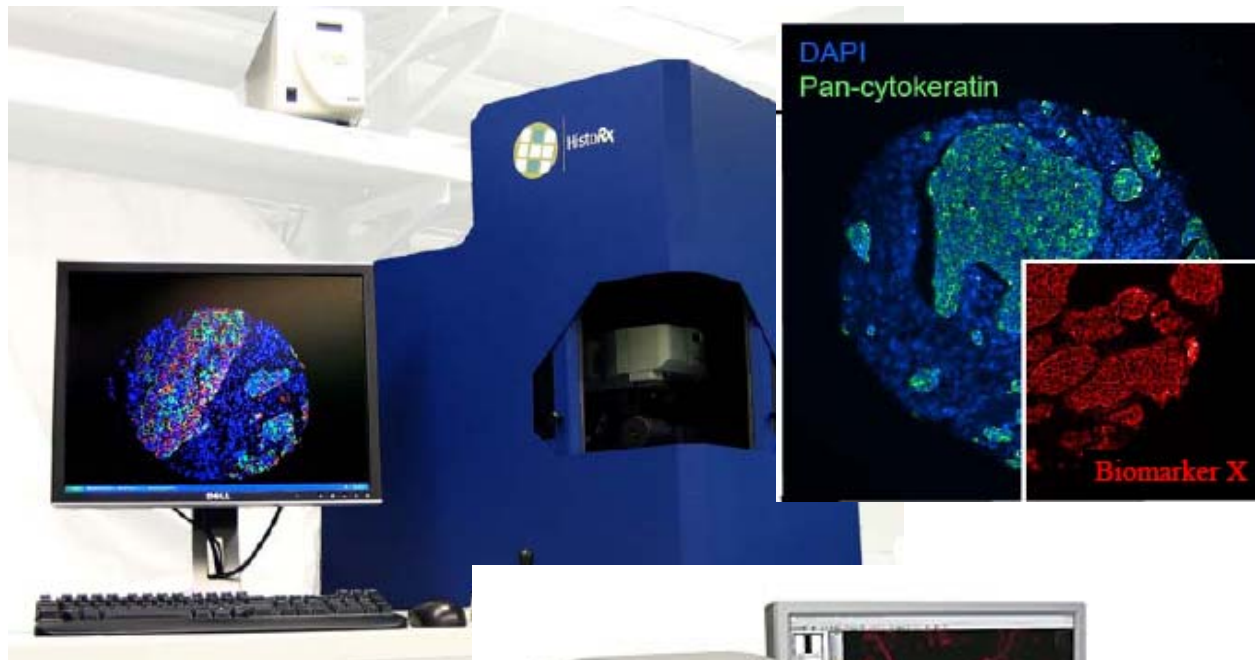


# Patients who are subtly “positive” behave like positives



# The HistoRx AQUA<sup>®</sup> platform

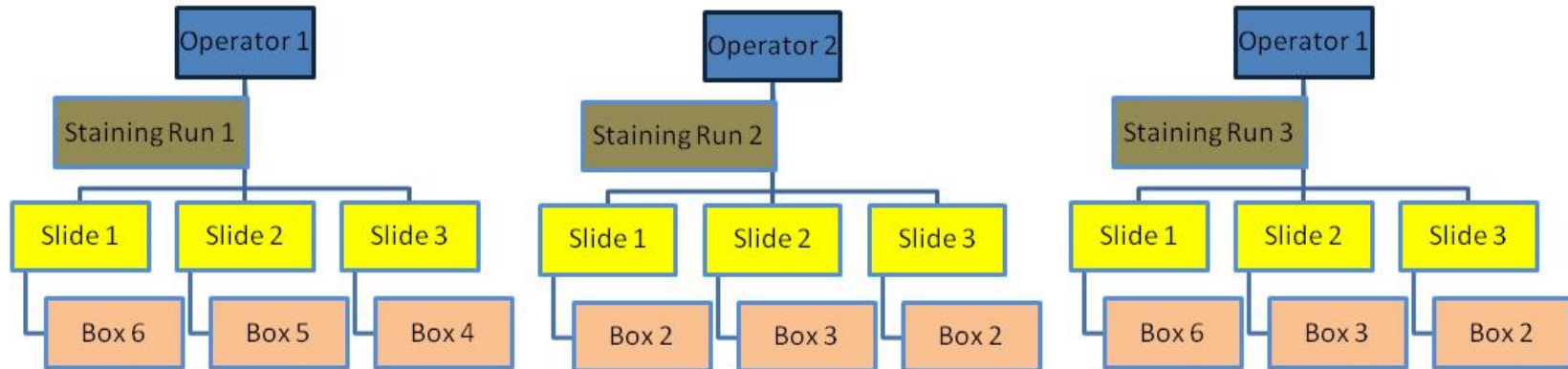
## Hardware, Software, & Reagents for Quantitative Pathology



- ❑ Automated Fluorescence Microscopy
  - Expanded dynamic range of measurement
  - Multi-parametric
- ❑ US Patent 7,219,016
- ❑ Commercially available with about 18 current placements worldwide
- ❑ AQUA<sup>®</sup> analysis software compatible with .tiff images
- ❑ In use by more than a dozen Pharma companies for drug development
- ❑ AQUA<sup>®</sup> software now available on Aperio Scanscope FL platform



# Precision Results (ER-alpha)



	Pearson R	Slope
Day 1 v. Day 2	.97	.97
Day 1 v. Day 3	.97	1.01
Day 2 v. Day 3	.98	1.04

**%CV = 4.2**

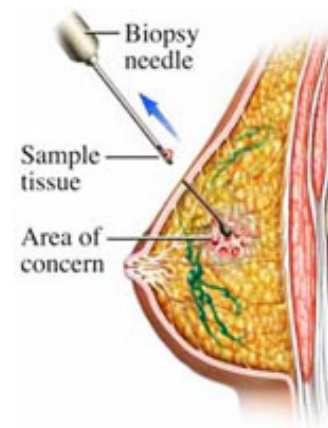
# Toward Intrinsic Controls for FFPE tissue

- How do we quantify protein expression on slides (AQUA)
- **Using AQUA to quantify the effects of pre-analytic variables**
- Progress toward an intrinsic control

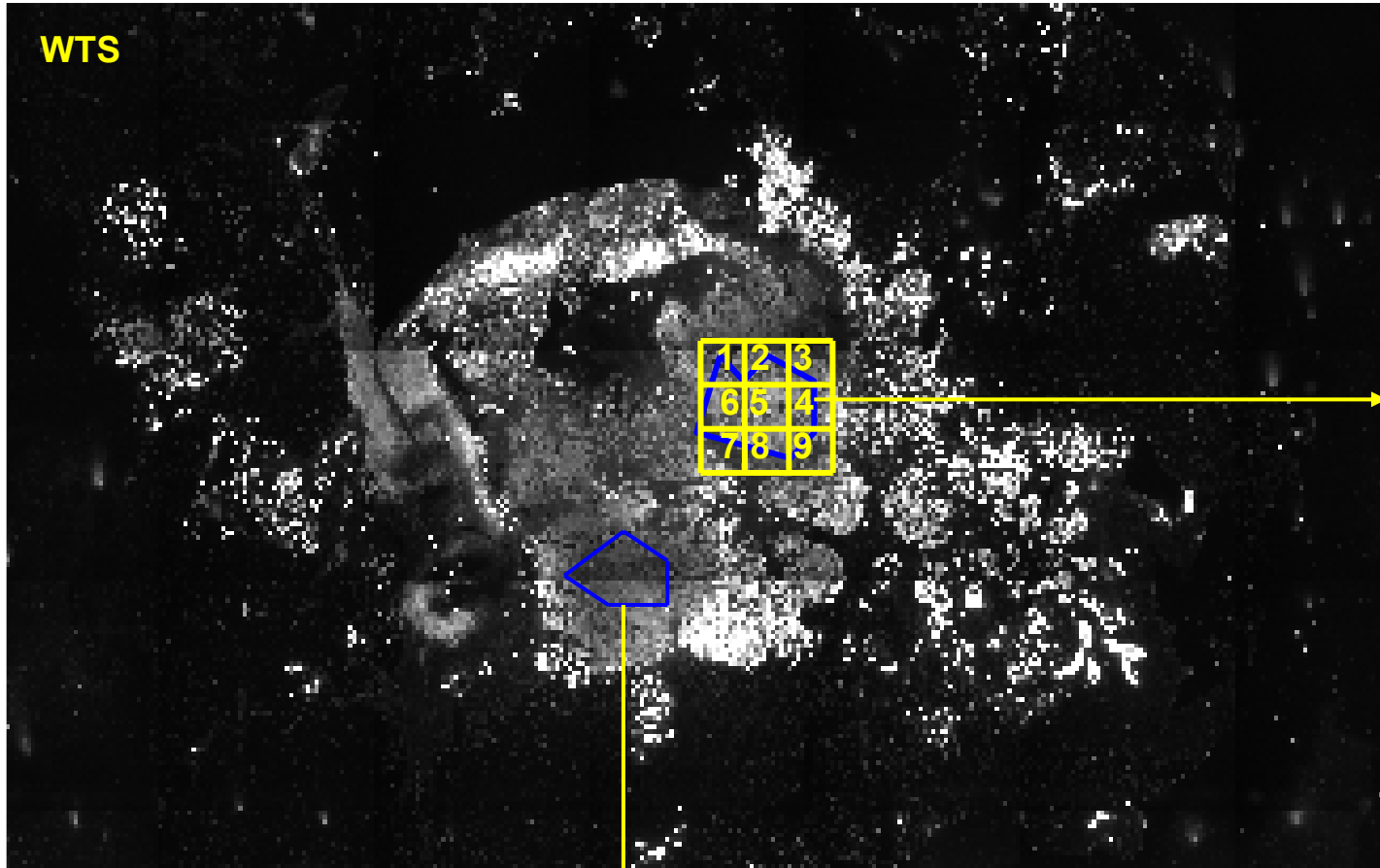
# Quantification of pre-analytic variables: Core Needle Biopsy vs. Resection specimen

## Advantages of Core Needle Biopsies (CNBs)

- Short time to fixation (short “cold ischemic time”)
- Rapid fixation due to small diameter
- Minimal surgical manipulation of tissue



WTS



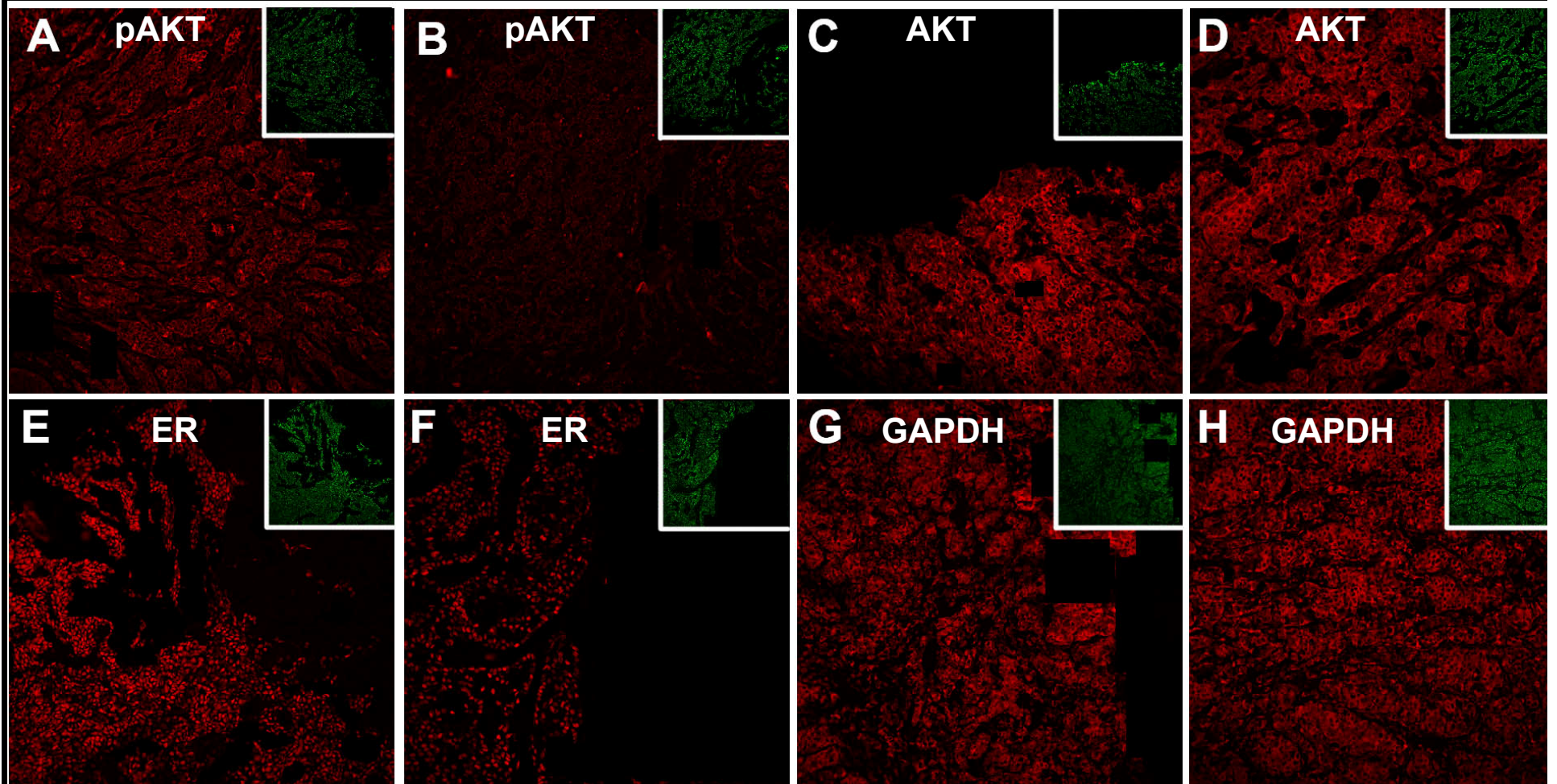
Field of View (FOV)

Region of Interest (ROI)

target AQUA is the average from multiple FOVs

Yalai Bai





**Bx**

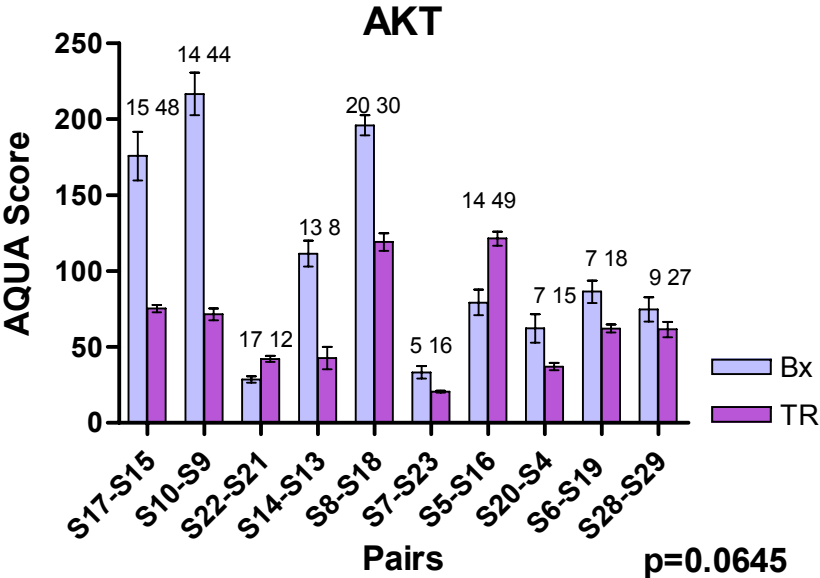
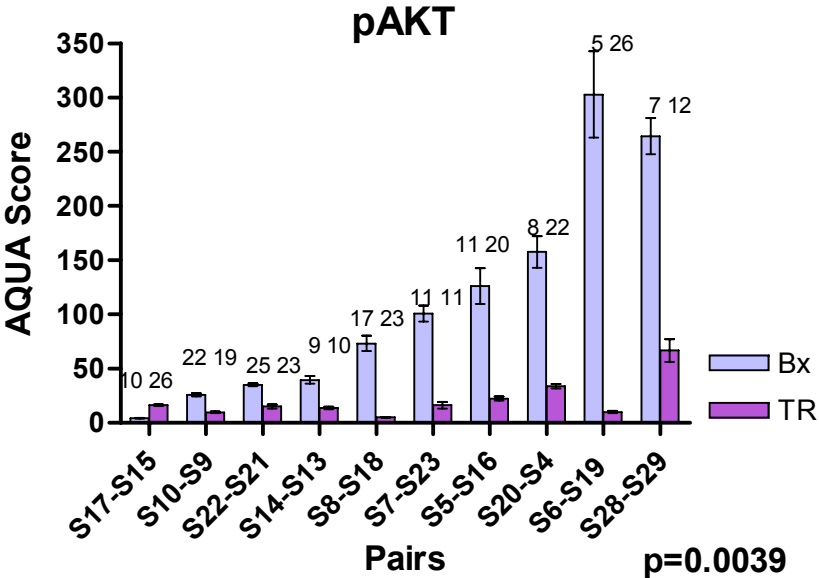
**TR**

**Bx**

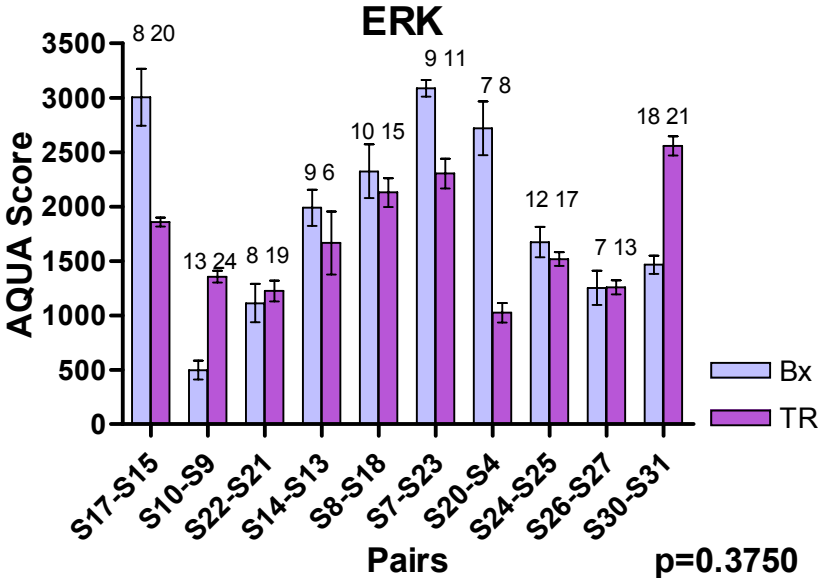
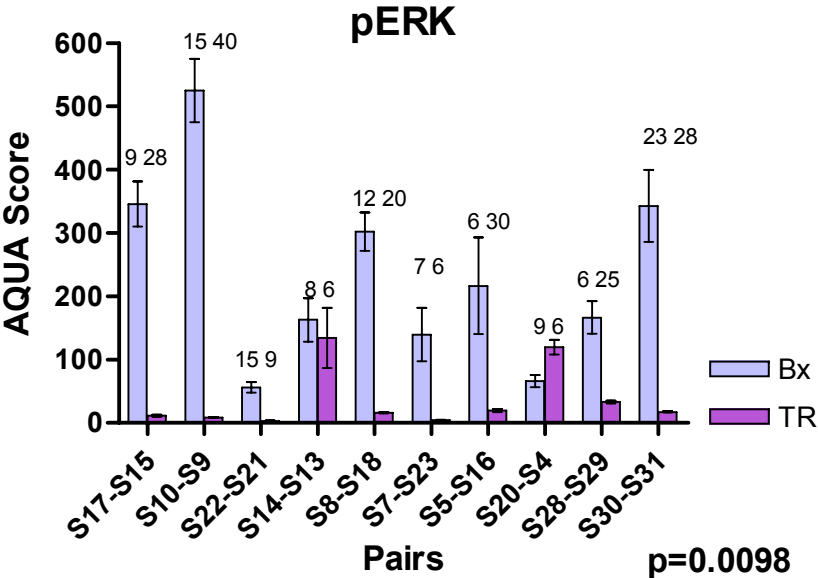
**TR**

**Biopsy: Bx**  
**Tumor Resection: TR**

# Lower Expression of pAKT in the resections than in CNBs

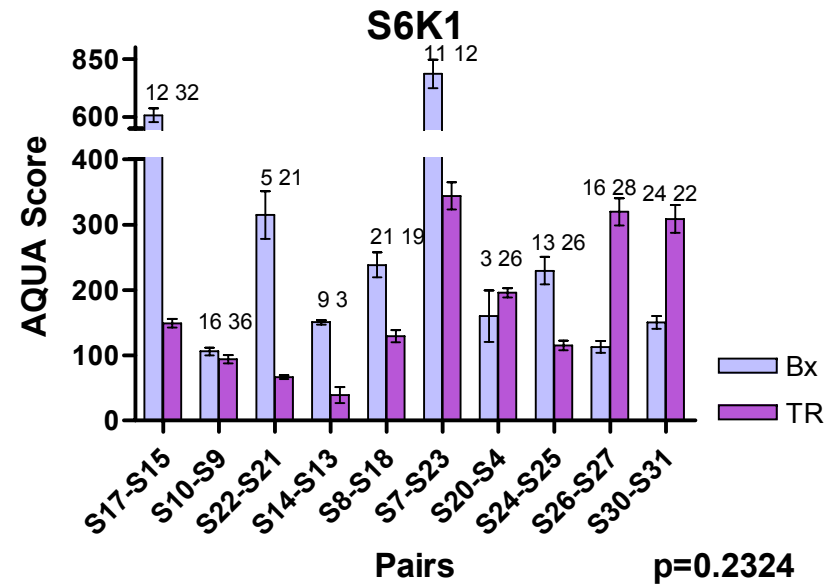
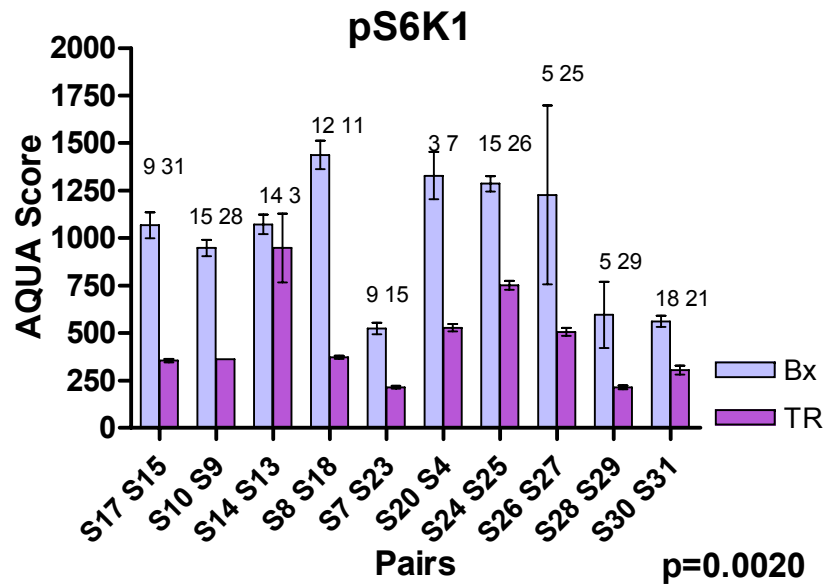


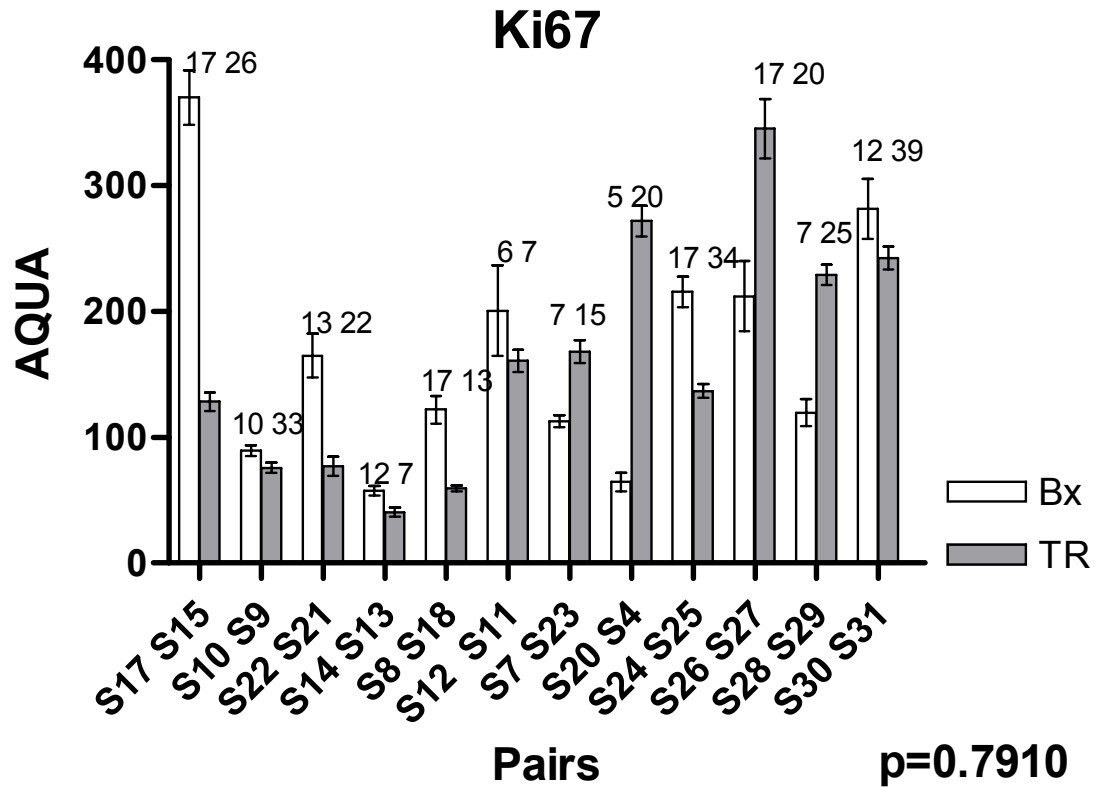
# Lower Expression of pERK in the resections than in CNBs



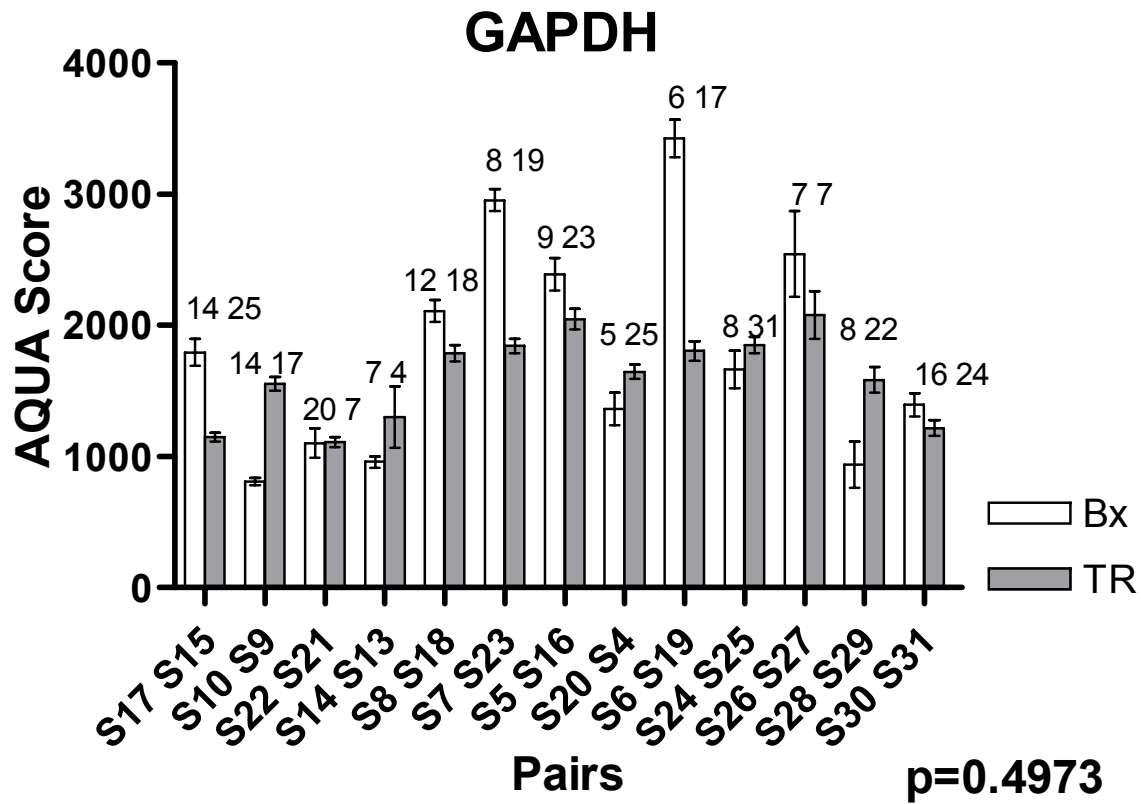
Yalai Bai

## Lower Expression of p-P70S6K in the resections than in CNBs

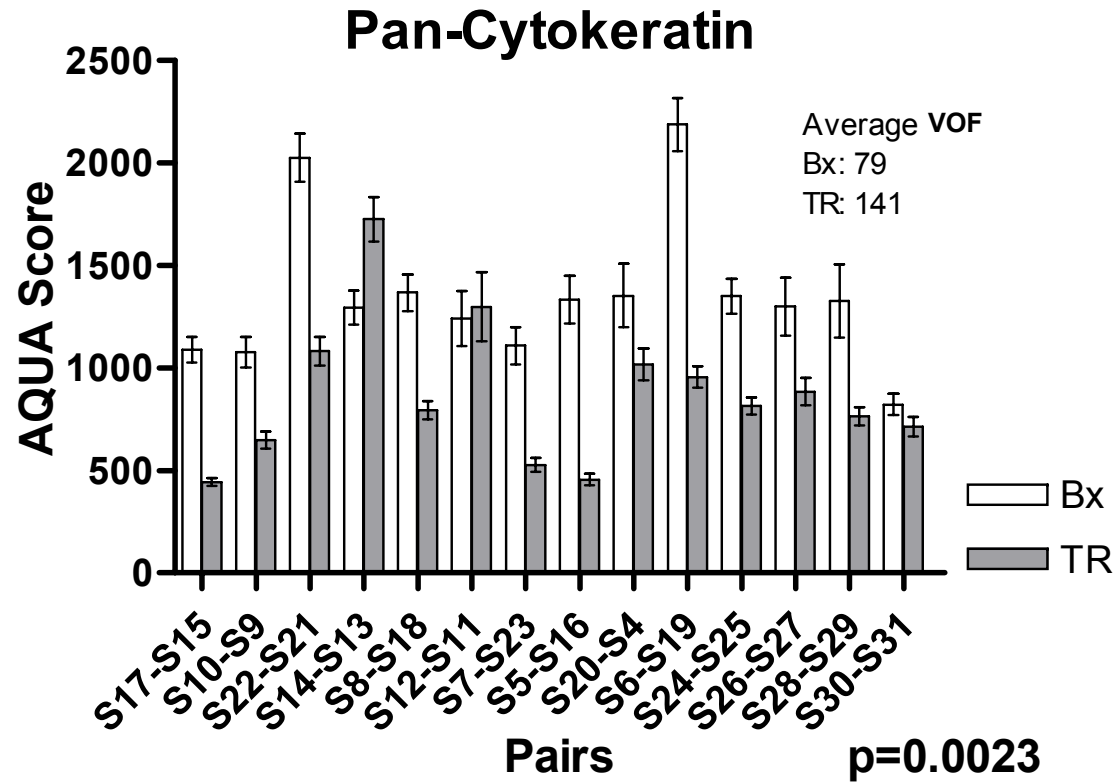




Decreased expression of Ki67 in 4/12 pairs  
 Increased expression of Ki67 in 4/12 pairs



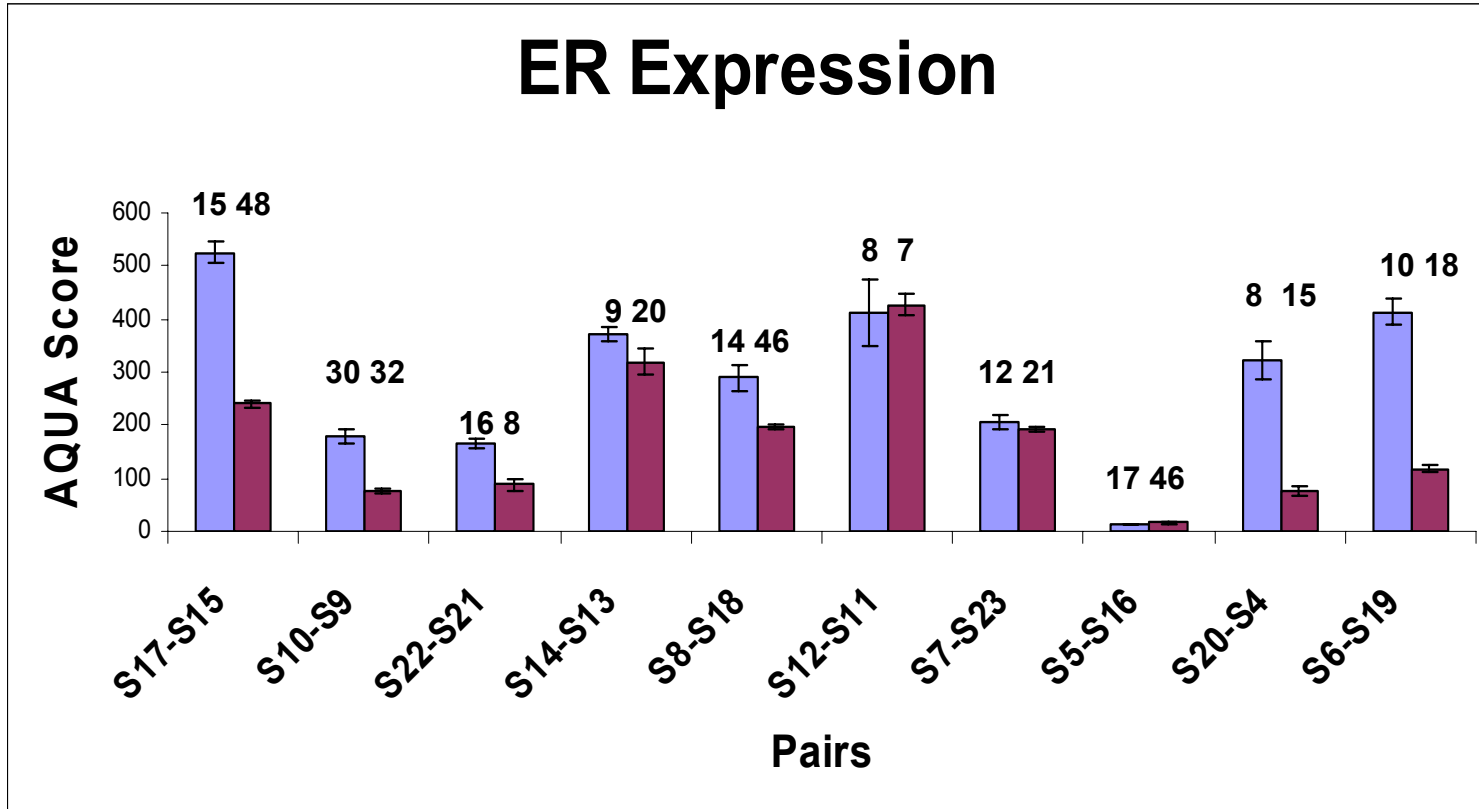
no apparent difference of GAPDH expression in 9/13 pairs



Decreased expression of Cytokeratin in 10/14 pairs

Yalai Bai

# Decreased Level of ER Expression In Surgical Resections

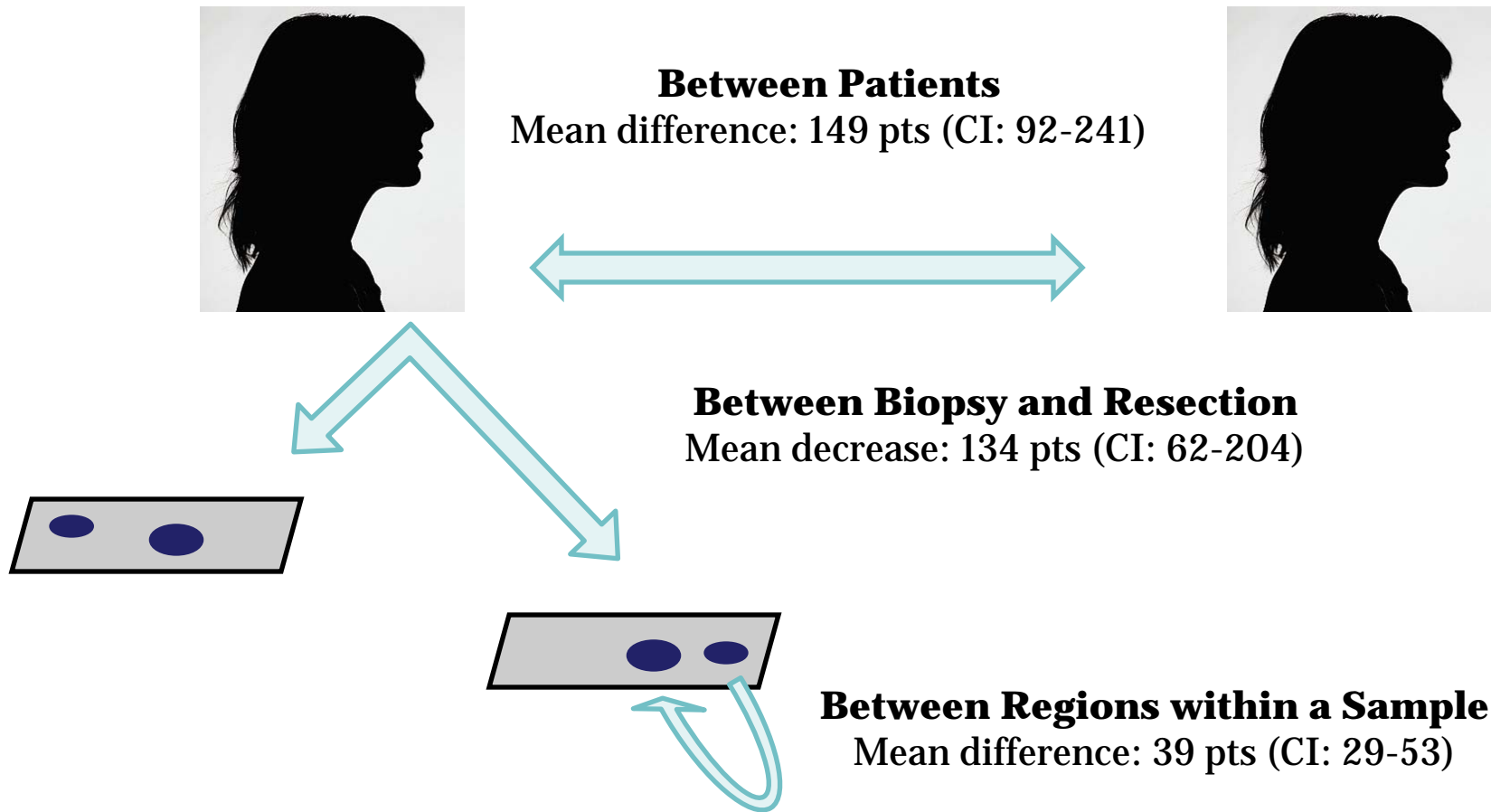


■ CNBs  
■ Tumor Resection

Wilcoxon Signed Rank test  $p=0.014$



# Results: Example of Estrogen Receptor



# Preliminary Conclusions From Mixed Effects Model

Protein	Amount of decrease as percent of biopsy score	Coefficient of variation for differences between individuals $\sigma / \mu$
ER	44%	0.53
ERK	Not significant	0.32
pERK	81%	0.81
pP70S6K	40%	0.34
pAKT	81%	0.87
AKT	37%	0.40
Ki67	Not significant	0.29
GAPDH	Not significant	0.28

# Summary of Pre-analytic Data

- There is good evidence for a range of epitope degradation with cold ischemic time
- Phospho-epitopes appear to be more sensitive than non-phospho epitopes
- Normalization may be possible, but method not yet determined
- **Need to find an intrinsic control for protein degradation**

# Toward Intrinsic Controls for FFPE tissue

- How do we quantify protein expression on slides (AQUA)
- Using AQUA to quantify the effects of pre-analytic variables
- **Progress toward an intrinsic control**

# Intrinsic Controls for FFPE tissue

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

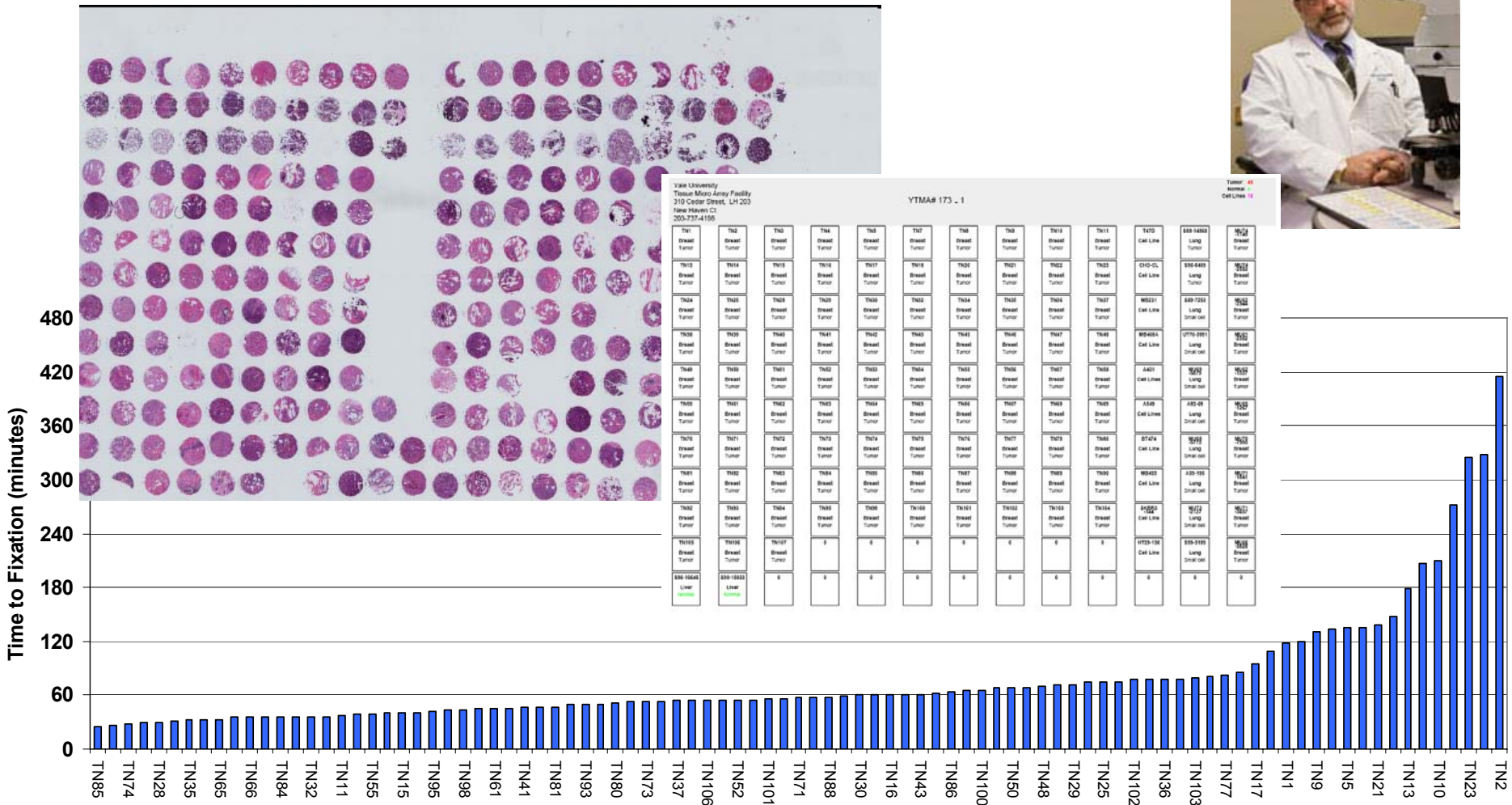
*Goal 2: Assessment of markers of cold ischemia (“housekeeping markers”) on discovery cohorts*

*Goal 3: Assessment of markers of hypoxia on discovery cohorts*

*Goal 4: Generation of a Multiplexed “Tissue Immunologic Competence” (TIC) Model 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*

# Construction of the Rochester Tissue Microarray (2x redundancy)



Two fold redundancy

N=125 , tumor=93, normal=2, cell lines=10 control breast tumor=10 ,control lung tumor = 10

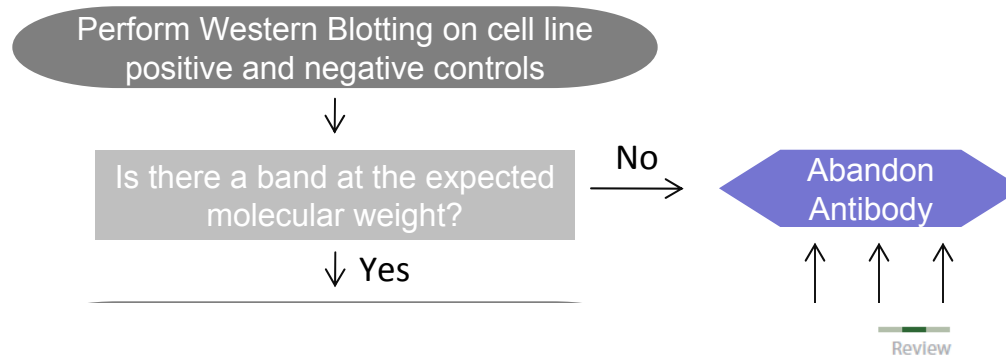
Collected by Dr. David Hicks and colleague, University of Rochester Medical Center

**Selection, titration and validation of markers  
(note, 7 completed of proposed 25-30)**

Symbol	Description	Antibody			Supplier
		Origin	Clone/Isotype	Catalog #	
<i>Markers of cold ischemia</i>					
ACTB	Beta-Actin	Rabbit	13E5/IgG	4970	CST, Danvers, MA
TUBB	Beta-Tubulin	Rabbit	9F3/IgG	2128	CST, Danvers, MA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Rabbit	14C10/IgG	2118	CST, Danvers, MA
HIST4	Histone H4	Mouse	L64C1	2935	CST, Danvers, MA
HIST3	Histone H3	Mouse	96C10/IgG1, kappa	3680	CST, Danvers, MA
HIST2A	Histone H2A	Mouse	L88A6/IgG1	3636	CST, Danvers, MA
<i>Markers of hypoxia</i>					
VEGF	Vascular Endothelial Growth Factor	Mouse	VG1/IgG1, kappa	M7273	Dako, Carpinteria, CA
CCND1	Cyclin D1	Rabbit	SP4/IgG	RM-9104	Thermo Fisher, Fremont, CA

Selection criteria: Abs have to be monoclonal, recommended for IHC and WB and validated on positive controls (shown in manufacturers datasheet)

## 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, Connecticut*

*BioTechniques* 48:XXX-XXX (March 2010) doi 10.2144/000113382

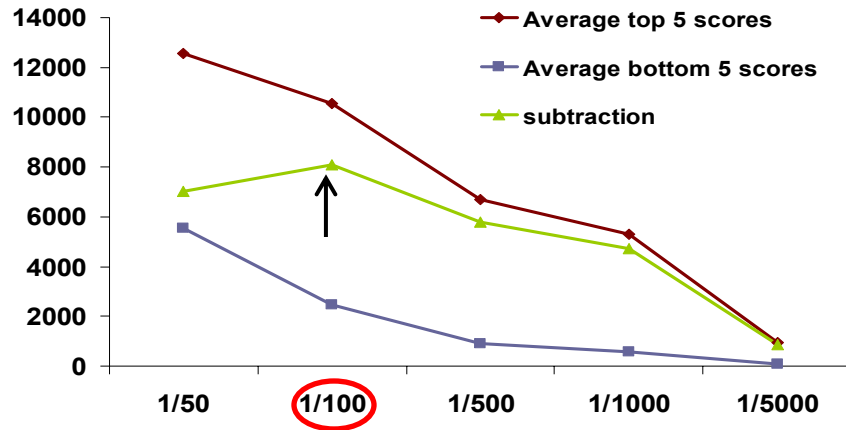
Keywords: Antibody; validation; immunohistochemistry; immunofluorescence

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.

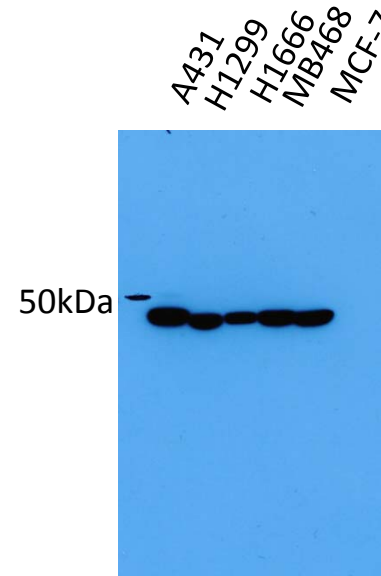


# Markers of cold ischemia: **Beta-actin**

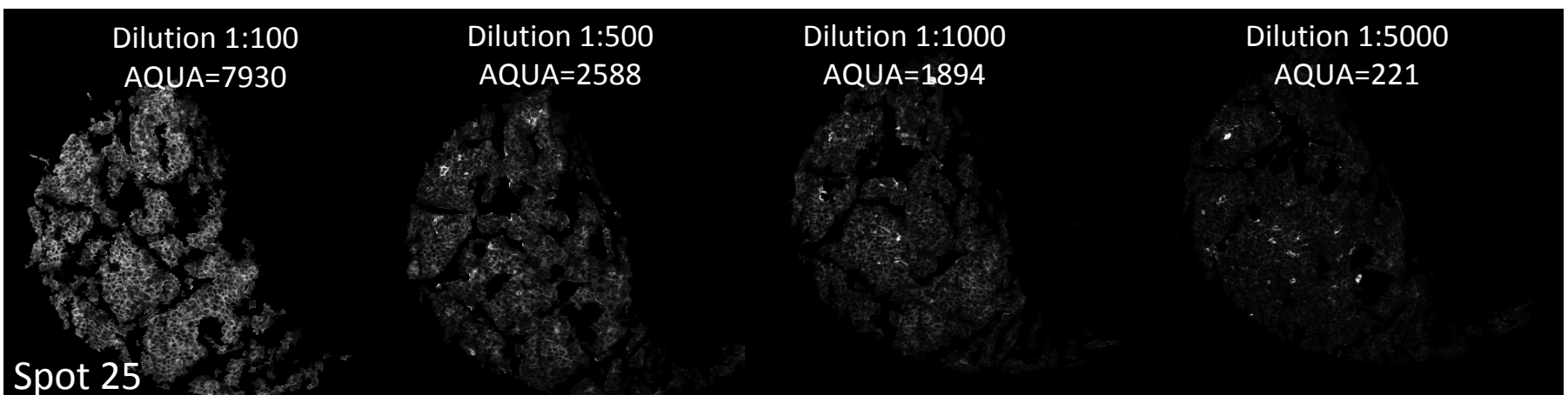
## Titer optimization: Expression Range Graph

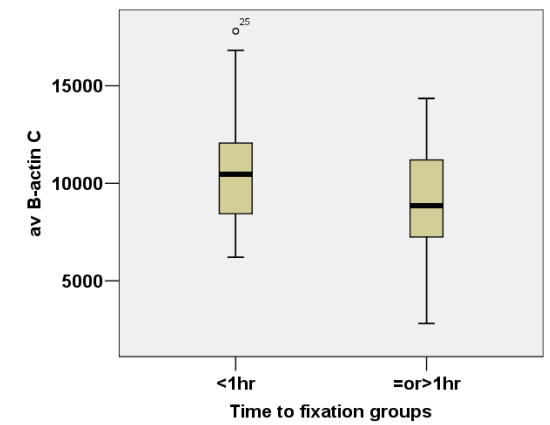
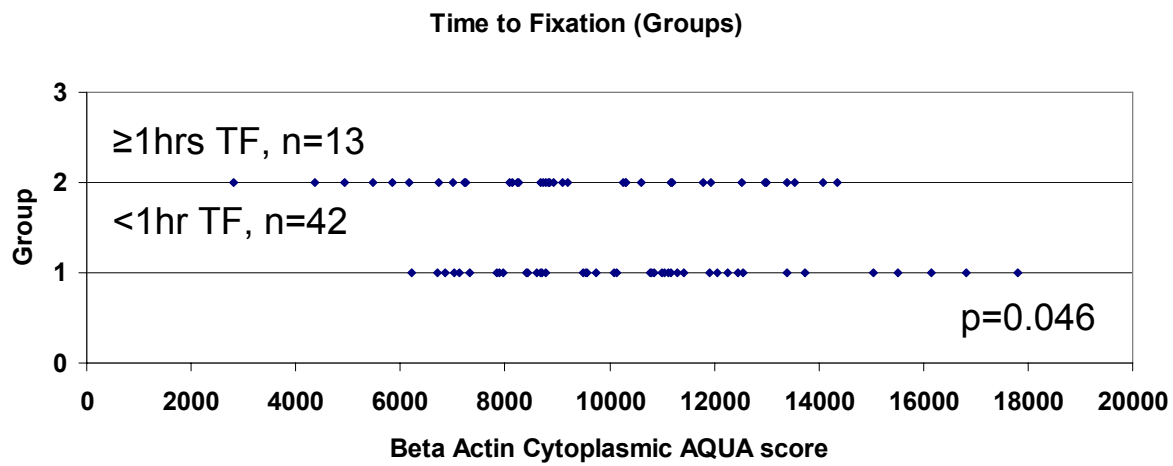
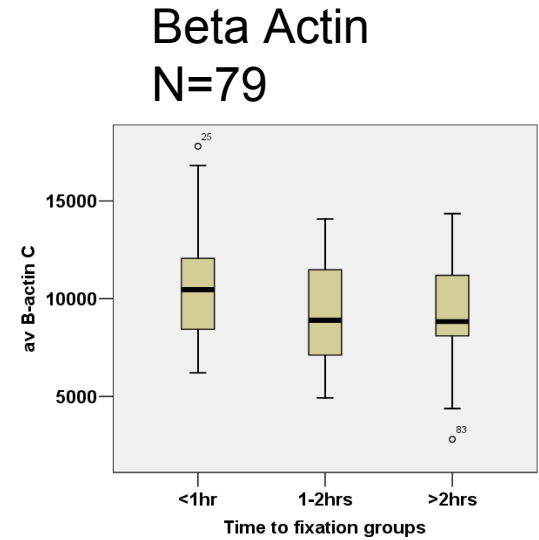
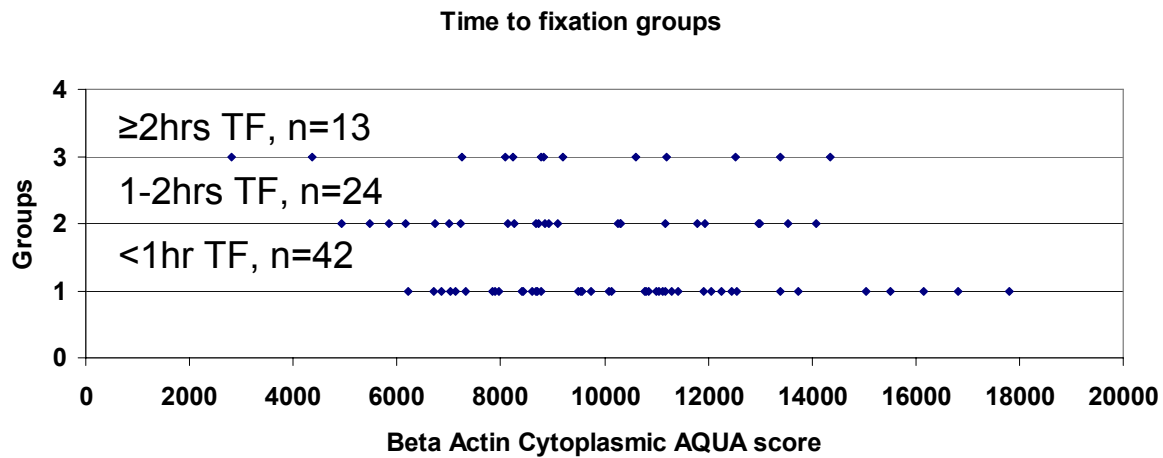


## Western Blotting

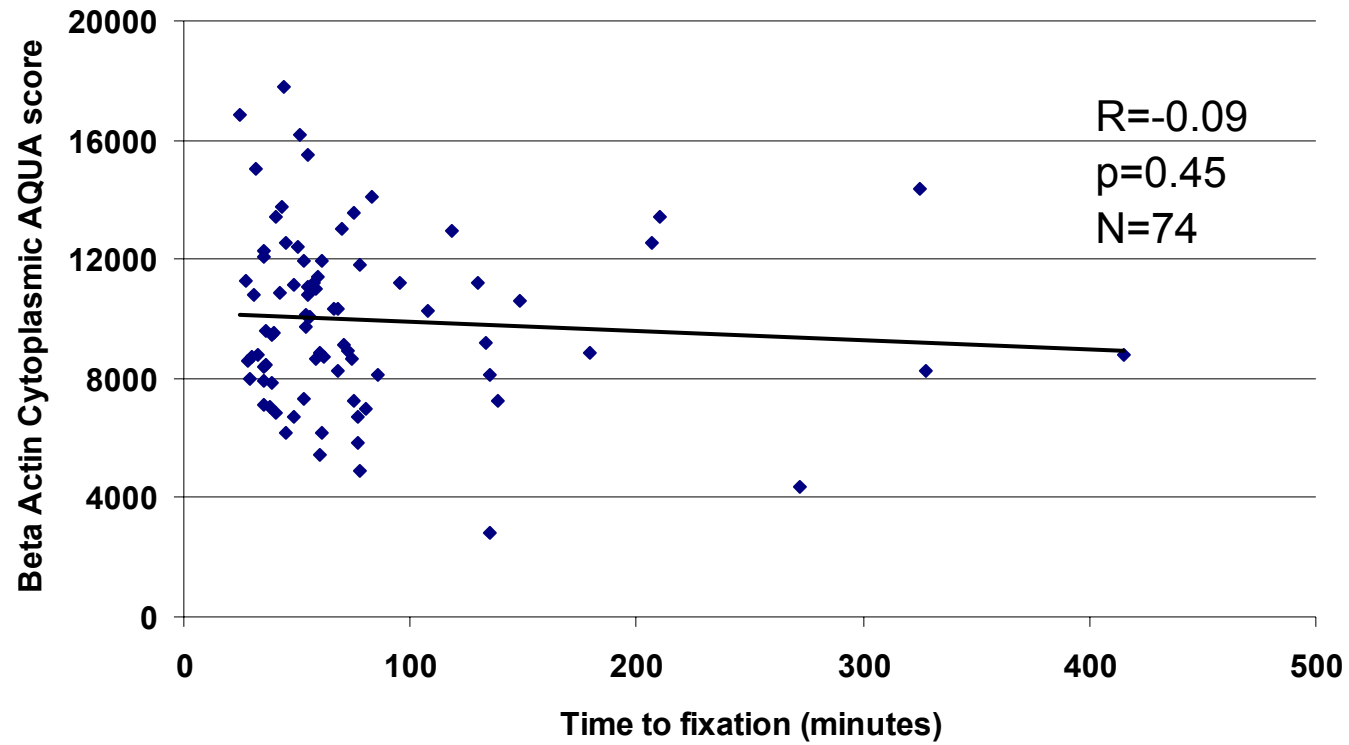


## Quantitative Immunofluorescence

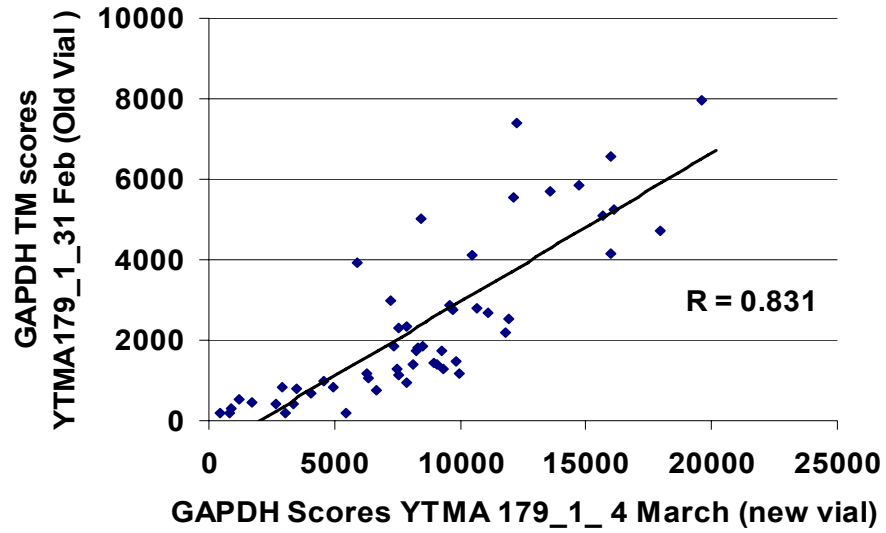




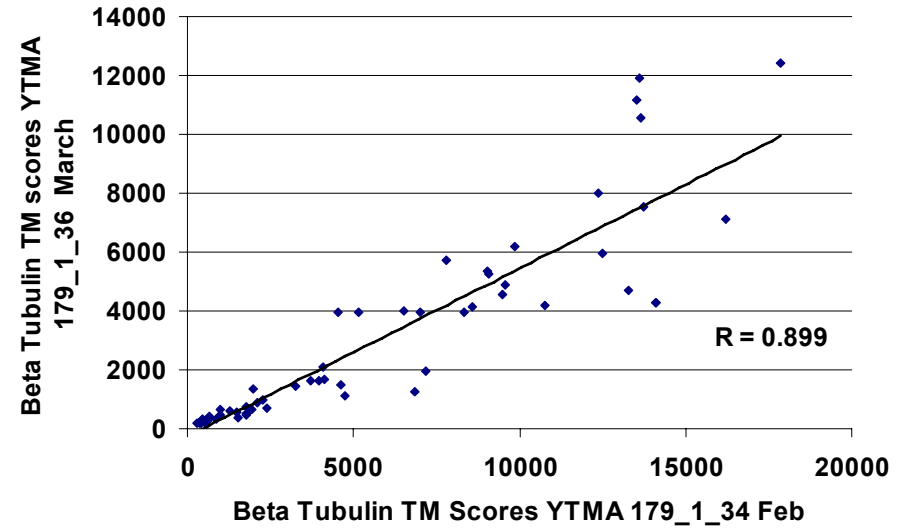
## Correlation between Beta-actin and time to fixation



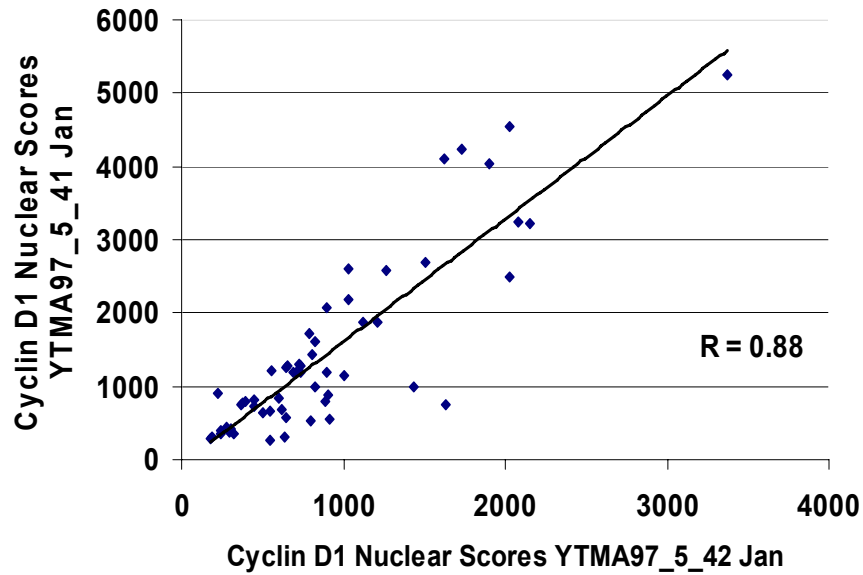
### GAPDH Reproducibility



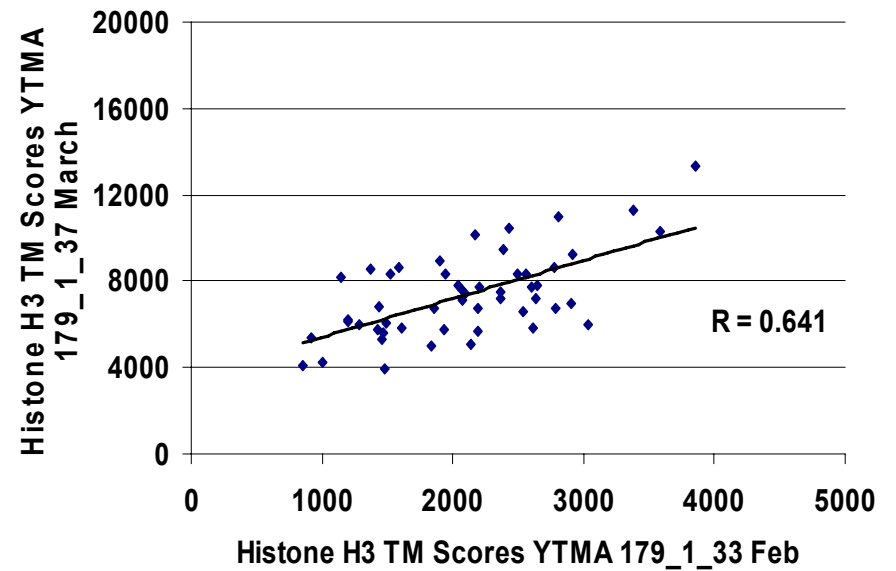
### Beta Tubulin Reproducibility



### Cyclin D1 Reproducibility

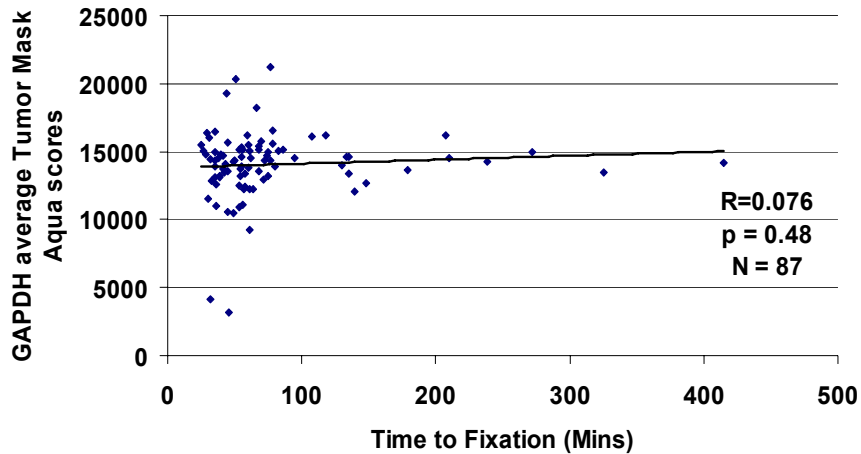


### Histone 3 Reproducibility

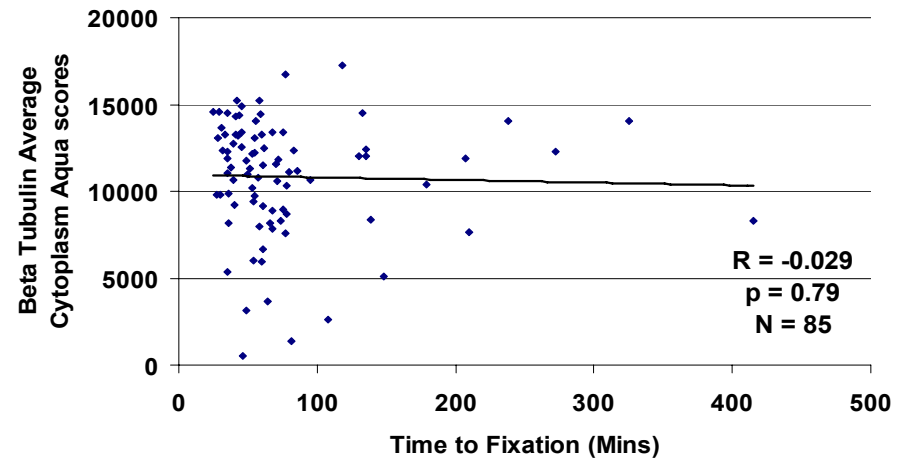


# Correlation between markers of cold ischemia and hypoxia with time to fixation

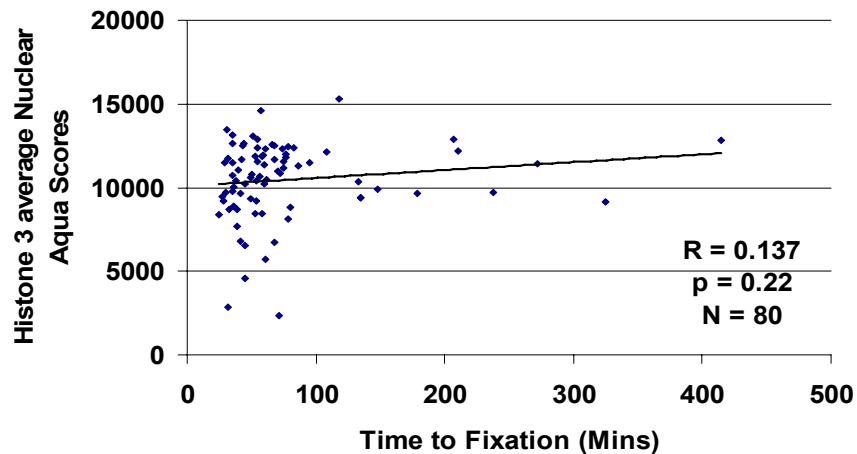
GAPDH - Tumor Mask



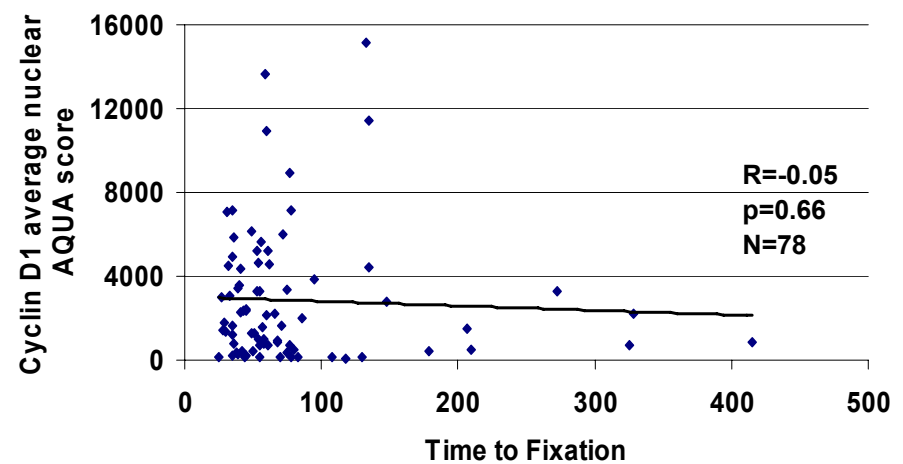
Beta Tubulin - Cytoplasm



Histone 3 - Nuclear

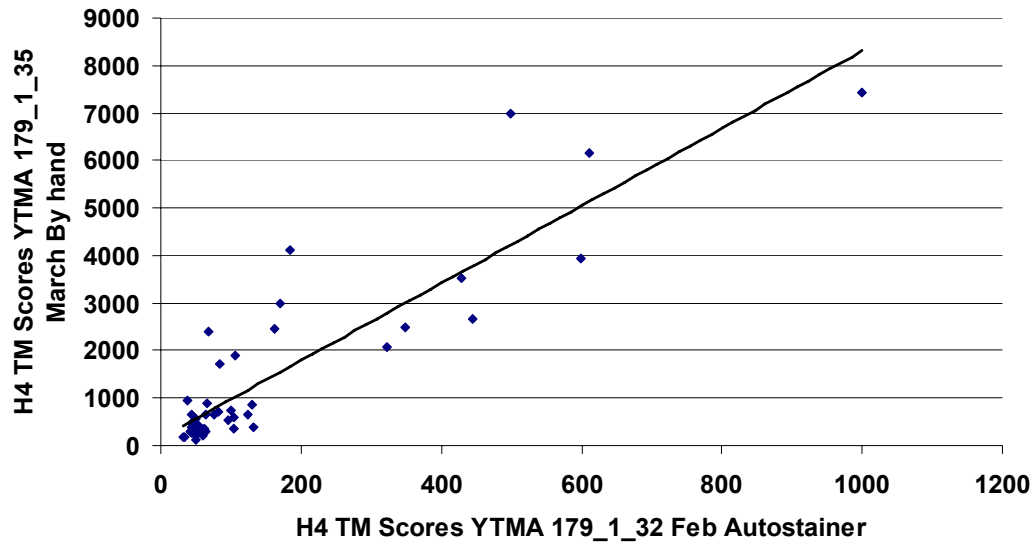


Cyclin D1-Nuclear



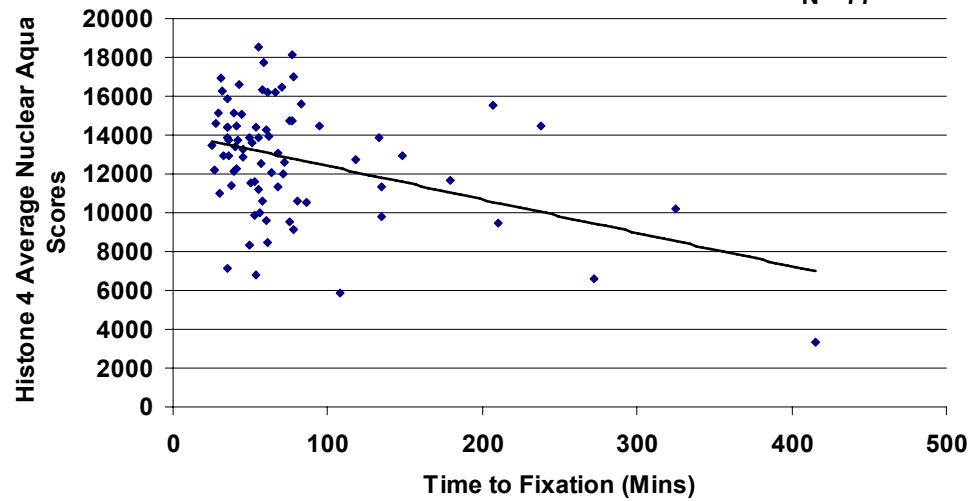
### Histone 4 Reproducibility

R = 0.891



### Histone 4 - Nuclear

R = -0.398  
p = <0.0001  
N = 77



# Preliminary summary of Intrinsic Controls data

- An ischemic time TMA for breast cancer has been built with tissue from David Hicks and the Rochester group
- 7 antibodies have been validated and tested
- Only Histone H4 appears to show degradation linear with time to fixation

# Thanks to:

## Rimm Group:

Valsamo (Elsa) Anagnostou  
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Huan Cheng  
Maria Baquero  
Alley Welsh  
Jason Hanna  
Jennifer Bordeaux  
Bill Bradley  
Larrisa Losovatsky  
Summar Siddiqui  
Liz Killiam  
Yalai Bai  
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## Yale Collaborators

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Ruth Halaban  
Steve Ariyan  
Daniel Boffa  
Catherine Sullivan  
Frank Detterbeck  
Lynn Tanoue  
Lyndsay Harris

## Yale Pathology Tissue Services

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Joe Salame      Aruna Madan  
Sudha Kumar    Emily Storch  
Peter Gershkovich



## Outside Yale Collaborators

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Gerold Bepler (Moffitt)  
Daniel Hayes and SWOG  
Elaine Alarid (UW)  
Bruce Haffty (CINJ)

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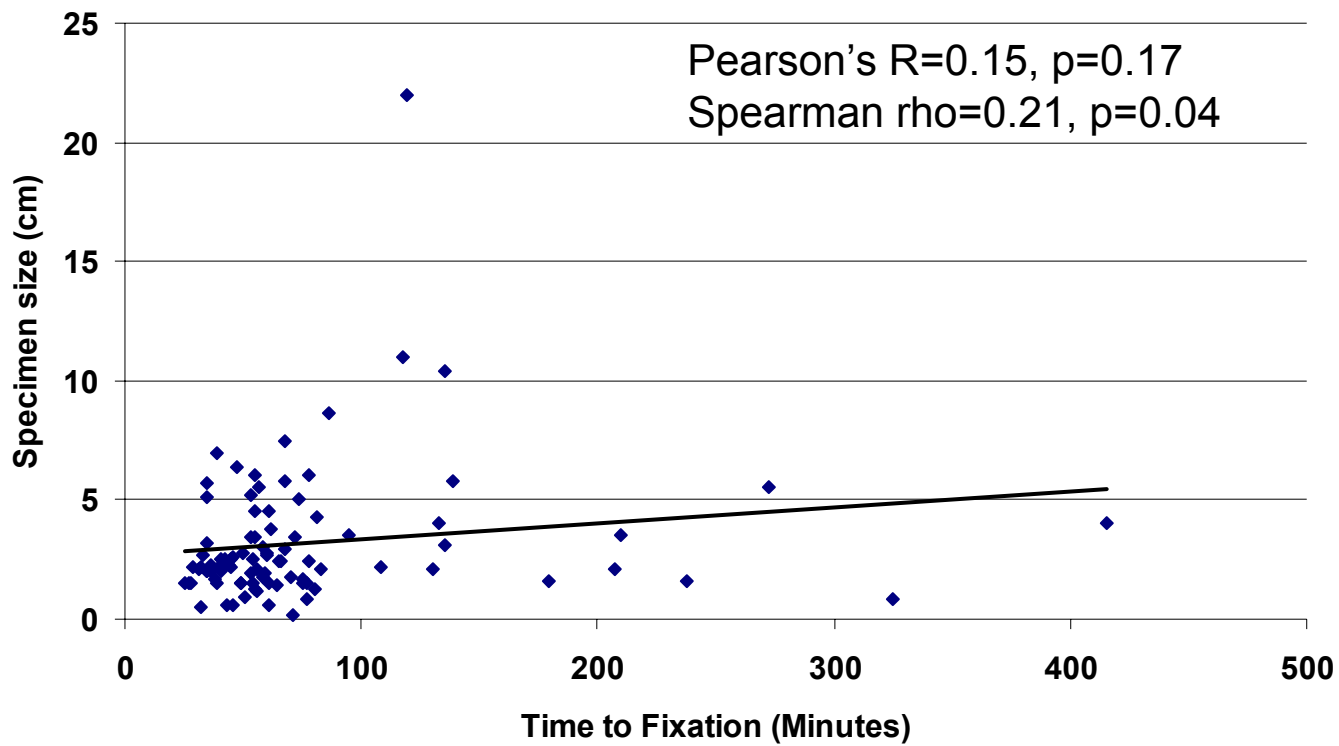


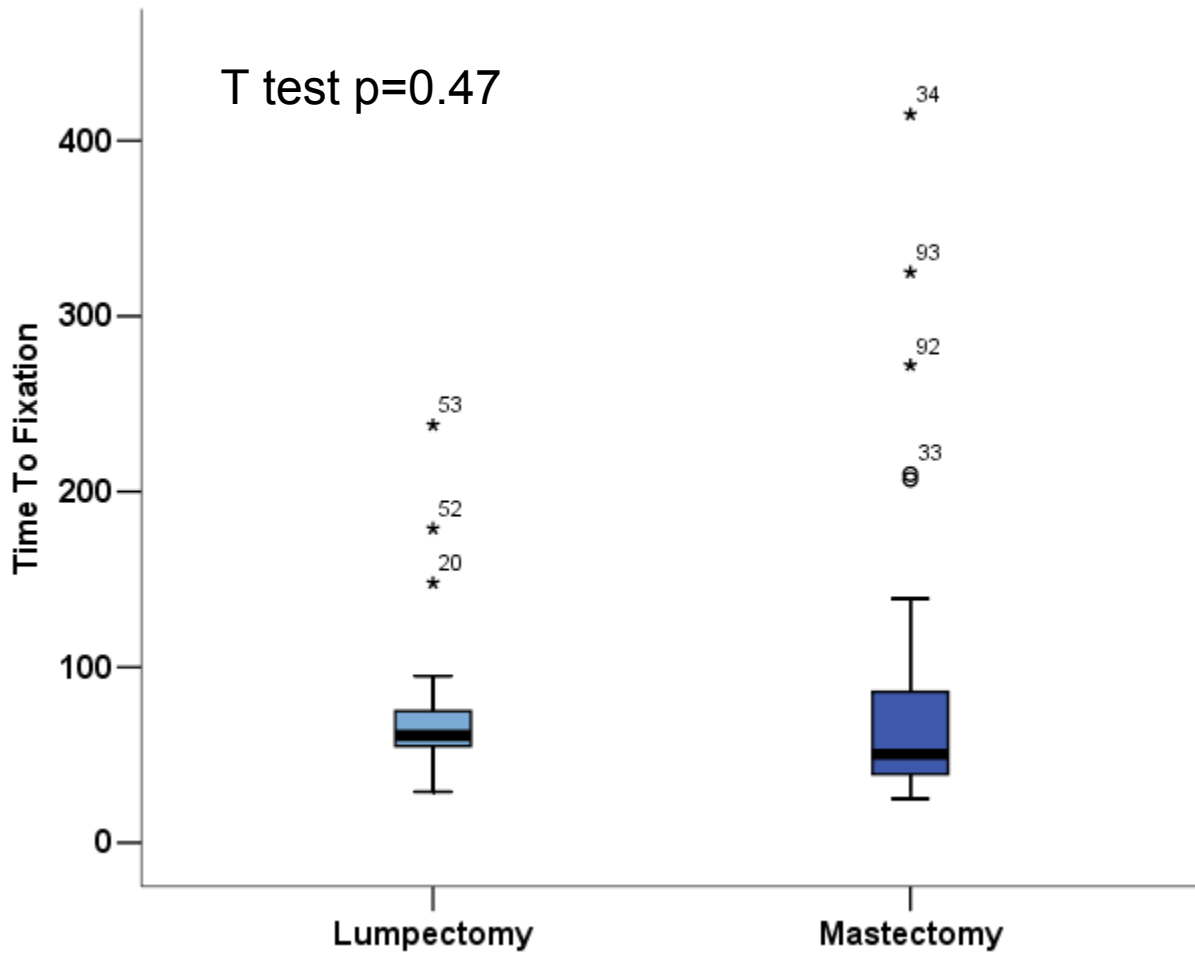


**Rimm Lab Summer '09**

**[www.tissuearray.org](http://www.tissuearray.org)**

## Correlation between specimen size and time to fixation



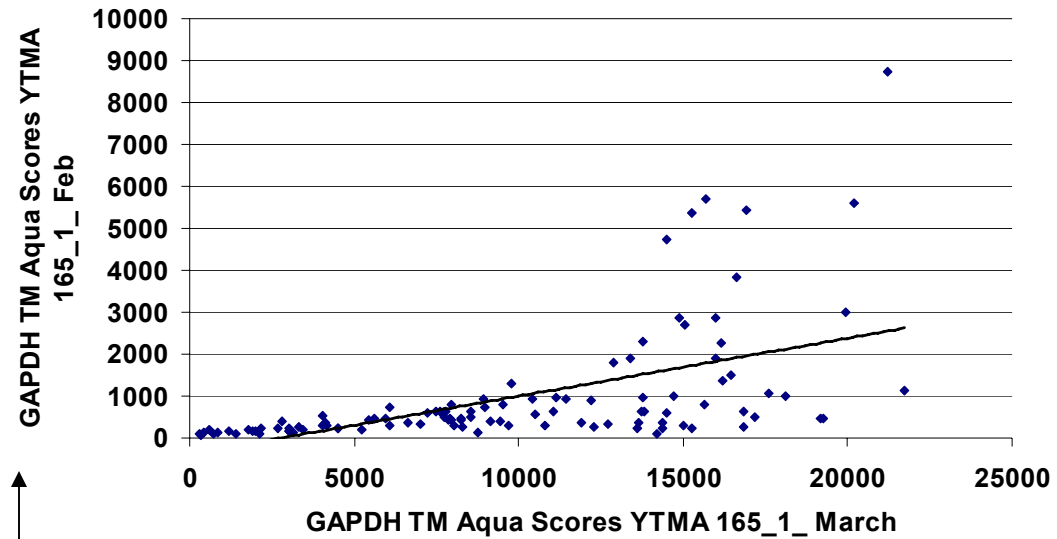


## Summary of Antibodies

<b>Antibody</b>	<b>Incubation Time</b>	<b>Titer Used for Staining</b>
<b>Beta actin</b>	<b>2 hours RT</b>	<b>1:100</b>
<b>Cyclin D1</b>	<b>30 mins RT</b>	<b>1:25</b>
<b>GAPDH</b>	<b>1 hour RT</b>	<b>1:50</b>
<b>Histone 3</b>	<b>1 hour RT</b>	<b>1:200</b>
<b>Histone 4</b>	<b>1 hour RT</b>	<b>1:200</b>
<b>Beta Tubulin</b>	<b>2 hours RT</b>	<b>1:100</b>

GAPDH Reproducibility (TIC Array) All Specimens

R = 0.550



**Autostainer Assay that might not be reliable: slides were not processed all together. They were kept in TBS for prolonged period of time.**

### Antibody loss over time

