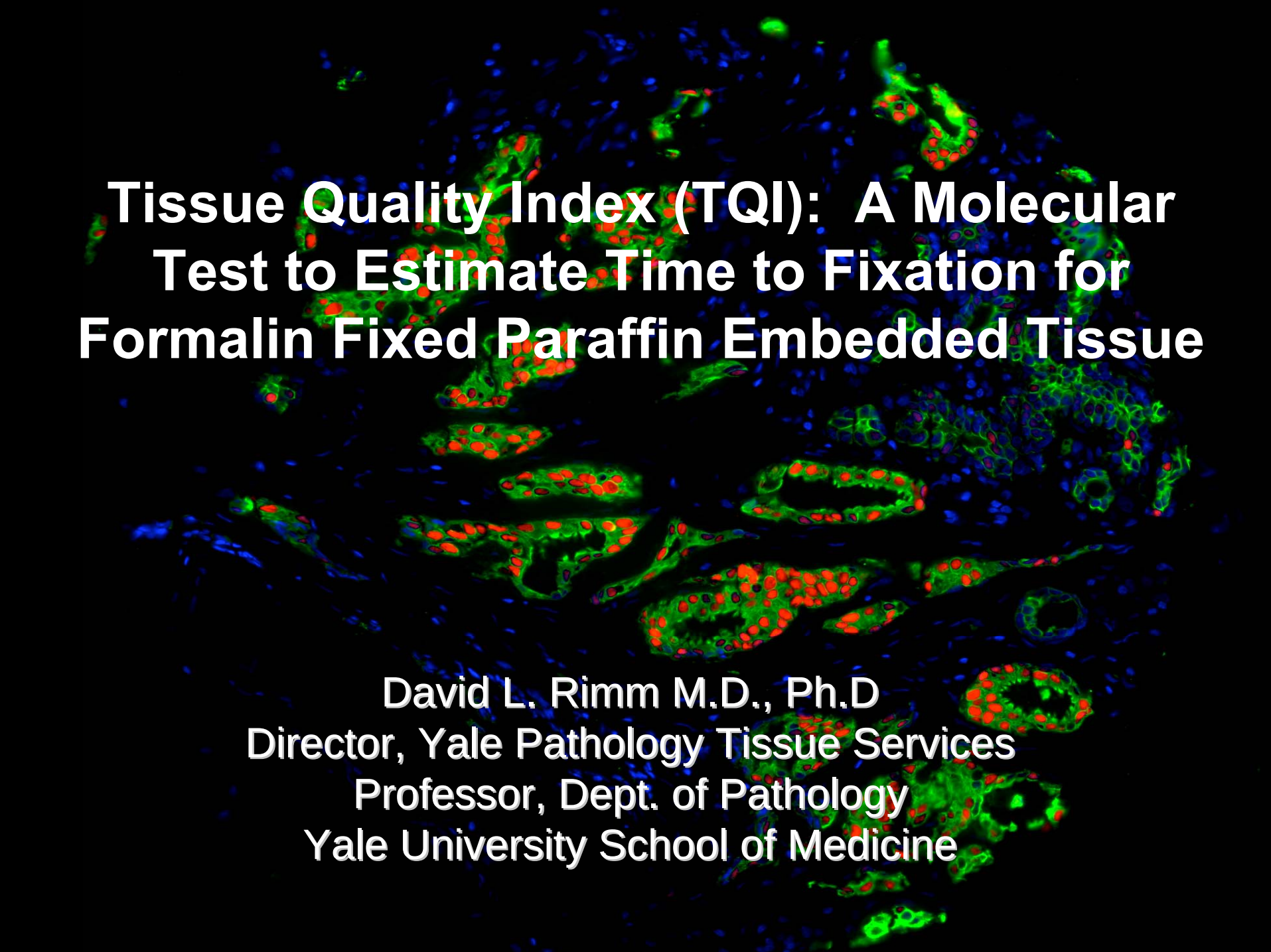


# Tissue Quality Index (TQI): A Molecular Test to Estimate Time to Fixation for Formalin Fixed Paraffin Embedded Tissue

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. HHSN26120080001E. 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 immuno-competence index)

# Extrinsic vs Intrinsic Controls

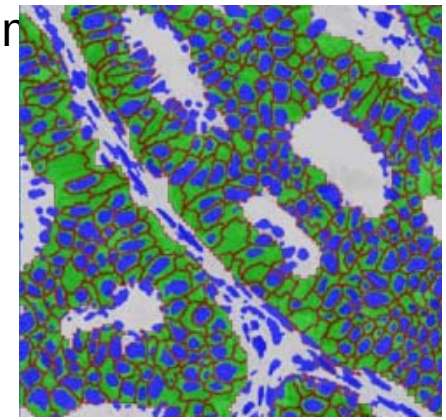
- Extrinsic controls; control for and standardize all the processes from the stainer through the analysis

- Intrinsic controls; control for and standardize all the processes from the patient to the stainer (pre-analytic varibles)

# Generating our solution to quantitative measurement of protein on slides

Available Software: Think like a human

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

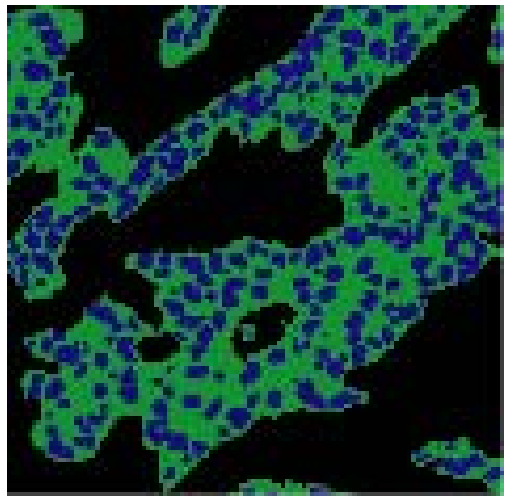


http://www.tissuestudio.com/

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

# AQUA®: 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)

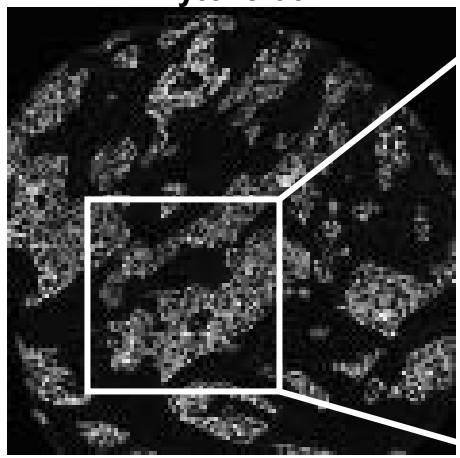
$$\text{Concentration} = \frac{\text{Numerator}}{\text{Denominator}} \Rightarrow \frac{\Sigma \text{ target intensity in compartment pixels}}{\Sigma \text{ compartment pixel area}} = \text{AQUA score}$$

**Step 3:** Calculate the AQUA score

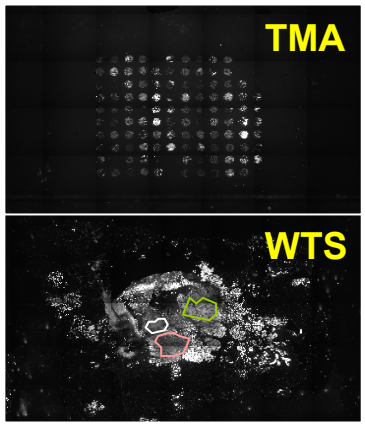
**Step 4:** Convert to absolute concentration or normalize to set of uniform standards

# Generating the AQUA® score

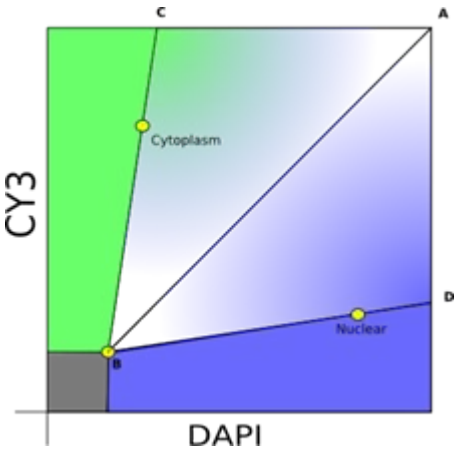
Cytokeratin



Tumor Mask

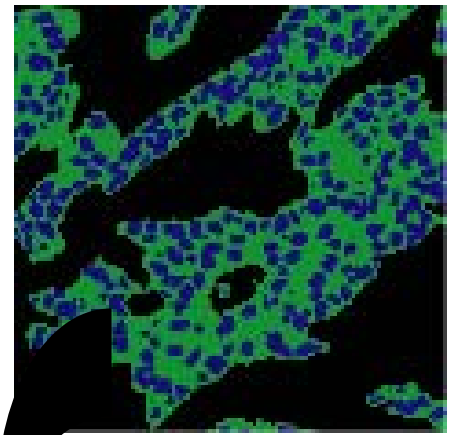


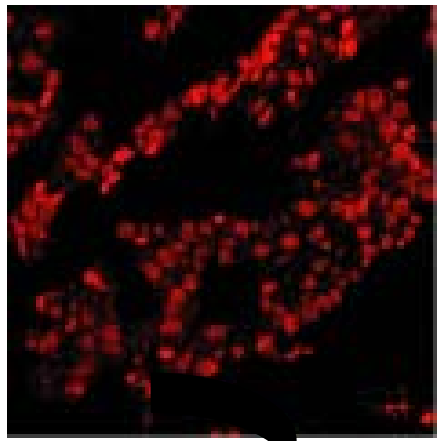

TMA-Tissue Microarray
WTS-Whole Tissue Section




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

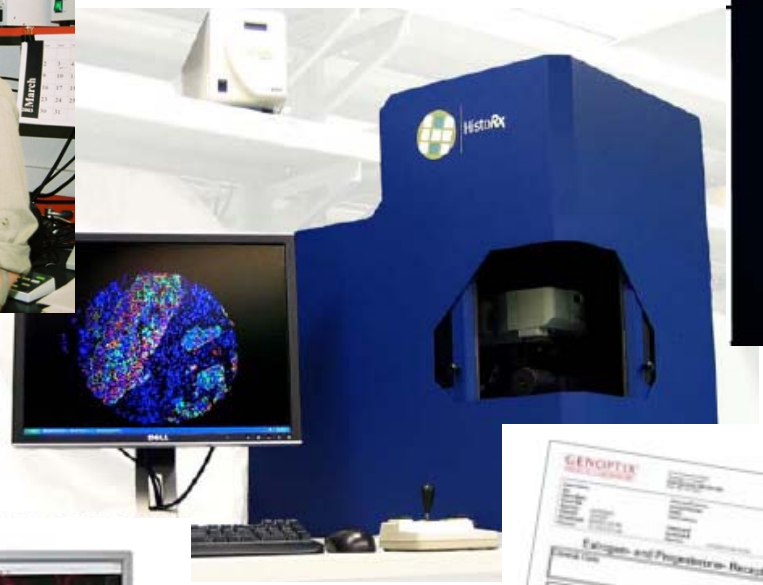
Estrogen Receptor

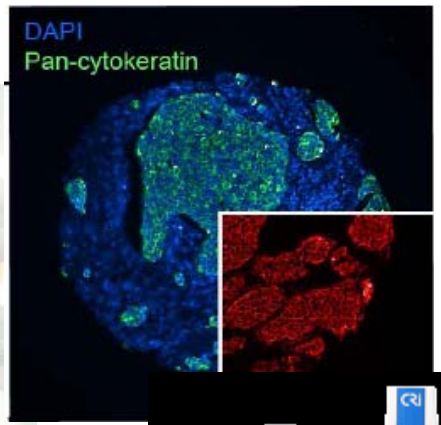




$$\frac{\Sigma \text{ target intensity in compartment pixels}}{\Sigma \text{ compartment pixel area}} = \text{AQUA score}$$

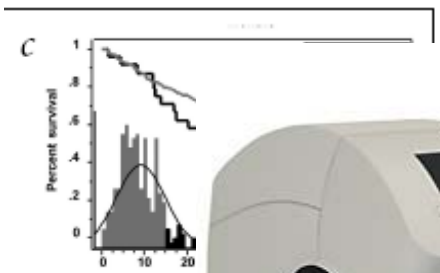
# Development and Commercialization Of a Quantitative Protein Measurement Technology (AQUA) from the lab to the patient






NATURE MEDICINE • VOLUME 8 • NUMBER 11 • NOVEMBER 2002

NEW TECHNOLOGY




NexCourse™ BCa

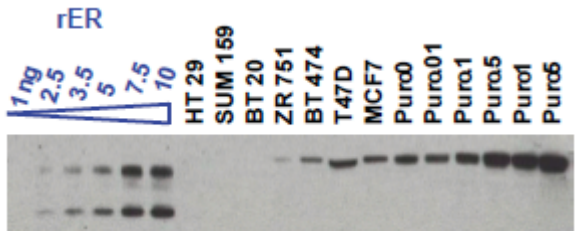
BREAST CANCER ASSAY BY AQUA® TECHNOLOGY

GENOPTIX®
MEDICAL LABORATORY

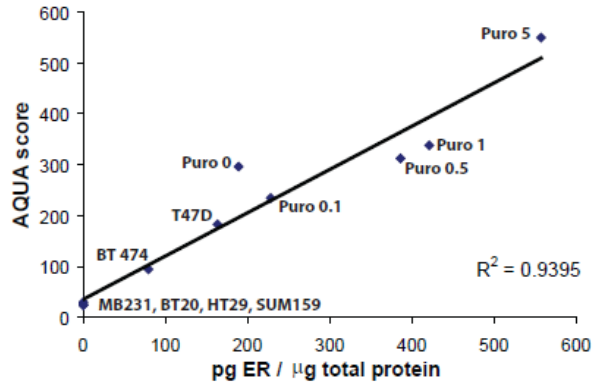
cell line panel
(*known* range of ER)

lysates

Western Blot alongside recombinant ER

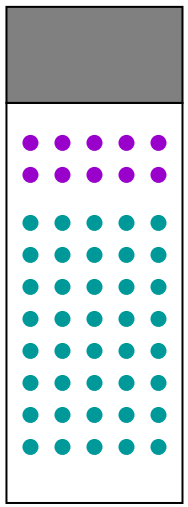



calculate ER in pg/μg




formalin-fixing & paraffin-embedding

40 patient controls
(range of ER)



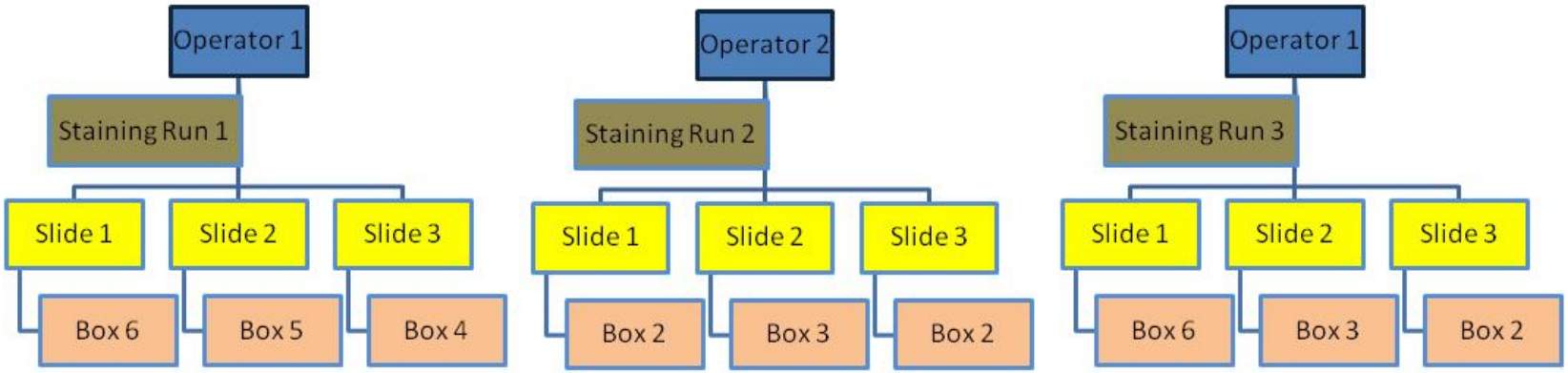
Control TMA

calculate ER as AQUA scores

1) Convert AQUA scores to pg/ug
2) Determine limit of detection for ER positivity

**Alley Welsh**

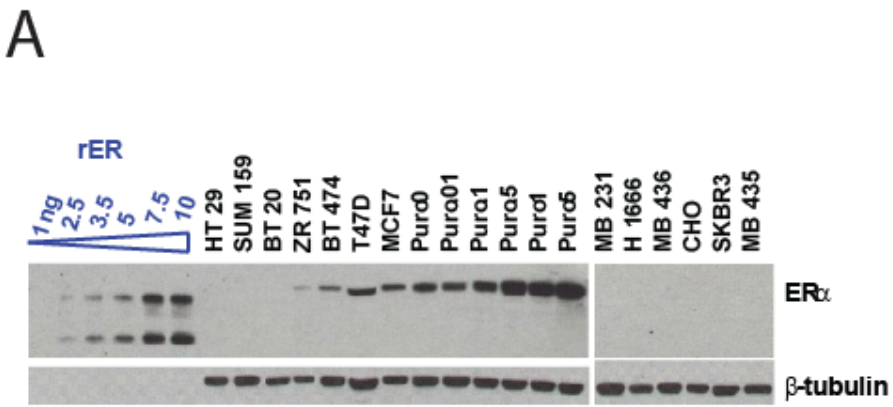
# Precision Results (ER-alpha)

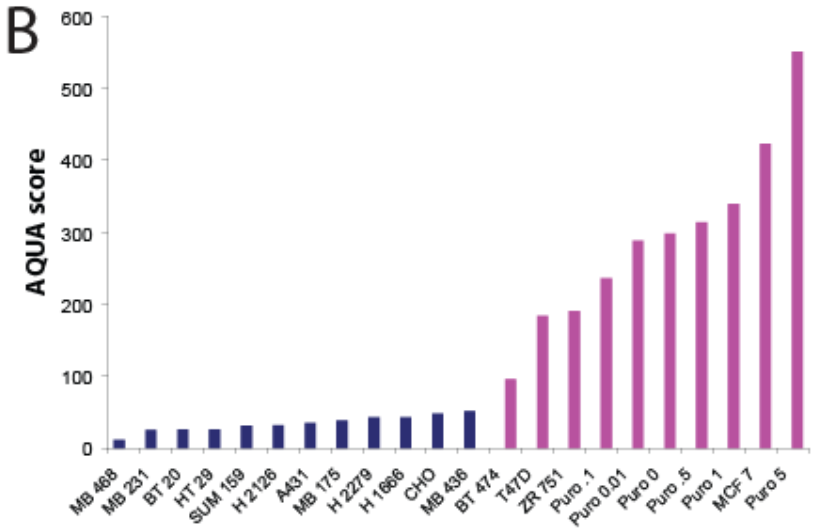


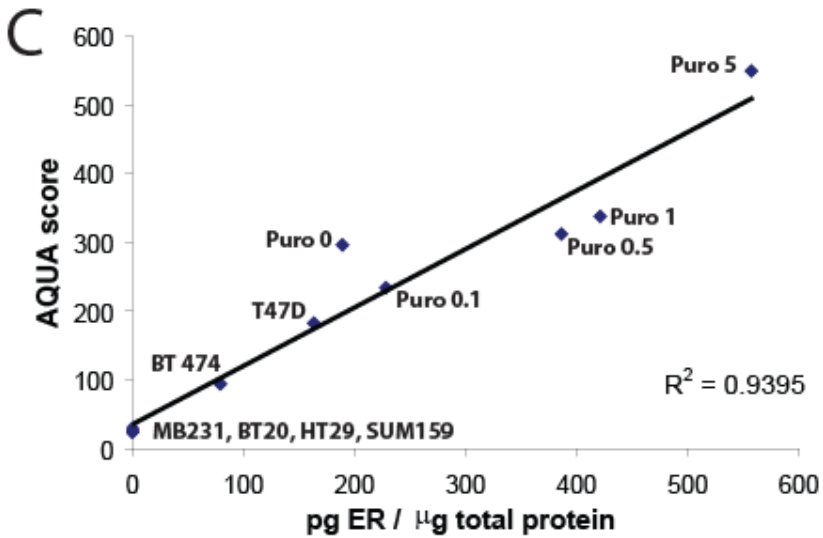

| | Pearson R | Slope |
|---|---|---|
| Day 1 v. Day 2 | .97 | .97 |
| Day 1 v. Day 3 | .97 | 1.01 |
| Day 2 v. Day 3 | .98 | 1.04 |

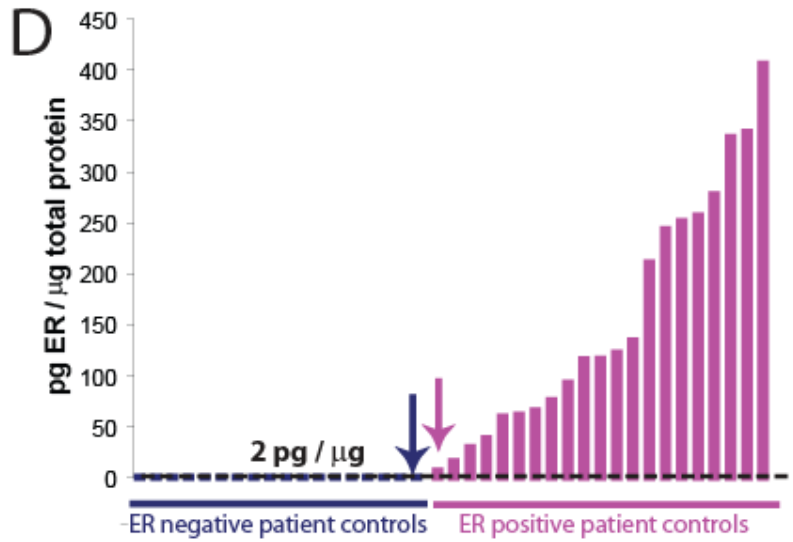
**%CV = 4.2**

Mark Gustavson and
Jason Christiansen



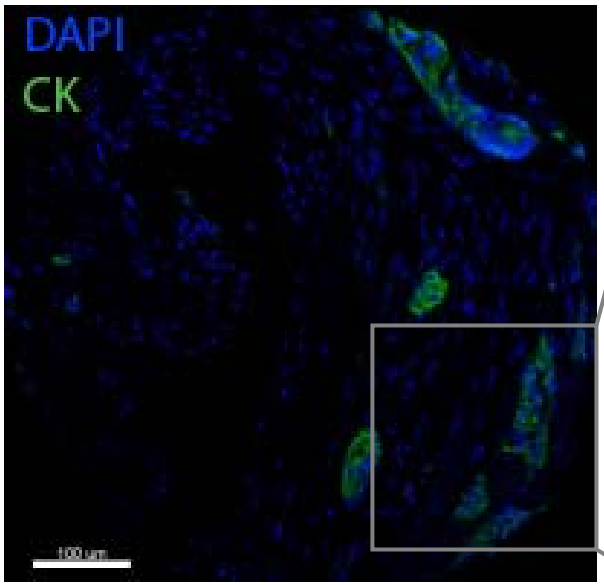



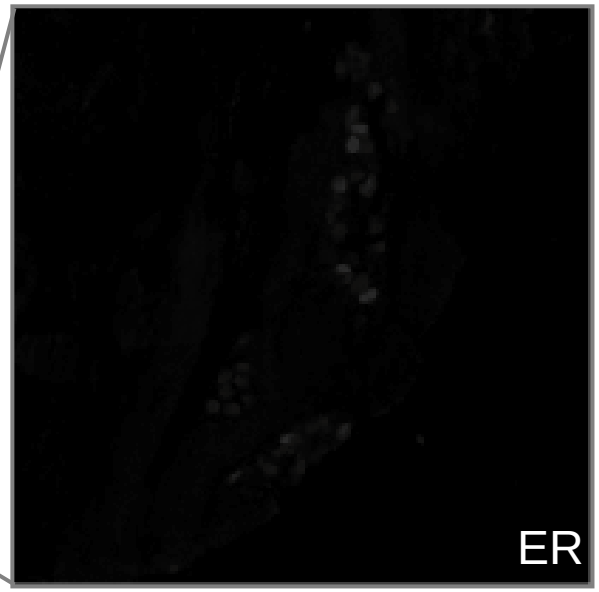

Standardized Index Array

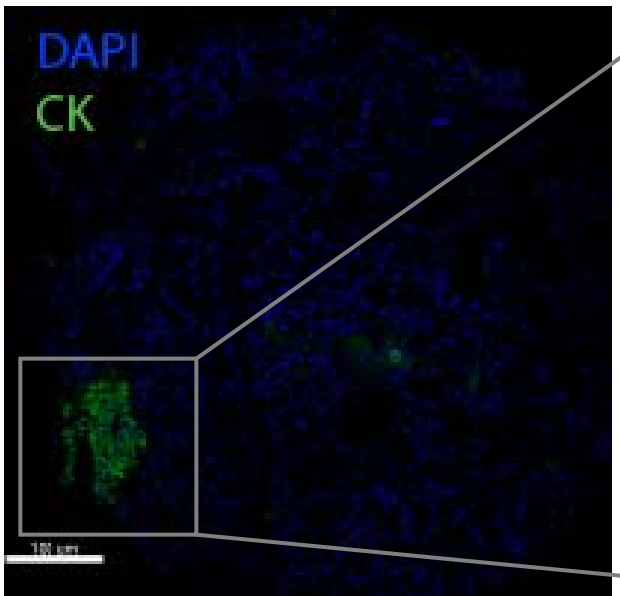
ER antibody used is 1D5

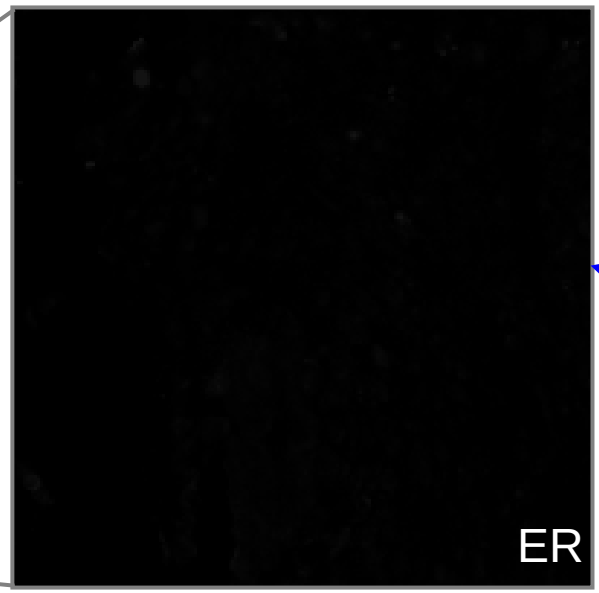
Alley Welsh

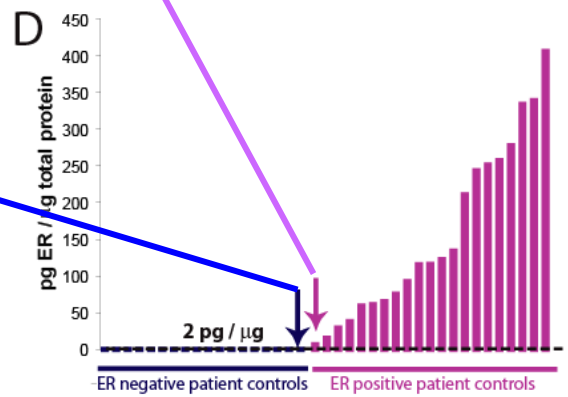
# Lowest positive vs. highest negative

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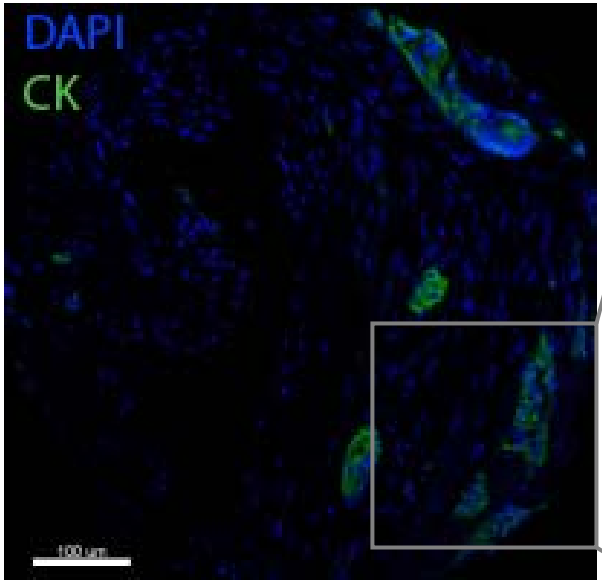
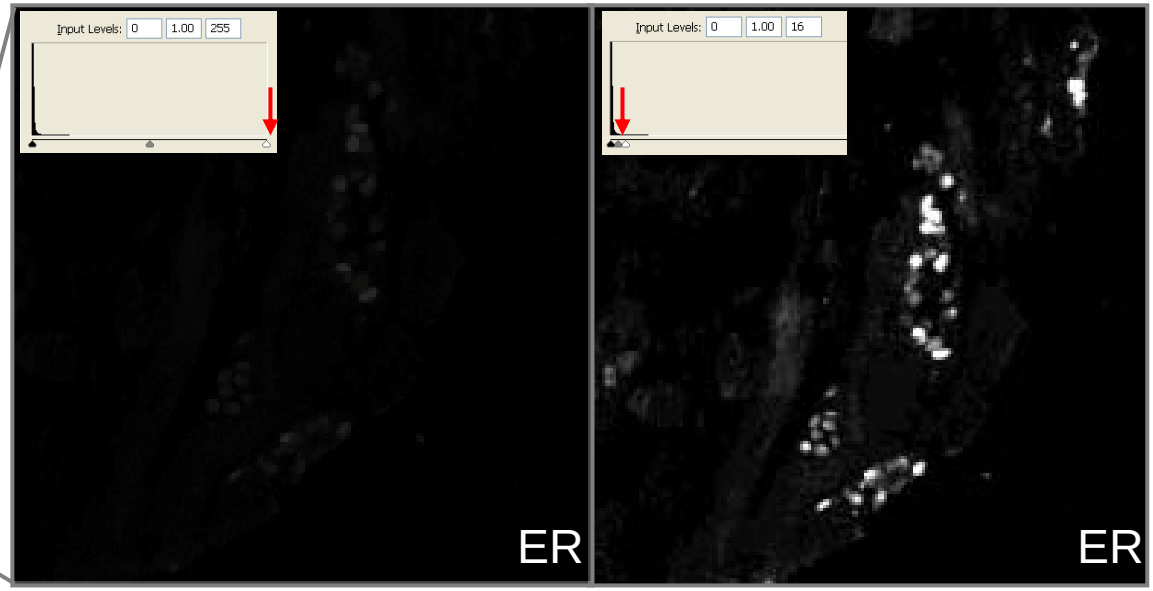
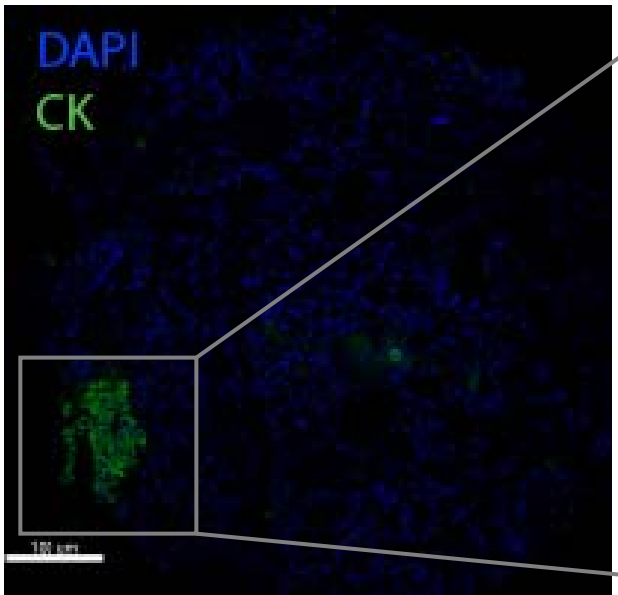
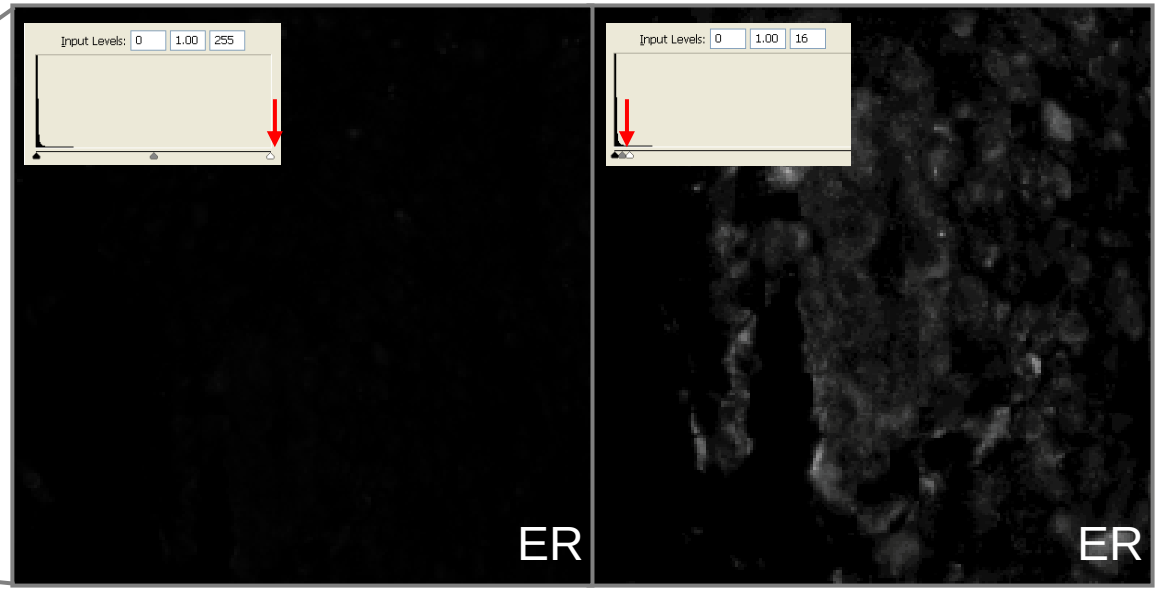
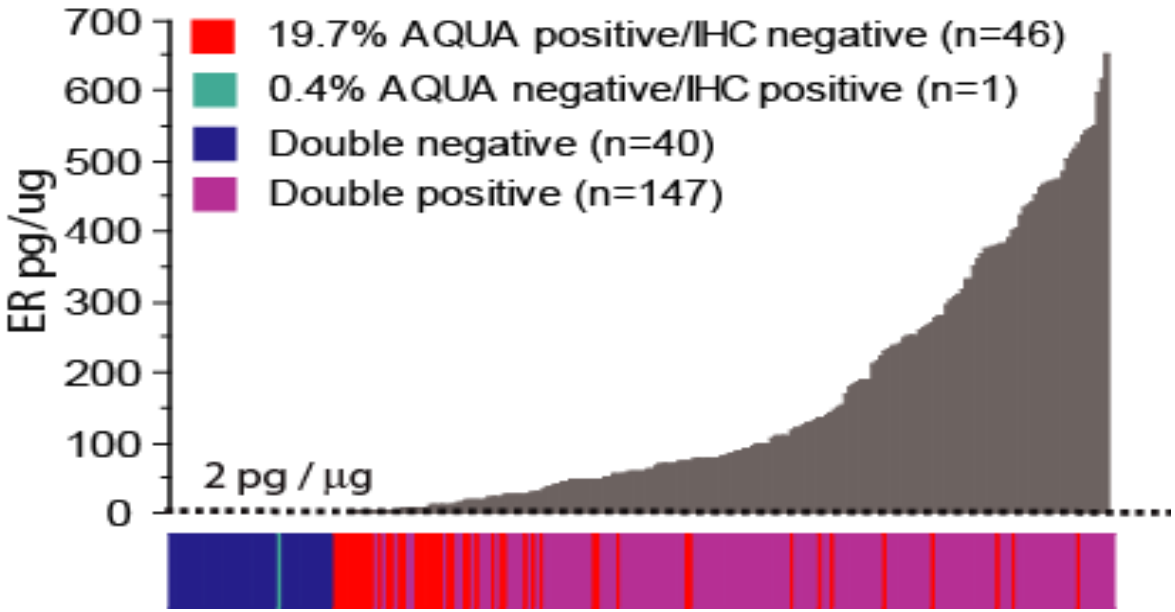

DAPI
CK
100 µm

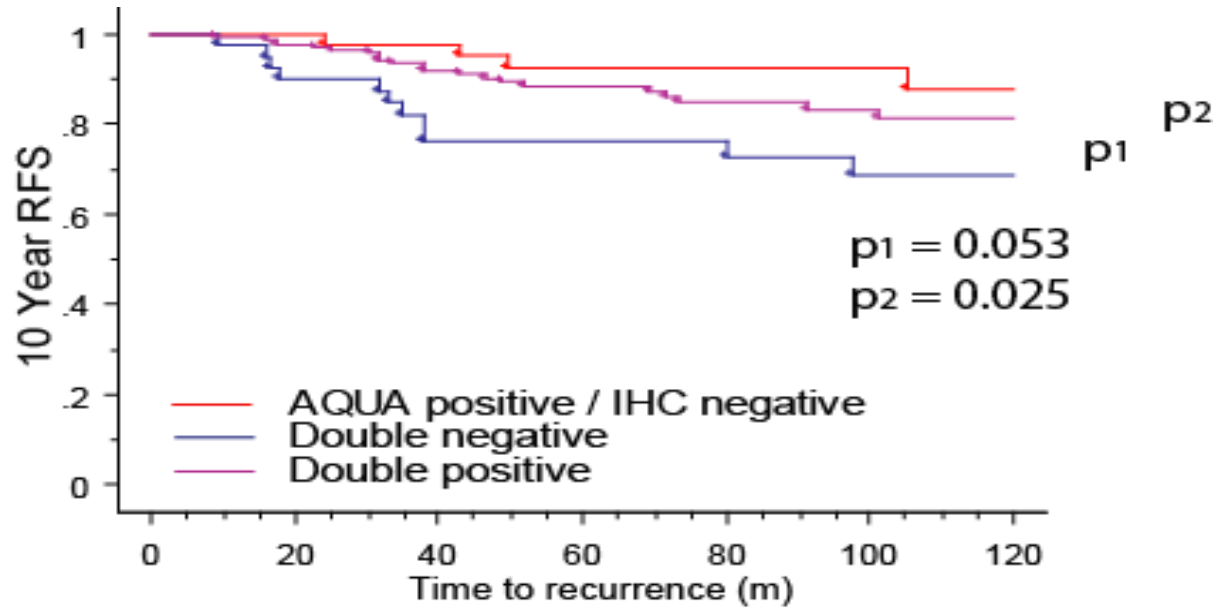

Input Levels: 0 1.00 255
Input Levels: 0 1.00 16
ER
ER


DAPI
CK
100 µm


Input Levels: 0 1.00 255
Input Levels: 0 1.00 16
ER
ER

# Discordant classification of ER status in YTMA 130 cohort



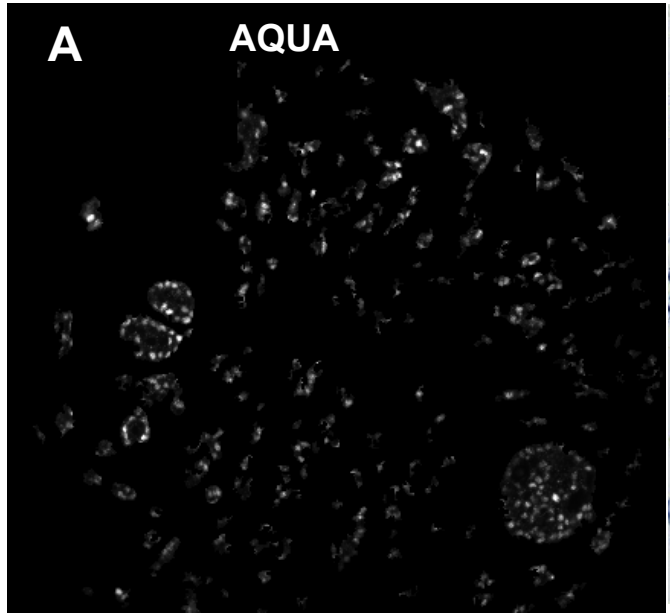



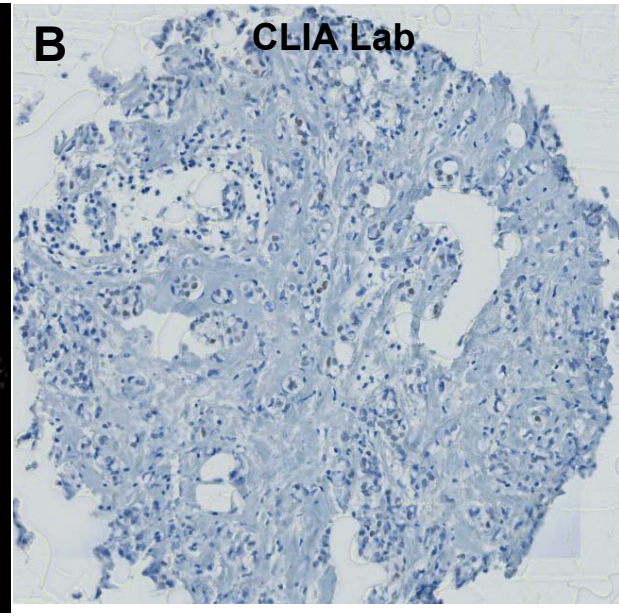

Welsh et al, JCO 2011

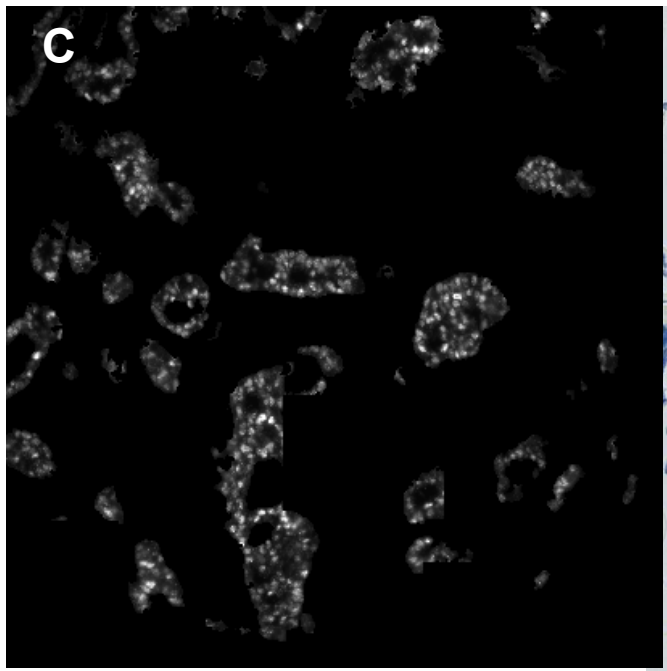
# Two example discordant cases

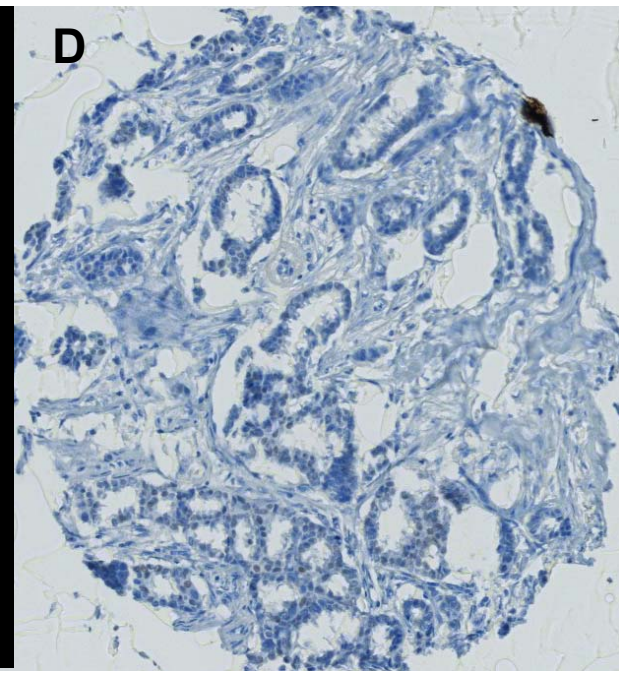
## What is the cause of the discordance?

- Is Q-IF more sensitive than IHC?
- Variation in DAB from lab-to-lab?
- Variation in Hematoxylin counterstain from lab to lab?




Alley Welsh

# The problem is the Hematoxylin

Polysciences, Inc.

*Corporate Headquarters*
*400 Valley Road*
*Warrington, PA 18976*
*1-800-523-2575*
*FAX 1-800-343-3291*
*Email: info@polysciences.com*
*www.polysciences.com*

*Europe - Germany*
*Polysciences Europe GmbH*
*Handelsstr. 3*
*D-69214 Eppelheim, Germany*
*(49) 6221-765767*
*FAX (49) 6221-764620*
*Email: info@polysciences.de*



## *Gill's Hematoxylin - Specific for Staining Nuclei*

***Three formulations for flexibility in nuclear staining.***

**Gill's Hematoxylin No. 1 for Cytology.** (Single Strength) Lower strength formulation, ideal for staining cytology.

**Gill's Hematoxylin No. 2 for Histology and Cytology.** (Double Strength) This intermediate formulation is used as a counterstain for immunohistochemistry (IHC) chromogens and routine Histology. It is excellent for more intense cytological staining.

**Gill's Hematoxylin No. 3 for Histology.** (Triple Strength) The strongest formulation of the stain provides greater intensity for histological staining of nuclei with shorter staining times.

entiation in an acid solution is unnecessary. Nucleoli are delicately stained so that their acidophilia may be seen. The colors of counterstains have no interference from nuclear staining with Gill's Hematoxylin formulas.

**Chemical Principles of Hematoxylin**

Hematoxylin is derived from the extract of logwood and is isolated as a mixture of hematoxylin and hematein. For effectiveness as a stain, hematoxylin must be oxidized to hematein, which is then combined with a metallic iron mordant to increase the selectivity of the stain for chromatin. Sodium iodate is a convenient oxidizing agent while aluminum sulfate is the mordant.[5] Acetic

# Hematoxylin Confounds Automation

Science Translational Medicine
AAAS

RESEARCH ARTICLE

IMAGING

## Systematic Analysis of Breast Cancer Morphology Uncovers Stromal Features Associated with Survival

Andrew H. Beck,[1,2]* Ankur R. Sangoi,[1,3] Samuel Leung,[4] Robert J. Marinelli,[5] Torsten O. Nielsen,[4] Marc J. van de Vijver,[6] Robert B. West,[1] Matt van de Rijn,[1] Daphne Koller[7]†

The C-Path system permits the quantification of thousands of morphologic features in breast cancer microscopic images facilitating the construction of a robust prognostic model and the discovery of new prognostically significant morphologic phenotypes in breast cancer.

Our results suggest that, prior to applying C-Path to images from a new institution that uses a different slide processing regimen, it may be useful to train the epithelial/stromal classifier on a subset of images from the new institution.

Should be “will be necessary”

# 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 OBBR Contract/Project

- Development of a Tissue Quality Index (TQI):
  - by developing a quantitative intrinsic control that can measure the degree of degradation of any FFPE sample.
  - Validation of the TQI
- Assessment of the effects of Time to Fixation on Common Markers using QIF
- Proof that result is the same using quantitative DAB-based IHC

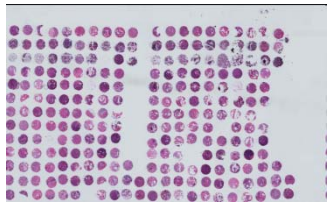
# Pre-Analytic Variables; Can we treat them as a black box?

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

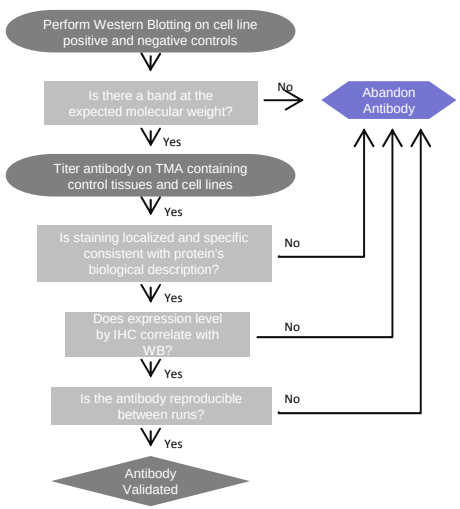
**Can we disqualify specimens for companion dx testing?**

# Approach

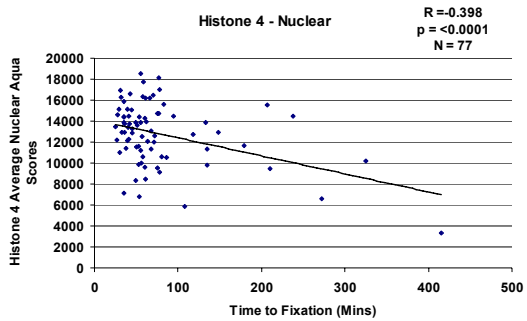
Generate Intrinsic Control cohorts



Select and validate potential antibodies/reagents



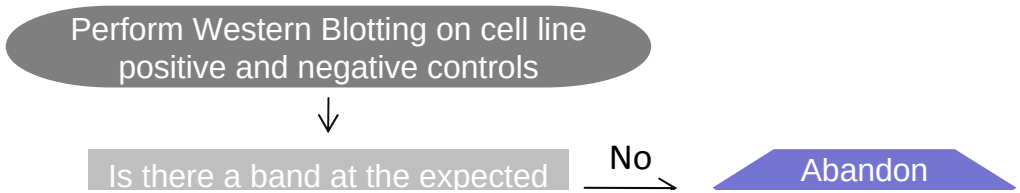

Test each reagent individually on Intrinsic Control Cohorts




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

Validate TQI

# Antibody Validation (Overview)




Review

## Antibody validation

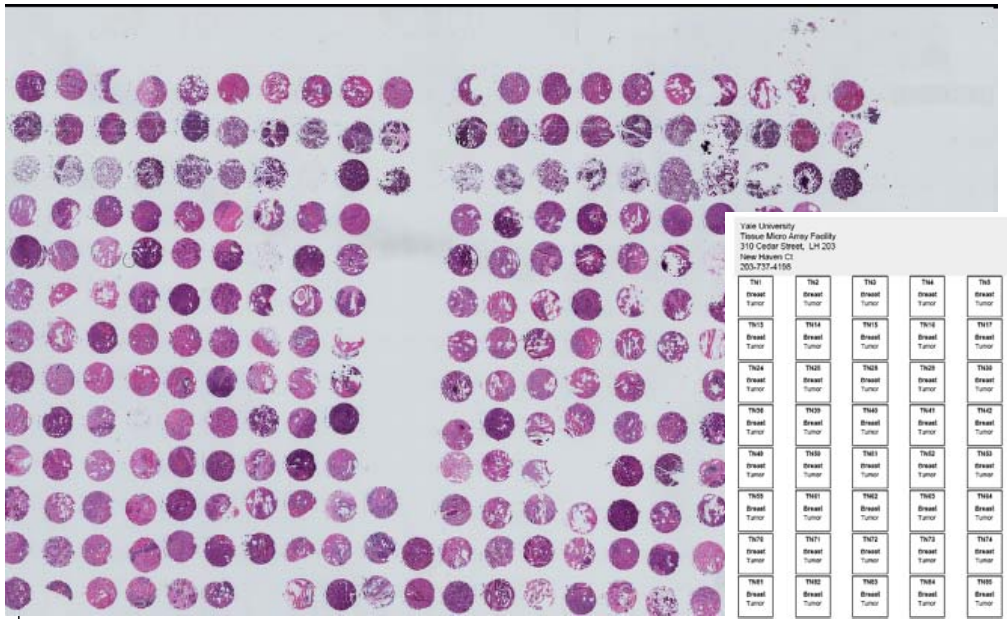
Jennifer Bordeaux, Allison W. Welsh, Seema Agarwal, Elizabeth Killiam, Maria T. Baquero, Jason A. Hanna, Valsamo K. Anagnostou, and David L. Rimm
*Department of Pathology, Yale University School of Medicine, New Haven, CT, USA*

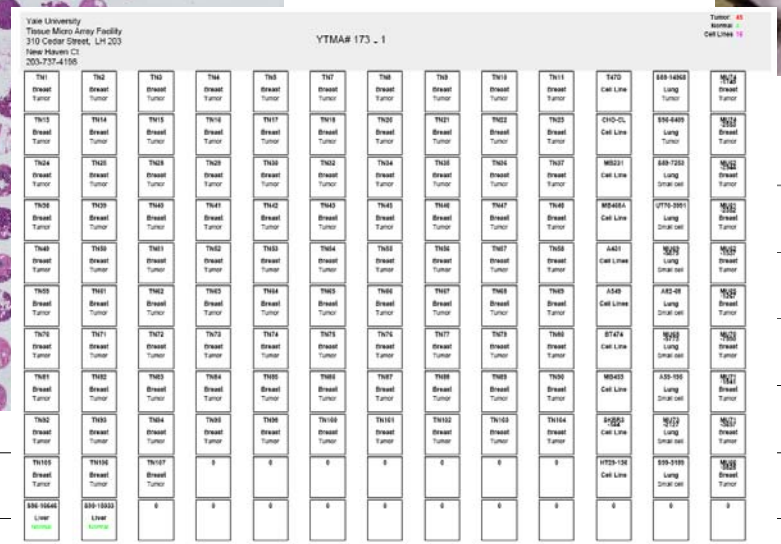
*BioTechniques* 48:197-209 (March 2010) doi 10.2144/000113382
Keywords: antibody; validation; immunohistochemistry; immunofluorescence

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.

Validated

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









Two fold redundancy

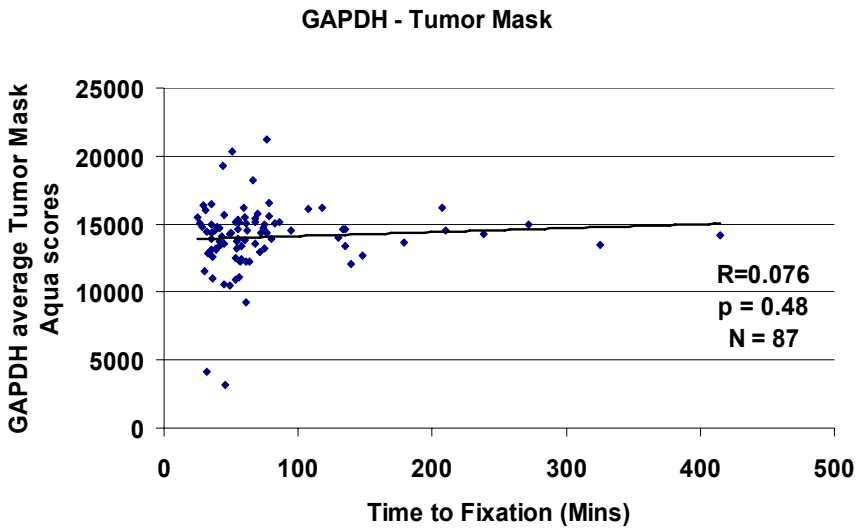
N=125 , tumor=93, normal=2, cell lines=10 control breast tumor=10 ,control lung tumor = 10

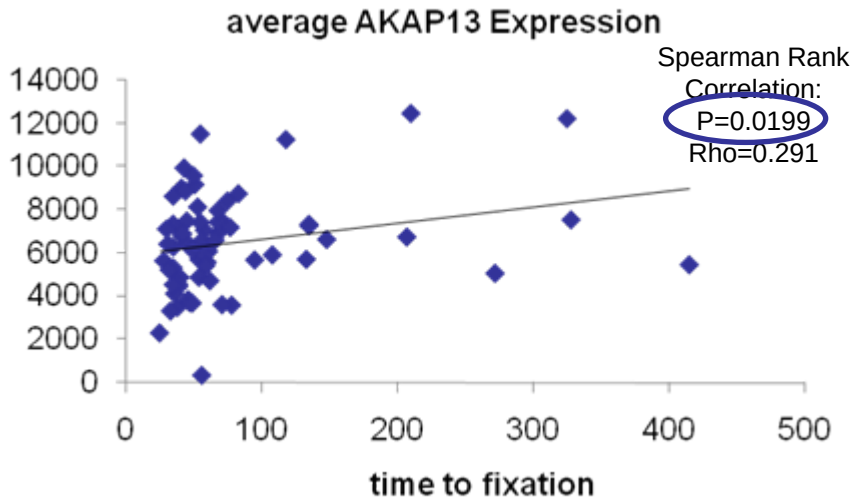
Collected by Dr. David Hicks and colleague, University of Rochester Medical Center

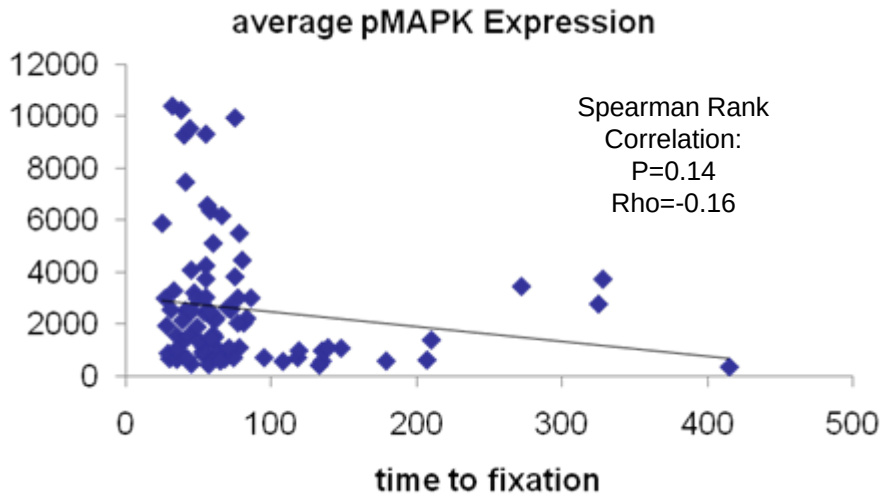
# Summary of markers, which were titrated and validated up to date:

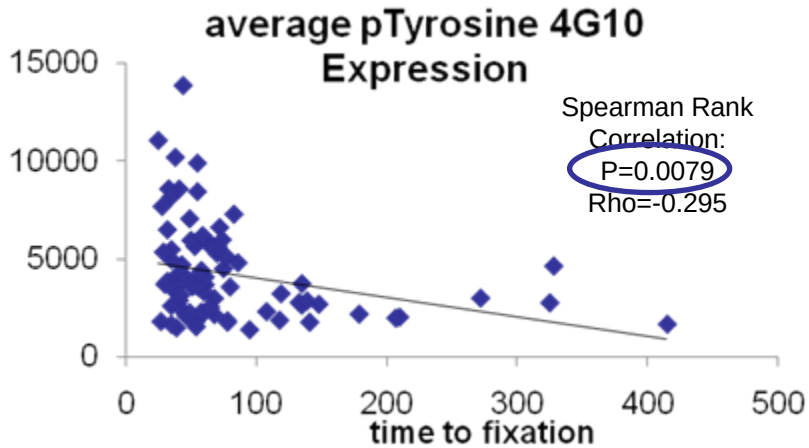
| Symbol | Description | Antibody | | | | Supplier |
|---|---|---|---|---|---|---|
| | | Origin | Clone/Isotype | Catalog # | Validated | |
| ***Markers of Cold Ischaemia*** | | | | | | |
| ACTB | Beta-Actin | Rabbit | 13E5/IgG | 13E5/IgG | 13E5/IgG | Cell Signaling Technology |
| TUBB | Beta-Tubulin | 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 | no | Cell Signaling Technology |
| RPL19 | Ribosomal Protein 19 | Mouse | IgG2a/K-12 | sc-100830 | no | Santa Cruz Biotechnology |
| RPL9 | Ribosomal Protein 9 | Mouse | IgG1/ST-7 | sc-100828 | no | Santa Cruz Biotechnology |
| RPS16 | Ribosomal Protein 16 | Rabbit | polyclonal | sc-102087 | no | Santa Cruz Biotechnology |
| LMNA/C | Lamin A/C | Rabbit | polyclonal | 2032 | yes | Cell Signaling Technology |
| LDHA | Lactat Dehydrogenase | Rabbit | IgG, C4B5 | 3582 | yes | Cell Signaling Technology |
| ERalpha | Estrogen Receptor alpha | Rabbit | SP1/IgG | RM-9101 | yes | Thermo Scientific |
| CK | Cytokeratin | Mouse | AE1/AE3/IgG1 | M3515 | yes | DAKO |
| CK | Cytokeratin | Rabbit | polyclonal | ZO622 | yes | DAKO |
| | | | | | | |
| Eosin | Shandon EosinY aqueous | | | 6766009 | yes | Thermo Electron Corporatior |
| | | | | | | |
| ***Markers of Hypoxia*** | | | | | | |
| VEGF | Vascular Endothelial Growth Factor | Mouse | VG1/IgG1, kappa | M7273 | no | DAKO |
| CCND1 | Cyclin D1 | Rabbit | IgG/SP4 | RM-9104 | yes | Thermo Fisher Fremont |
| Caspase | Cleaved Caspase 3 (Asp175) | Rabbit | polyclonal | 9661 | yes | Cell Signaling Technology |
| HIF1 | Hypoxia Inducible Factor 1 | Rabbit | polyclonal | NB 100-449 | yes | Novus Biological |
| AKAP13 | A-kinase anchoring protein13 | Mouse | IgG2a/ZX-18 | sc-81902 | yes | Santa Cruz Biotechnology |
| CDC42 | | Mouse | IgG3/B-8 | 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/R-16 | 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 | no | Lifespan Biosciences |
| CA9 | Carbonic Anhydrase IX | Rabbit | polyclonal(aa581-592) | LS-B273 | no | Lifespan Biosciences |
| Cleaved Caspase 8 | Cleaved Caspase 8 | Rabbit | IgG, 18C8 | 9496 | in progress | Cell Signaling Technology |
| | | | | | | |
| ***Markers of phosphorylated proteins*** | | | | | | |
| pAKT 473 | phospho-Akt (ser473) | Rabbit | D9E/IgG | 4060 | yes | Cell Signaling Technology |
| pAKT 308 | Phosho-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 |
| Anti-Phosphotyrosine | 4G10 Anti-Phosphotyrosine | Mouse | IgG2b | 05-1050 | yes | Millipore |
| Anti-Phosphoserine | 4A4 Anti-Phosphserine | Mouse | IgG1/4A4 | 05-1000 | no | Millipore |
| Anti-Phosphoserine/threonine/tyrosine | Anti-Phosphoserine/threonine/tyrosine | Mouse | IgG1/spm101 | AB15556 | no | abcam |
| p53 | Anti-Human p53 protein | Mouse | IgG2b. DO-7 | M7001 | in progress | DAKO |
| | | | | | | |
| ***Markers of posttranslational modification*** | | | | | | |
| Sumo1 | small ubiquitin related modifier 1 | Rabbit | Y299/IgG | ab32058 | yes | abcam |
| Acetylated-Lysine | proteins posttranslat. Modified by acetylation | Rabbit | polyclonal, purified | 9441 | yes | Cell Signaling Technology |
| NEDD8 | neural precursor cell-expr. devel. Downreg. protein9 | Rabbit | IgG, 19E3 | 2754 | yes | Cell Signaling Technology |

# Change in expression as a function of time to fixation



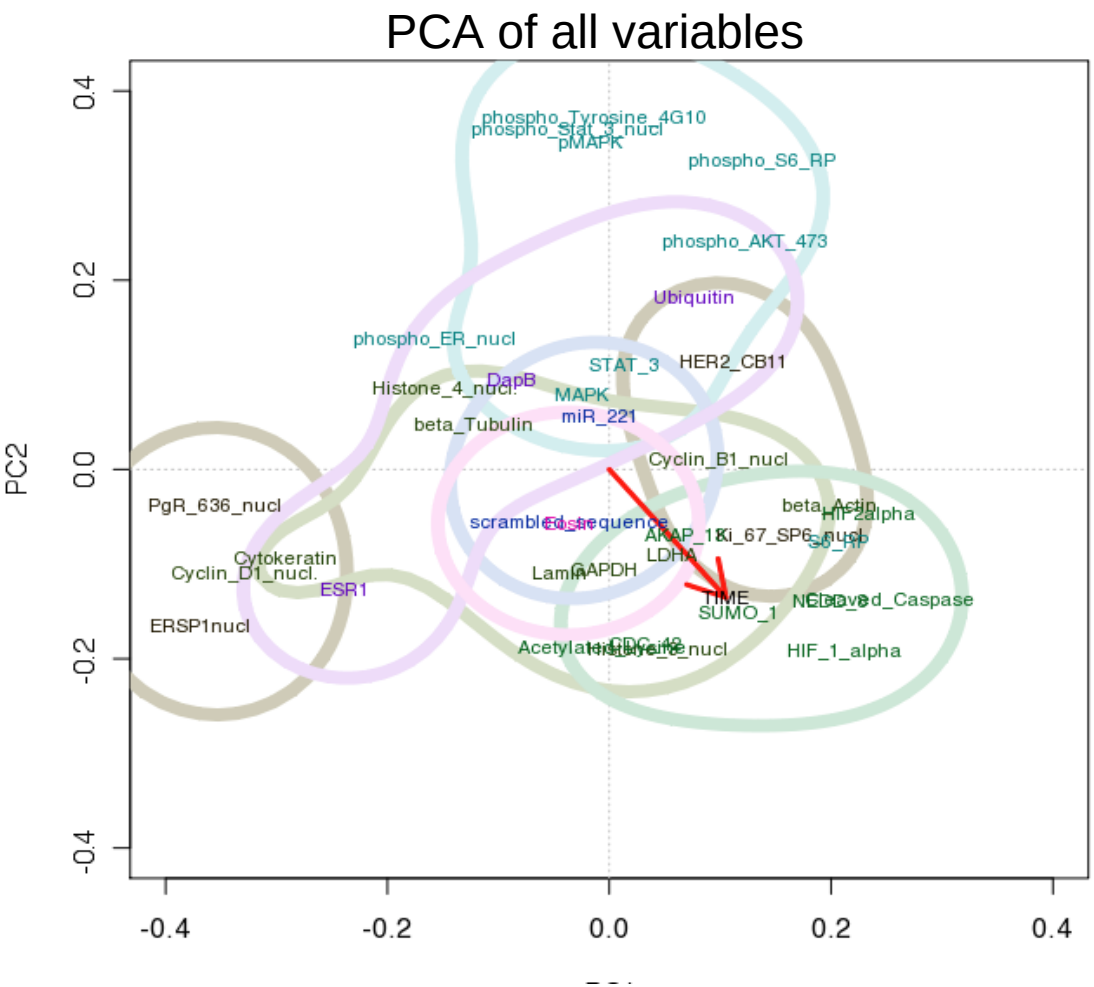



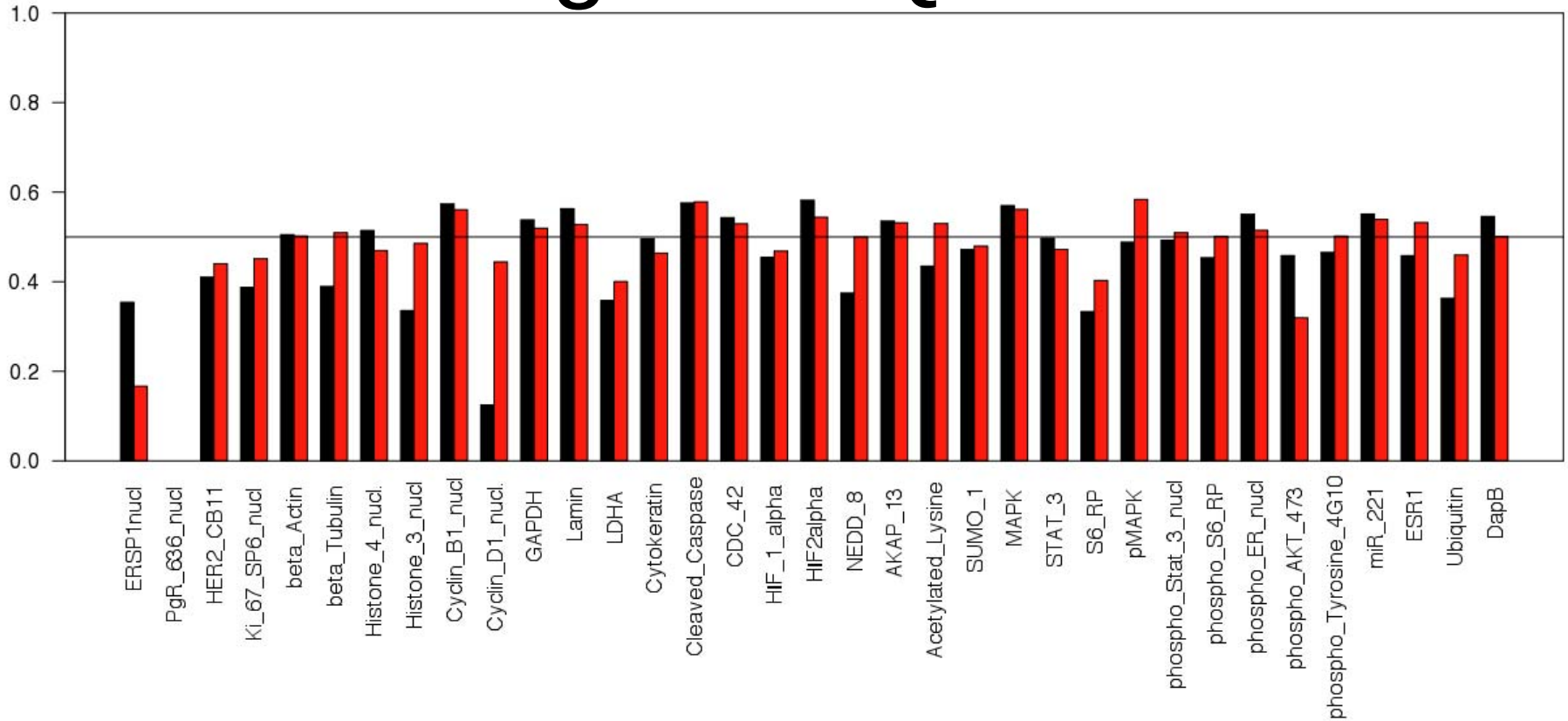

**Veronique Neumeister**

# Building the TQI Model




1. Select the two variables that are most positively correlated with TIME and the two variables that are most negatively correlated with TIME.
2. Define the sum of the first two variables “X1” and the sum of the last two variables as “X2”.
3. We predict a sample to be fresher than 60 minutes if X2>X1.
4. If X1>X2, then sample is predicted to be collected more than 60 minutes after resection.

# Building the TQI Model




We repeated the procedure 500 times and we computed the average sensitivity (black bars) and the average specificity (red bars) for each marker. Values above the 0.5 threshold indicate that the marker is performing better than a random classifier.

Sensitivity and specificity were assessed in the time interval between 30 minutes and 100 minutes

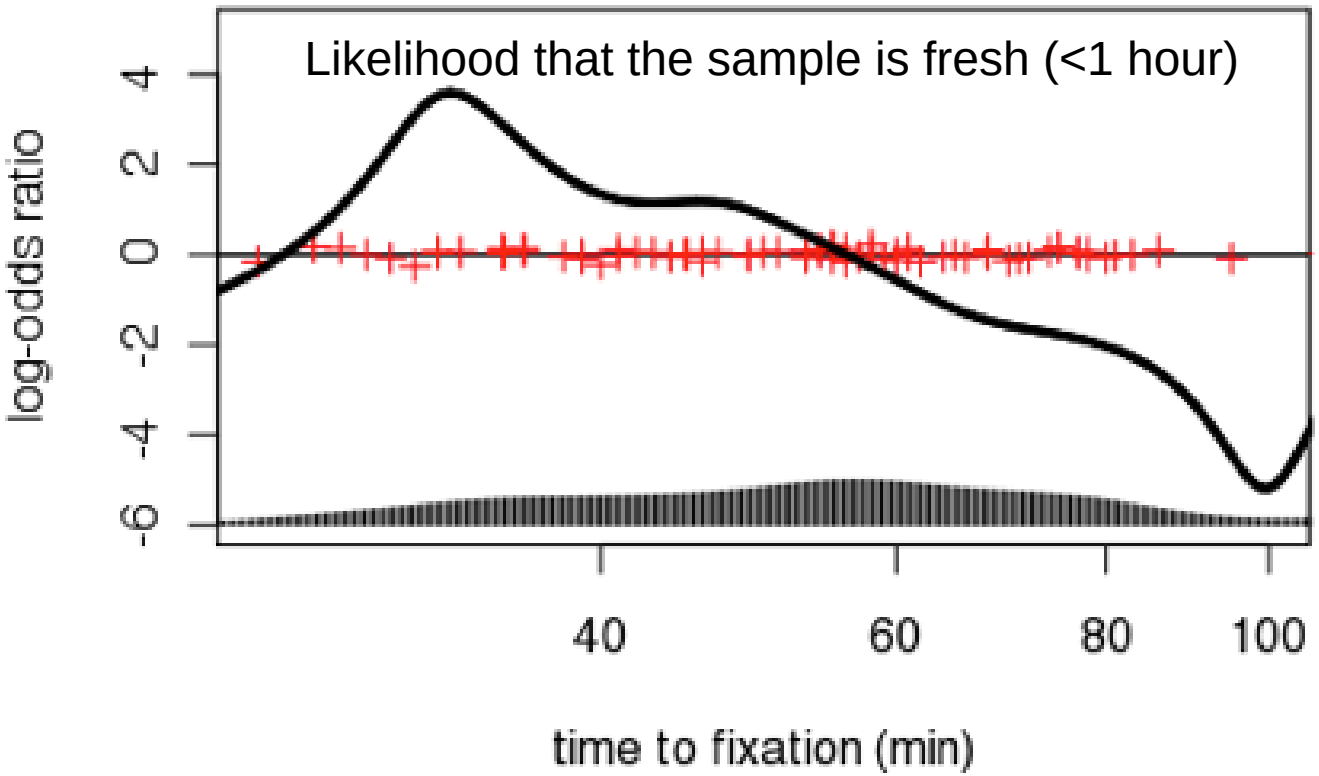
**Fabio Parisi and Yuval Kluger**

# TQI Model Construction

Best Model from full data training:

**X1*=Lamin+Hif2a**
**X2=MAPK+miR221**

* AKAP13 is a candidate substitute for X1 in case of technical issues in measuring Lamin or Hif2a.



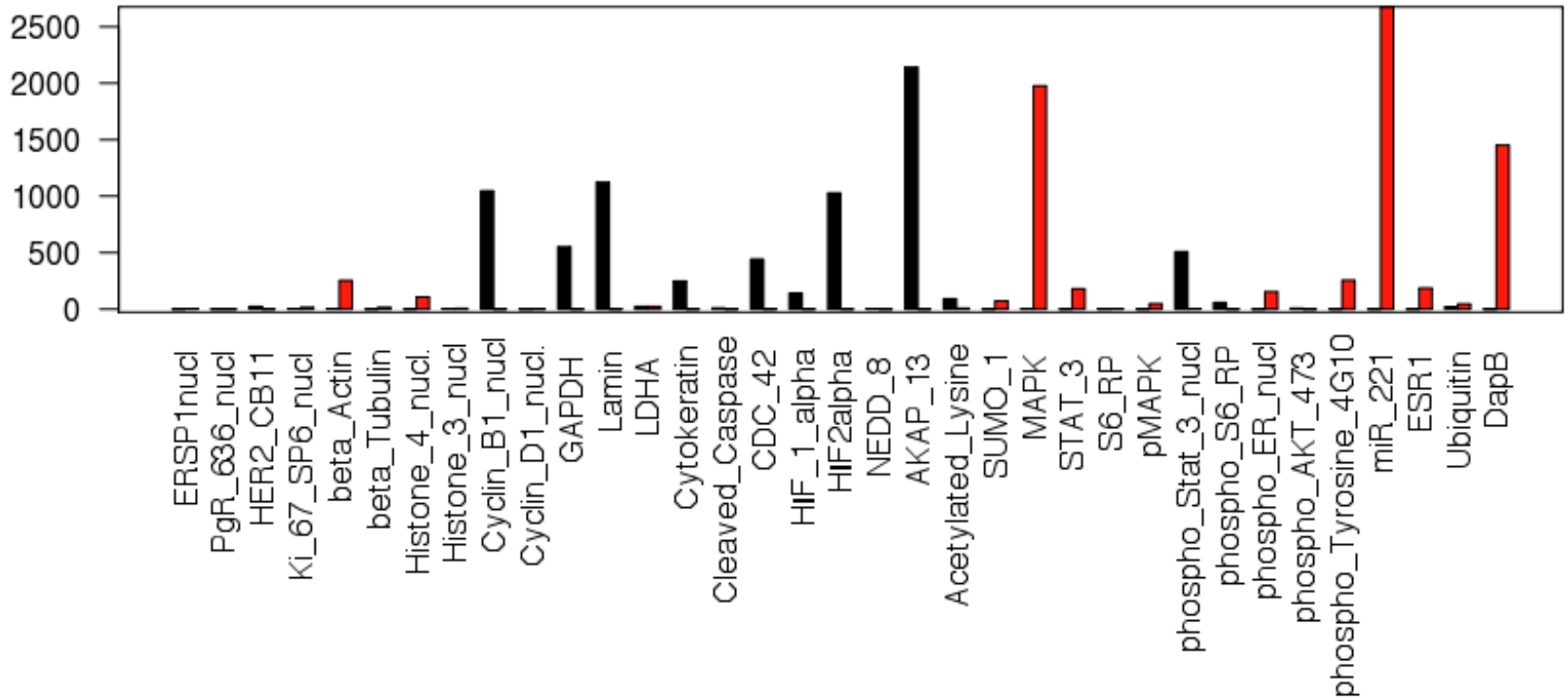

Analysis restricted to the interval between 30minutes and 100minutes, = 77% of the total observations

- Histogram of density of observations, bottom of the plot

-Times of each observation in the interval, red crosses.

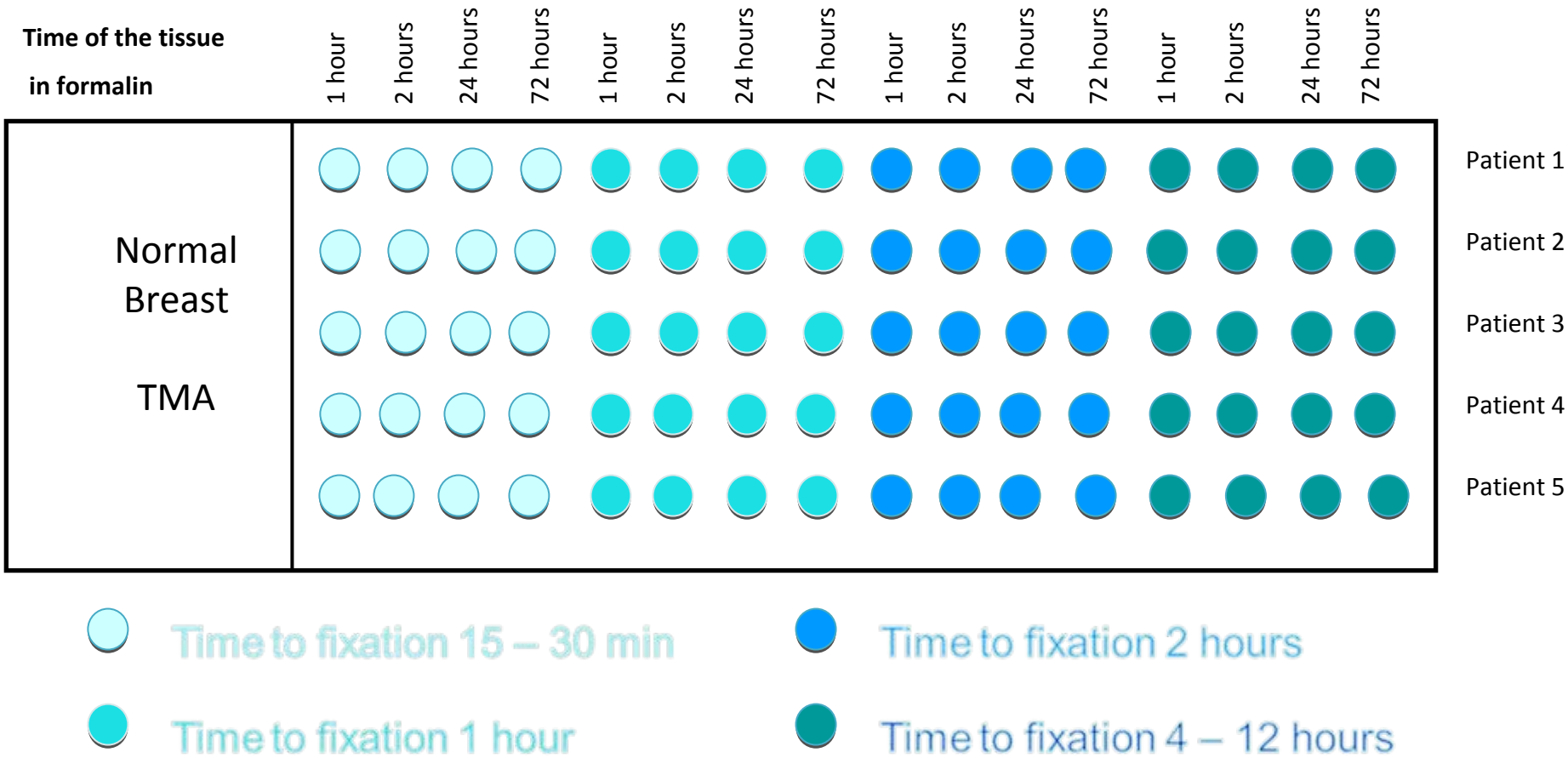
**Fabio Parisi and Yuval Kluger**

# TQI Model Assessment




The variables selected in the model trained on the full data exhibited performances that were among the highest in the dataset.

# Validation of the TQI
## (TMA under construction)

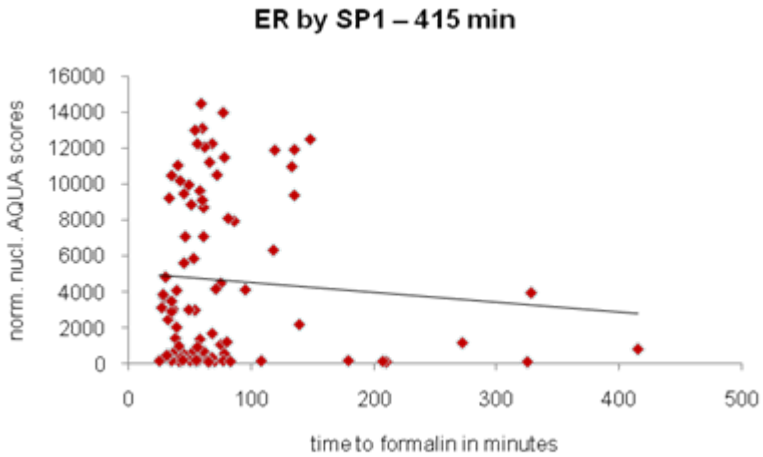


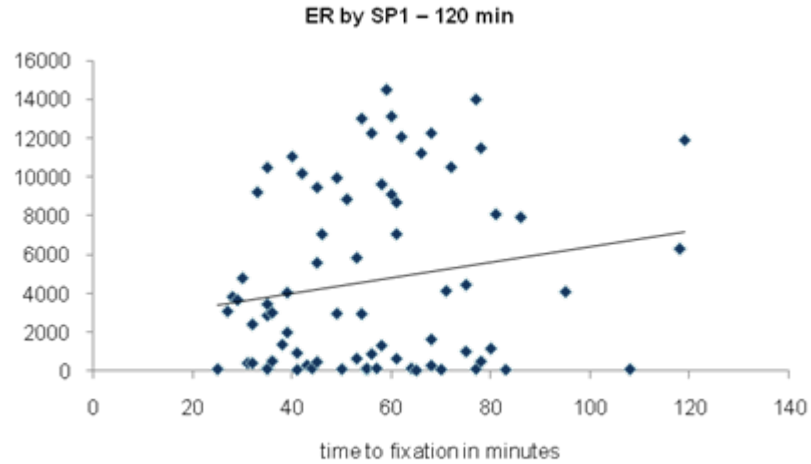

**Normal Breast TMA**

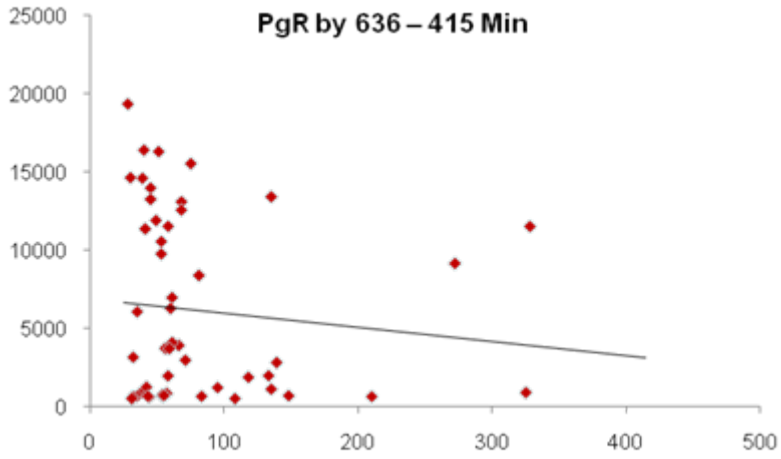
# Goals of our OBBR Contract/Project

- Development of a Tissue Quality Index (TQI):
  - by developing a quantitative intrinsic control that can measure the degree of degradation of any FFPE sample.
  - Validation of the TQI
- Assessment of the effects of Time to Fixation on Common Markers using QIF
- Proof that result is the same using quantitative DAB-based IHC

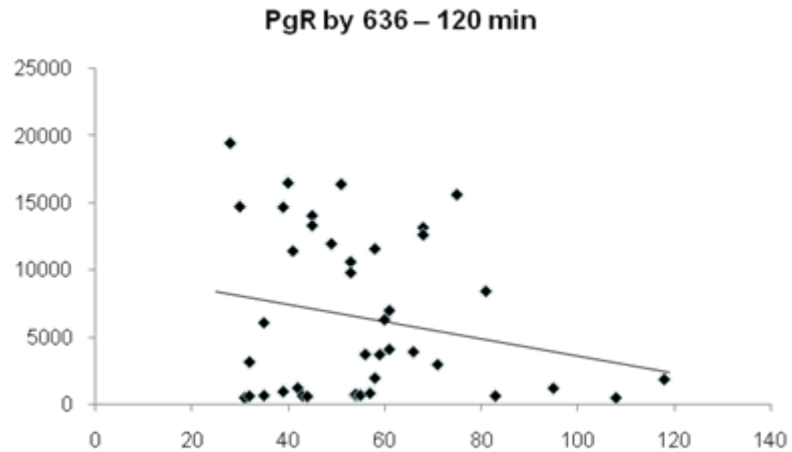
# QIF measurement of ER, PgR, HER2, and Ki67 on TTF TMA



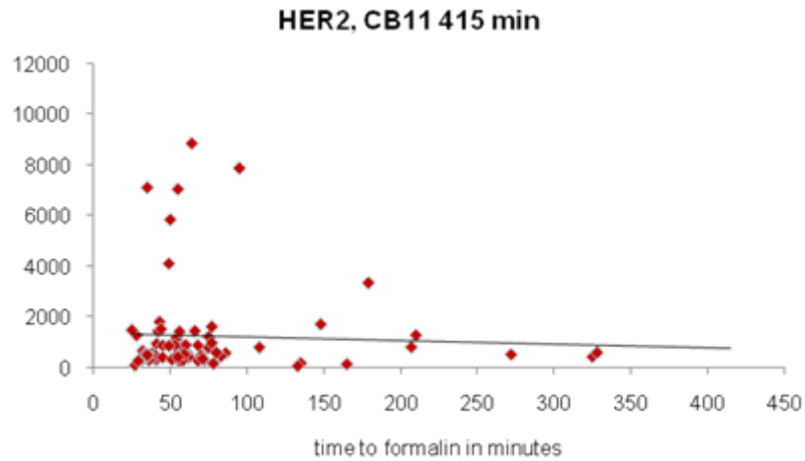



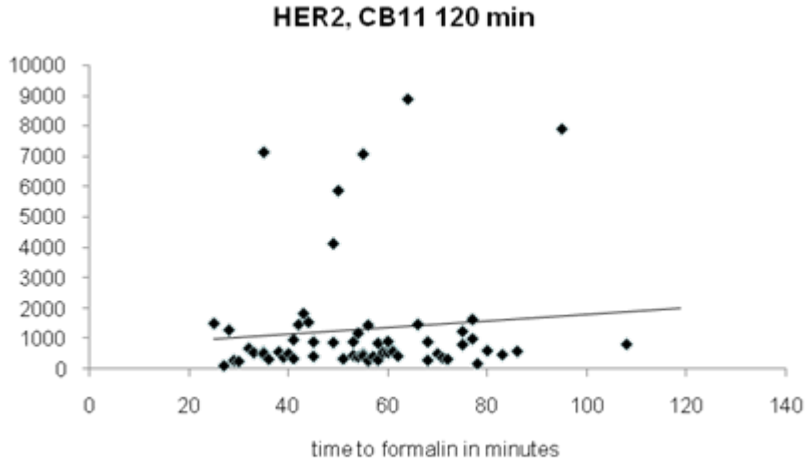

time to formalin in minutes

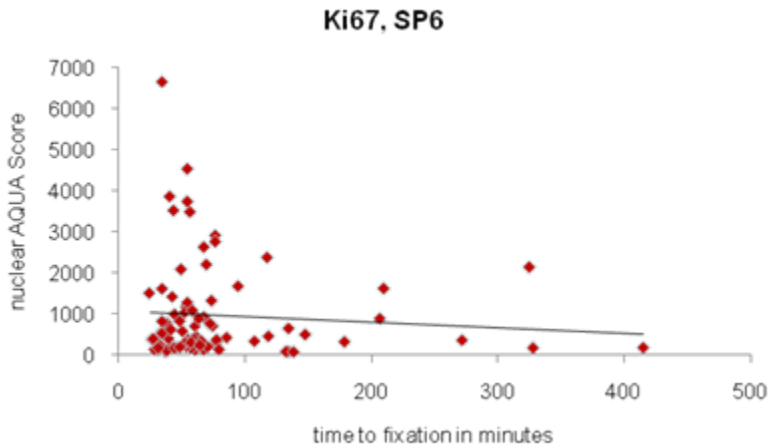


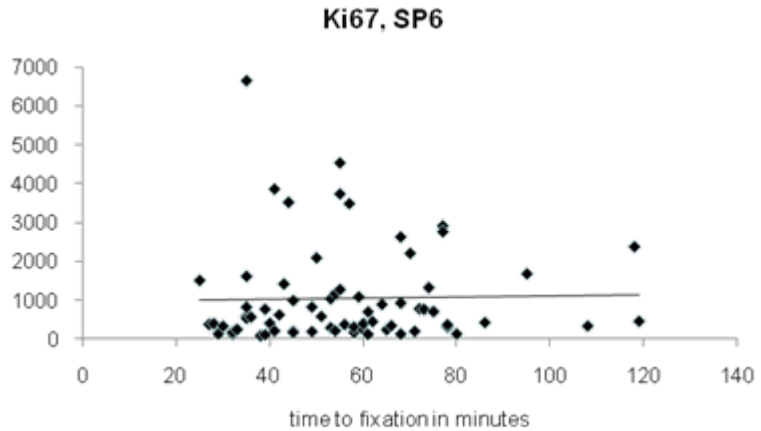

time to formalin in minutes

# QIF measurement of ER, PgR, HER2, and Ki67 on TTF TMA



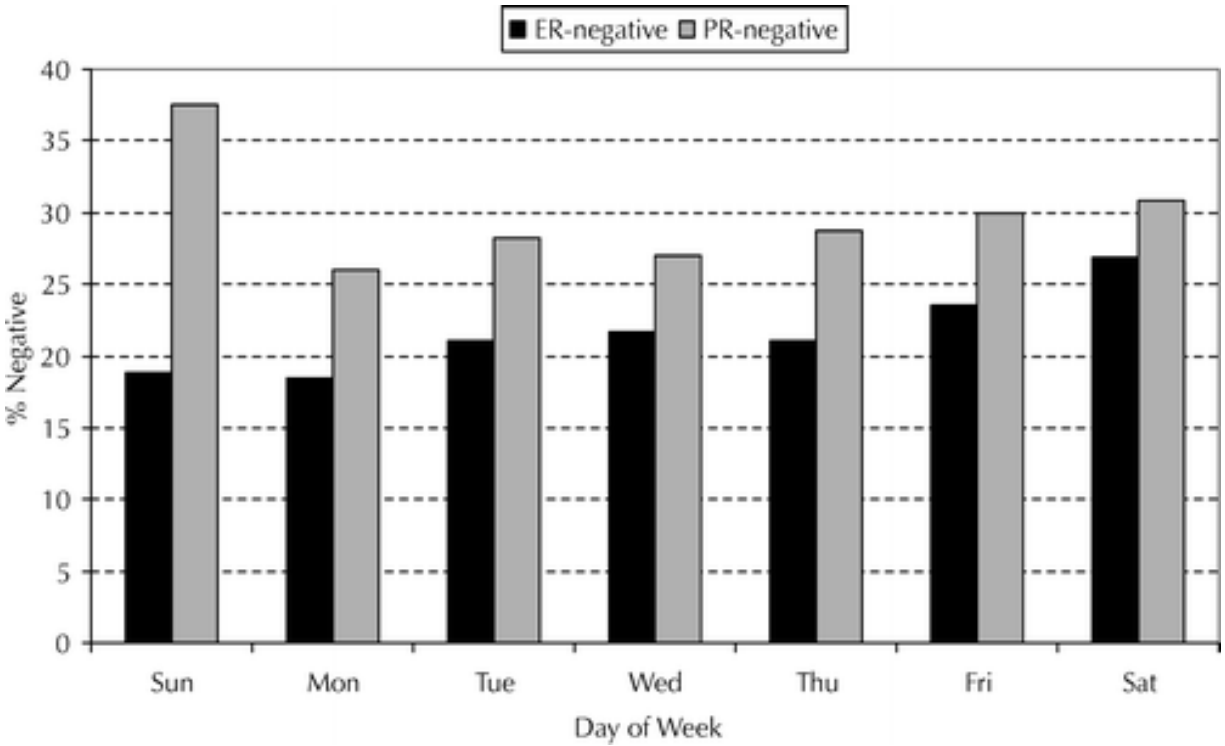



# Conclusions: Assessment of the Effects of Time to Fixation on Common Markers

- No significant loss of expression for ER, PgR, HER2 or Ki67 within 120 minutes (under-powered for longer time points)
- For complete details see Poster by Neumeister et al.
- Arguably, we have tested the wrong time window; it appears that loss occurs after 120 minutes




5077 women with breast cancer among the study hospitals were tested for ER/PR between 1997 and 2003 in central lab

Frequency of ER and PR negative test results by day of surgery

| Day | Cases | ER-Negative | PR-Negative |
|---|---|---|---|
| Sunday | 16 | 3 | 6 |
| Monday | 1252 | 230 | 325 |
| Tuesday | 1176 | 248 | 332 |
| Wednesday | 784 | 170 | 212 |
| Thursday | 904 | 191 | 259 |
| Friday | 919 | 216 | 276 |
| Saturday | 26 | 7 | 8 |
| **System** | **5077** | **1065** | **1418** |

Abbreviations: ER, estrogen receptor; PR, progesterone receptor.

Frequency of ER/PR negativity significantly increased with each day of the week, both for ER ($P = 0.03$) and PR ($P = 0.059$ for trends).

Hammond et al. Arch Pathol Lab Med. 2010;134:606-612

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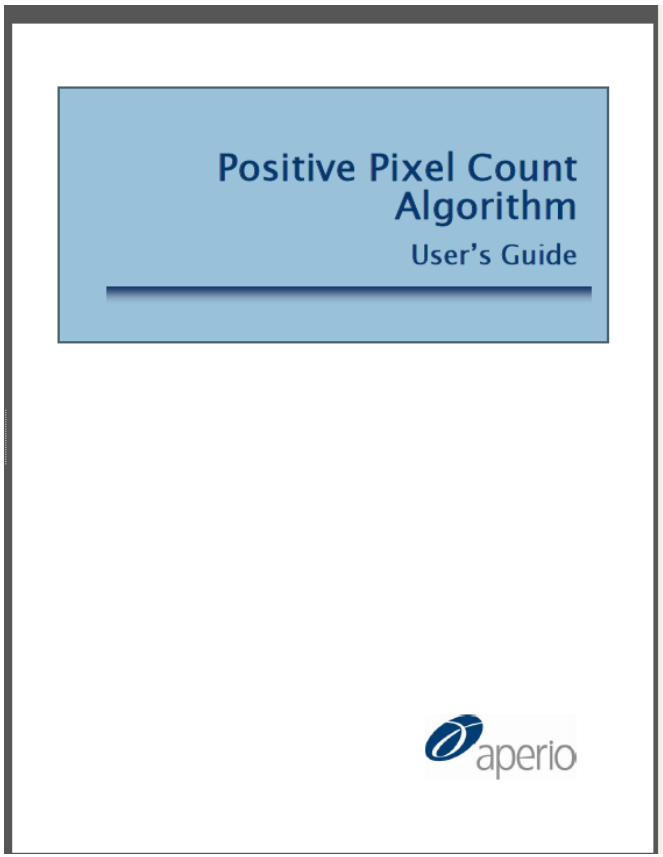
Automated scoring systems and algorithms for DAB stain …….




## The Positive Pixel Count Algorithm

The Positive Pixel Count algorithm can be used to quantify the amount of a specific stain present in a scanned slide image. You will specify a color (range of hues and saturation) and three intensity ranges (weak, positive, and strong). For pixels which satisfy the color specification, the algorithm counts the number and intensity-sum in each intensity range, along with three additional quantities:

average intensity, ratio of strong/total number, and average intensity of weak+positive pixels.

The algorithm has a set of default input parameters when first selected—these inputs have been pre-configured for Brown color quantification in the three intensity ranges (220-175, 175-100, and 100-0). Pixels which are stained, but do not fall into the positive-color specification, are considered negative stained pixels—these pixels are counted as well, so that the fraction of positive to total stained pixels is determined.

- Requires binning into 3 categories by intensity range
- Requires user-defined selection of region of interest (no masking capacity as used here)

# IHC Nuclear Image Analysis Algorithm:

IHC Nuclear Image Analysis

User's Guide

aperio

This algorithm is based on a cell feature detection method. Optical density is then measured in the nuclei. Based on the intensity, nuclear staining is classified as negative (0), weak positive (1+), medium (2+) or strong positive (3+).

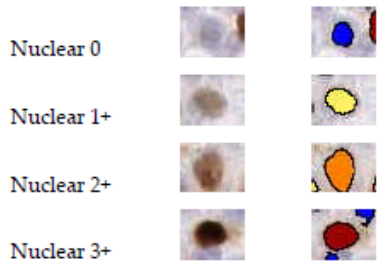


aperio

## Algorithm Description

Prior to running the algorithm, a qualified pathologist needs to use the ImageScope annotation tools to outline a set of tumor-cell only regions that are representative of the tumor.

The IHC Nuclear Image Analysis algorithm detects the nuclear staining for a target chromogen for the individual cells in those regions and quantifies their intensity. Nuclear staining classified as 0, 1+, 2+ and 3+ is based on nuclear staining intensity. A nucleus is classified 0 when it has no nuclear staining. A nucleus is classified 1+ when it has weak nuclear staining. A nucleus is classified 2+ when it has moderate nuclear staining. A nucleus is classified 3+ when it has intense nuclear staining. Based on the percentages of 0, 1+, 2+ and 3+ nuclei, the percentage of positive stained nuclei as a percentage of 0 to 100% and the average staining intensity of the positive nuclei as a score of 0, 1+, 2+ or 3+ is determined.






## Final Score

The IHC Nuclear Image Analysis is intended to be used as an aid to a pathologist. It is the responsibility of the pathologist to provide the final score based on his/her qualitative assessment and the quantitative information provided by the IHC Nuclear Image Analysis algorithm.

22. **The pathologist determines the final percentage of positive nuclei and average staining intensity of positive nuclei.**

Annotation

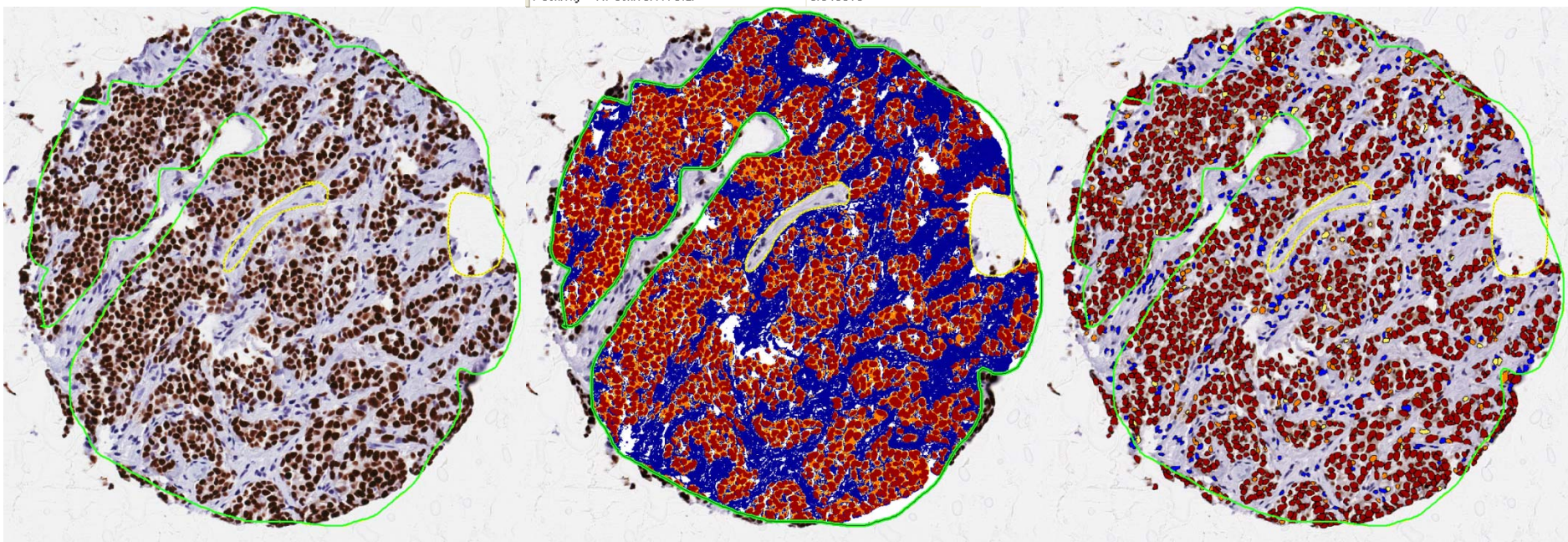
Positive Pixel Count

Nuclear Algorithm

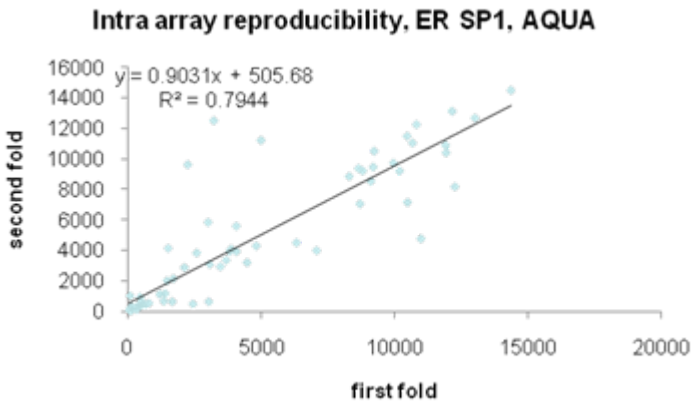
of a TMA spot, stained for ER SP1

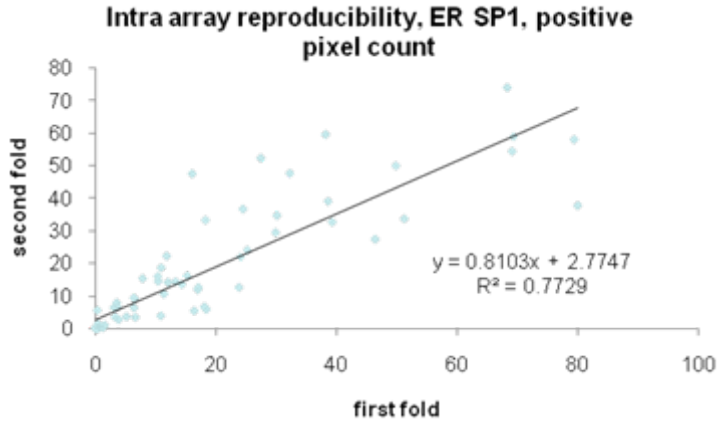
| Algorithm | Positive Pixel Count v9 |
|---|---|
| Date | 2012/01/26 |
| StartTime | 03:28:11 AM |
| EndTime | 03:28:12 AM |
| Status | 0 |
| StatusDescription | |
| Nwp = Number of Weak Positive | 50202. |
| Np = Number of Positive | 142108. |
| Nsp = Number of Strong Positive | 353940. |
| Iwp = Total Intensity of Weak Positive | 9653366. |
| Ip = Total Intensity of Positive | 19860575. |
| Isp = Total Intensity of Strong Positive | 13294586. |
| Iavg = (Iwp+Ip+Isp)/(Nwp+Np+Nsp) | 78.368 |
| Nsr = Nsp/(Nwp+Np+Nsp) | 0.647945 |
| Iwavg= (Iwp+Ip)/(Nwp+Np) | 153.471 |
| Nn = Number of Negative | 459702. |
| In = Total Intensity of Negative | 80647809. |
| NTotal = Total Number (Positive+Negative) | 1005952. |
| ATotal = Total Area (millimeter-squared) | 0.24827925454847999 |
| Positivity = NPositive/NTotal | 0.543018 |

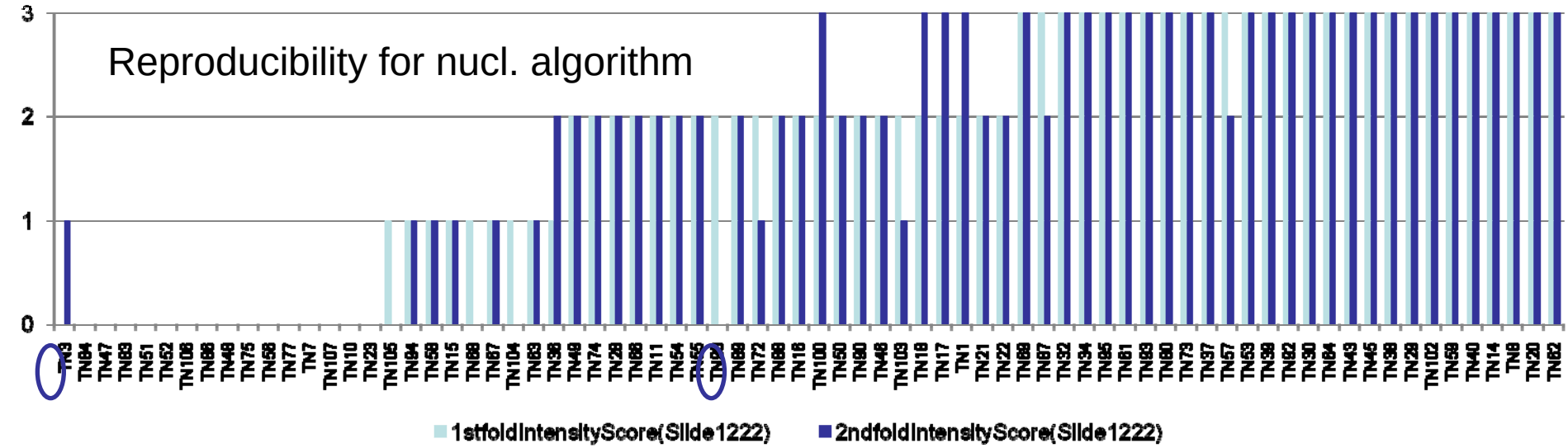
| Algorithm | Nuclear v9 |
|---|---|
| Date | 2012/01/17 |
| StartTime | 06:42:53 AM |
| EndTime | 06:42:58 AM |
| Status | 0 |
| StatusDescription | |
| Percent Positive Nuclei | 84.6154 |
| Intensity Score | 3 |
| (3+) Percent Nuclei | 75.2275 |
| (2+) Percent Nuclei | 6.69975 |
| (1+) Percent Nuclei | 2.68817 |
| (0+) Percent Nuclei | 15.3846 |
| Average Positive Intensity | 130.888 |
| Average Negative Intensity | 237.629 |
| (3+) Nuclei | 1819 |
| (2+) Nuclei | 162 |
| (1+) Nuclei | 65 |
| (0+) Nuclei | 372 |
| Total Nuclei | 2418 |

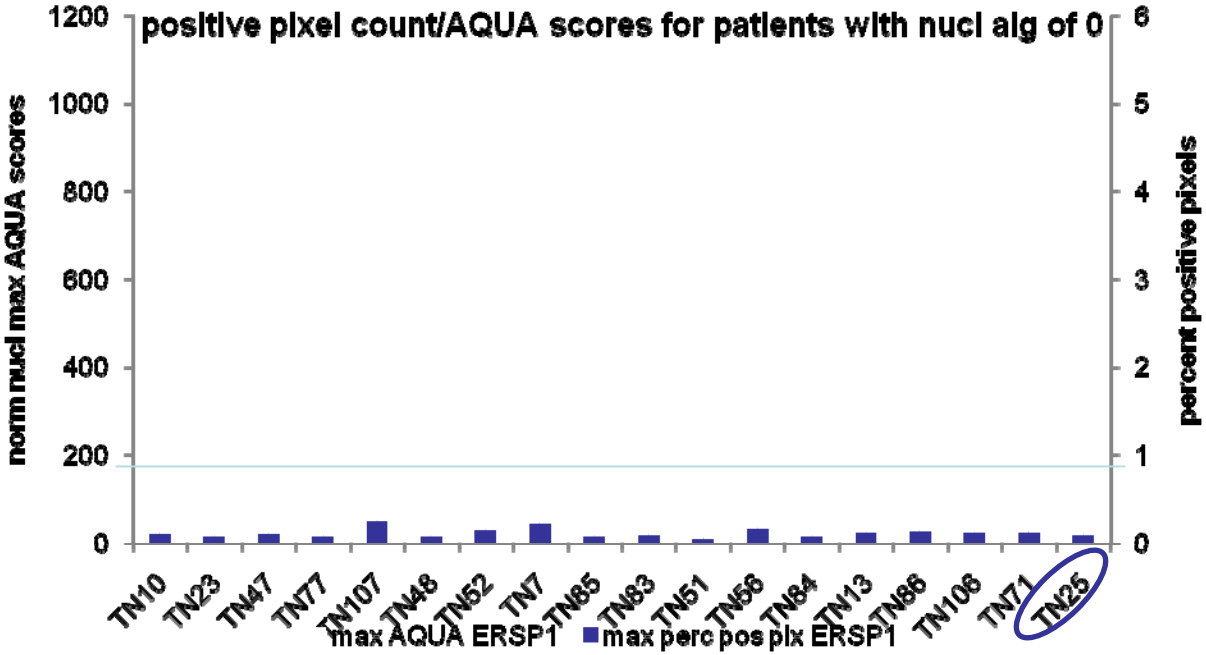


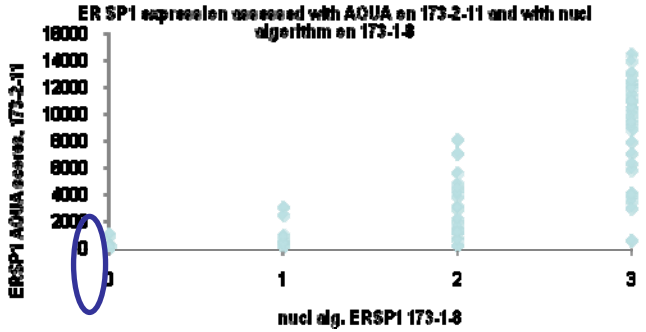
Intra array reproducibility on the time to fixation array for ER SP1 – Assessed with IF/AQUA and IHC/positive pixel count and nucl. algorithm



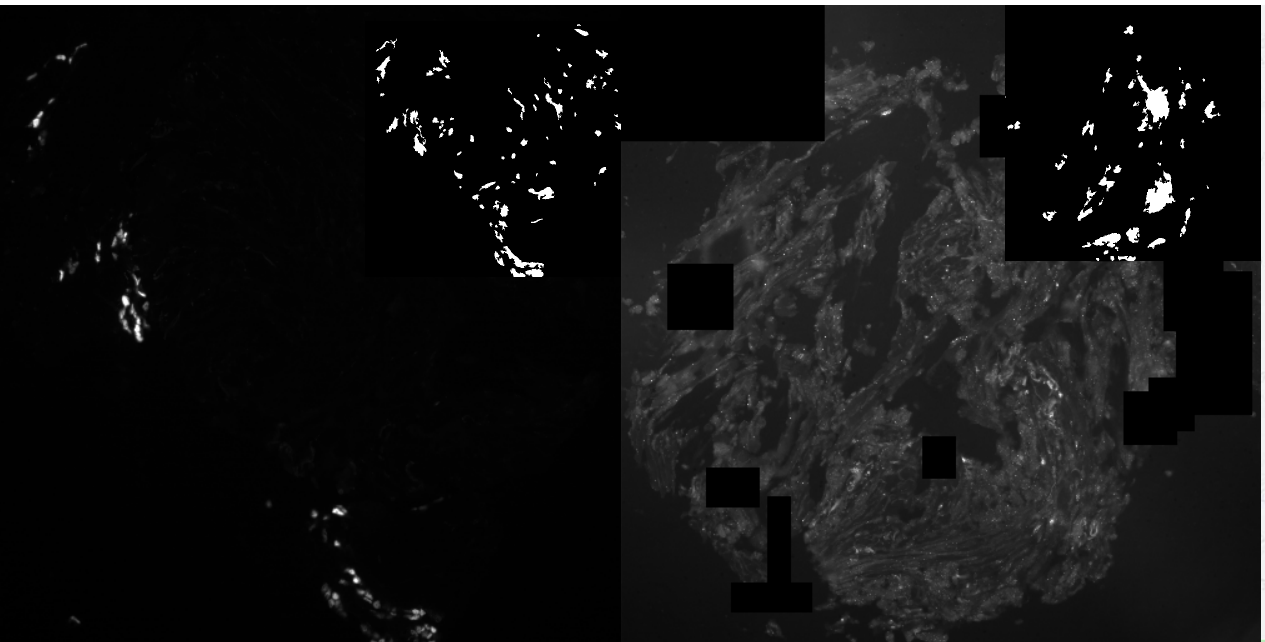



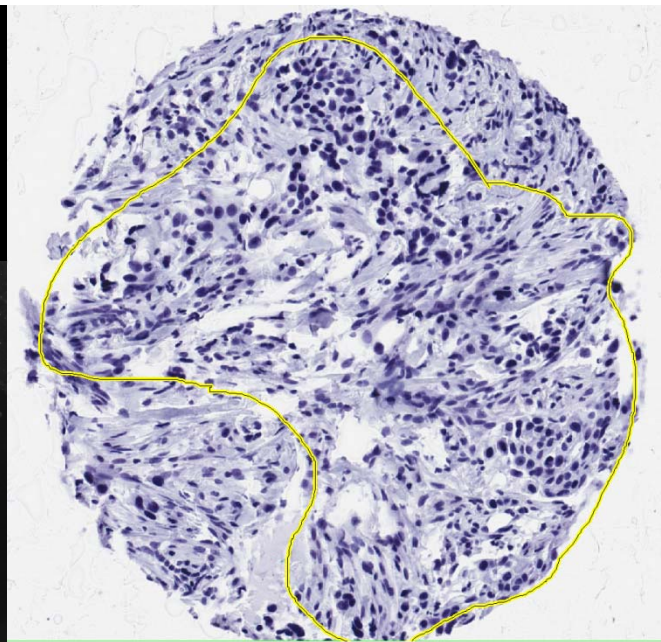



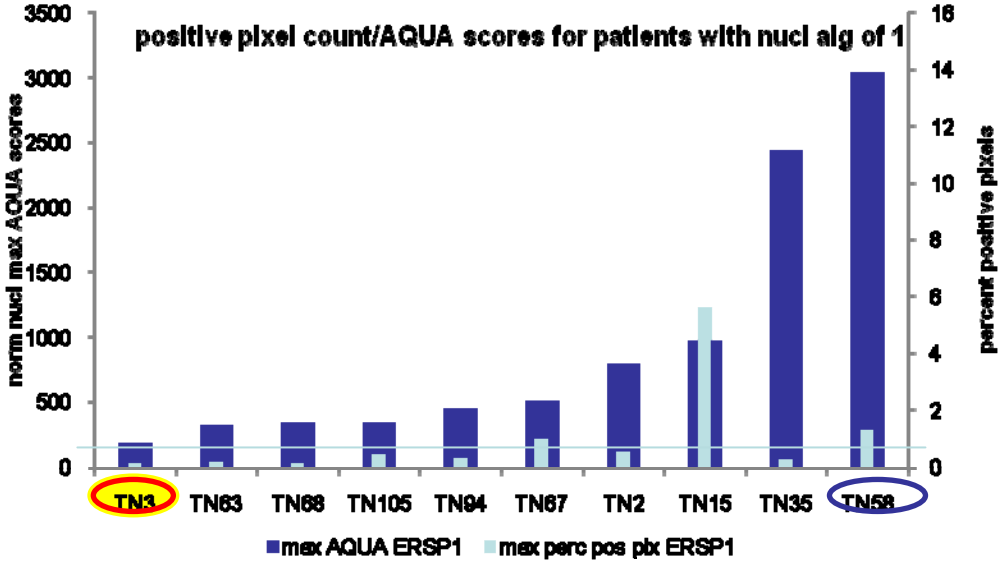


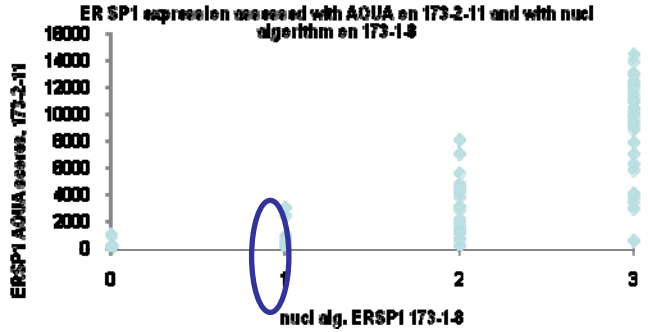




**TN25:** spot 251 – AQUA 1040 spot 262 – AQUA 86

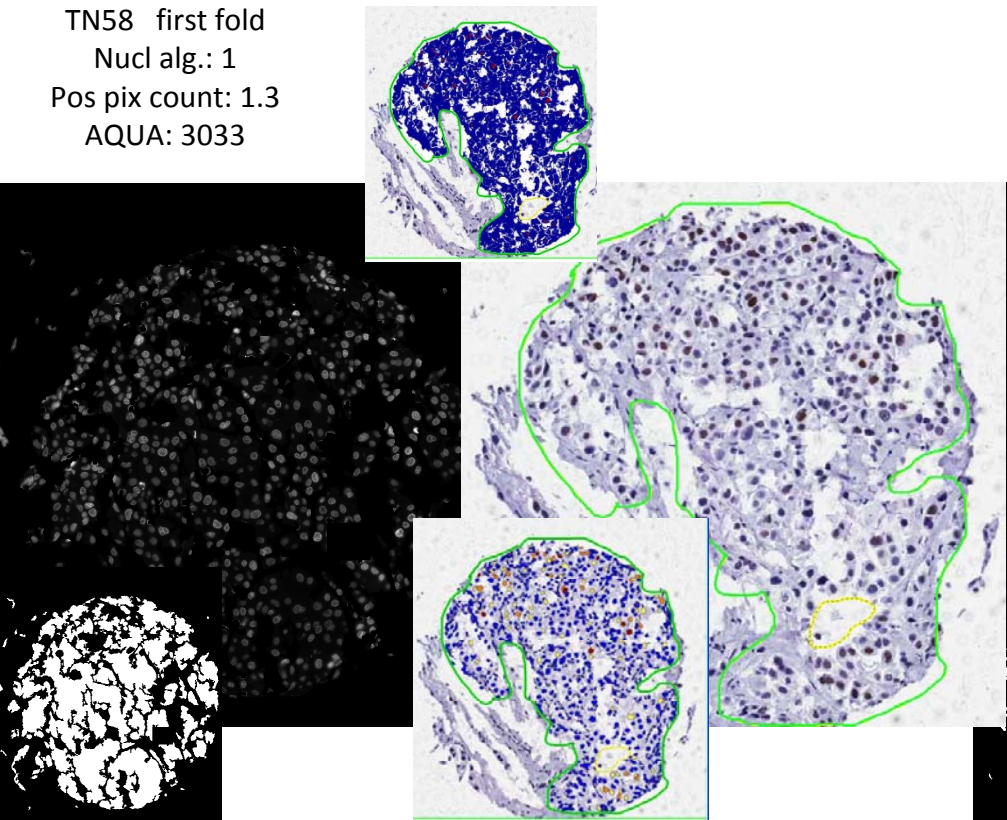




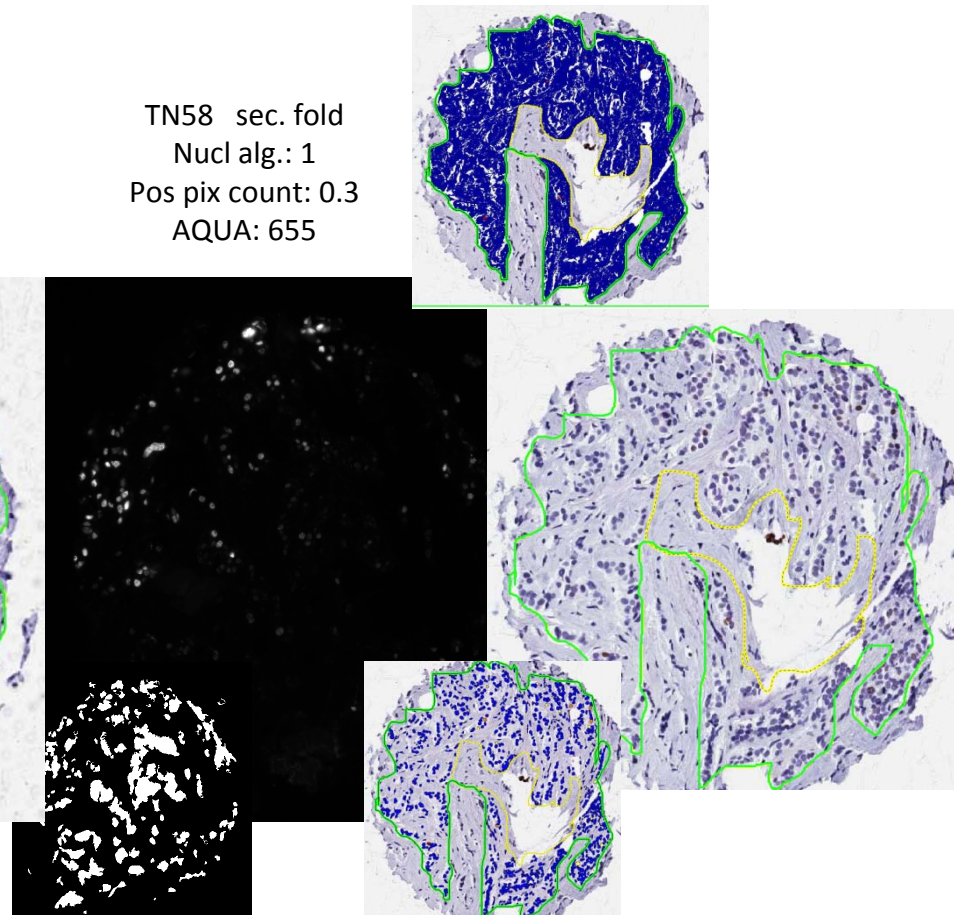


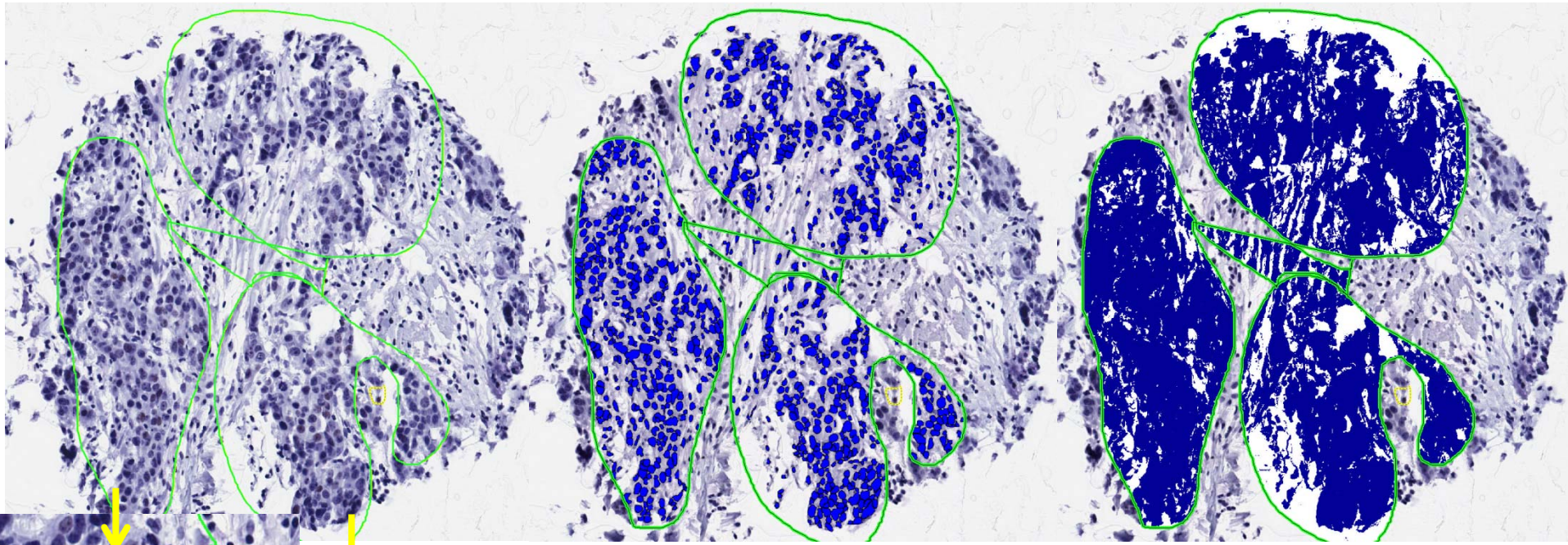



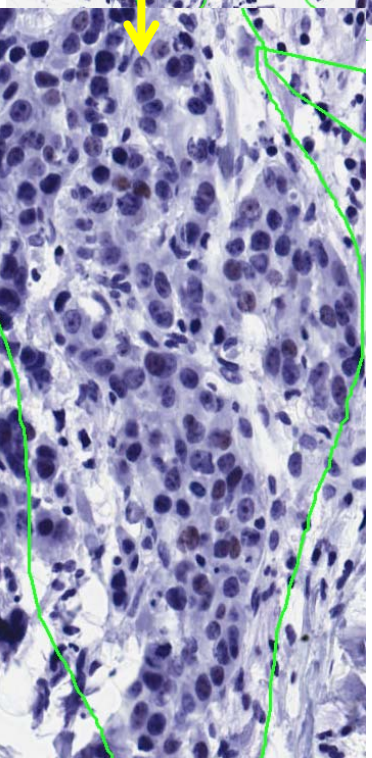

TN58 first fold
Nucl alg.: 1
Pos pix count: 1.3
AQUA: 3033

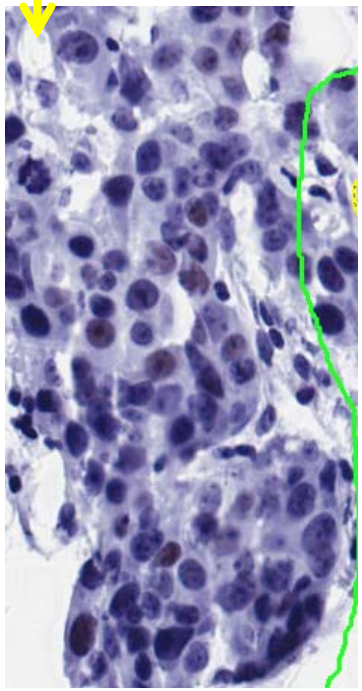


TN58 sec. fold
Nucl alg.: 1
Pos pix count: 0.3
AQUA: 655







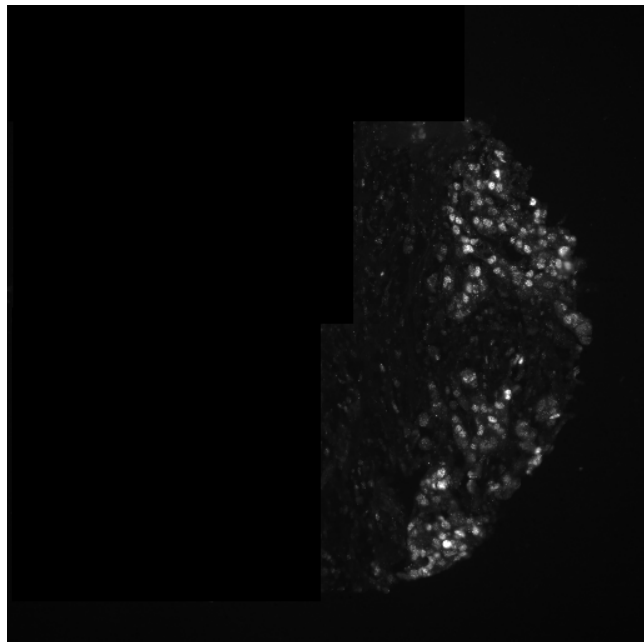


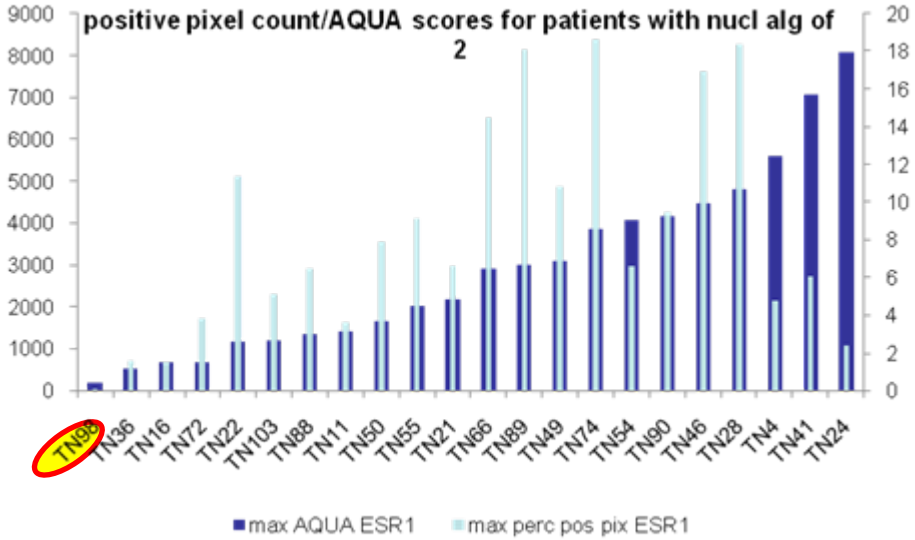
TN3 first fold

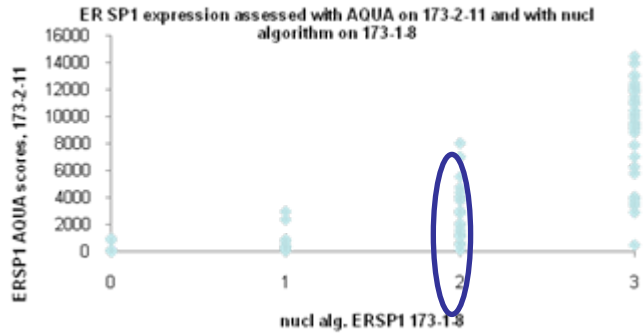
Nucl alg.: 0

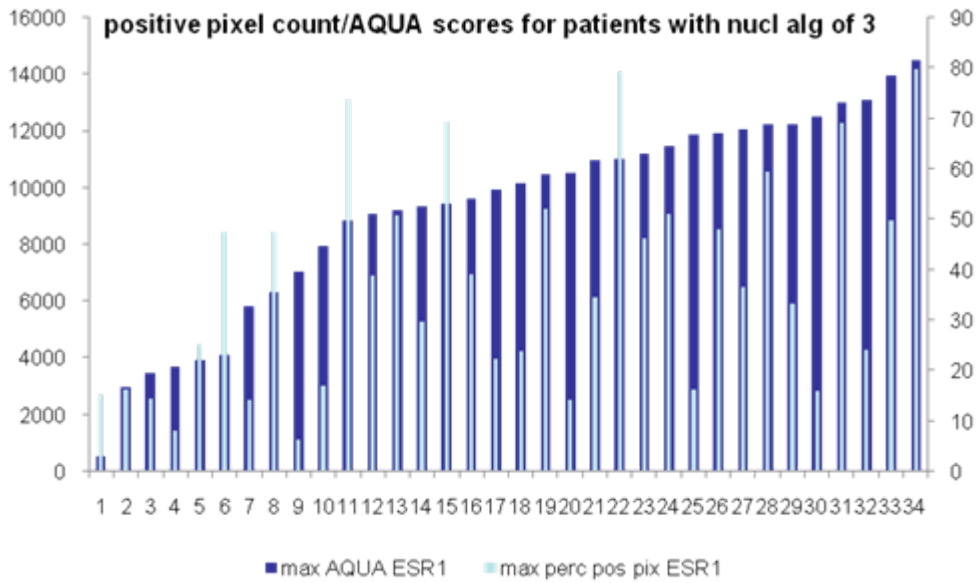
Pos pix count: 0.09

AQUA: 176

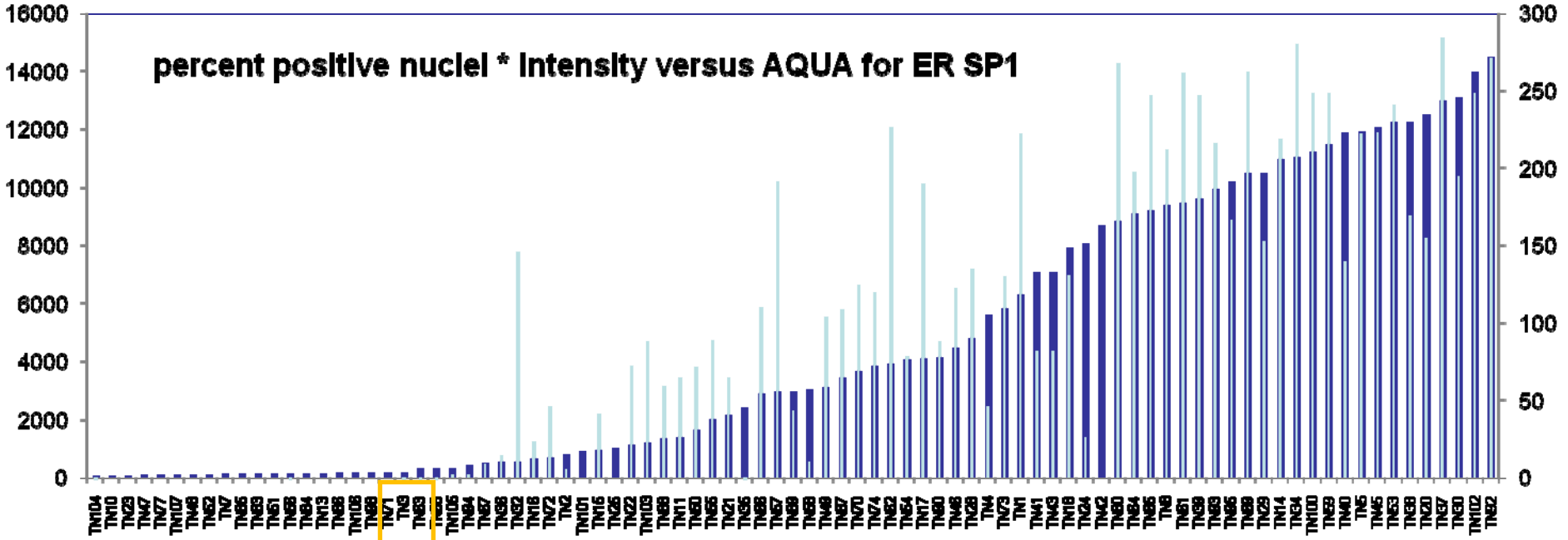



positive pixel count/AQUA scores for patients with nucl alg of 2
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
20
18
16
14
12
10
8
6
4
2
0
TN98
TN36
TN16
TN72
TN22
TN103
TN88
TN11
TN50
TN55
TN21
TN66
TN89
TN49
TN74
TN54
TN90
TN46
TN28
TN4
TN41
TN24
max AQUA ESR1
max perc pos pix ESR1

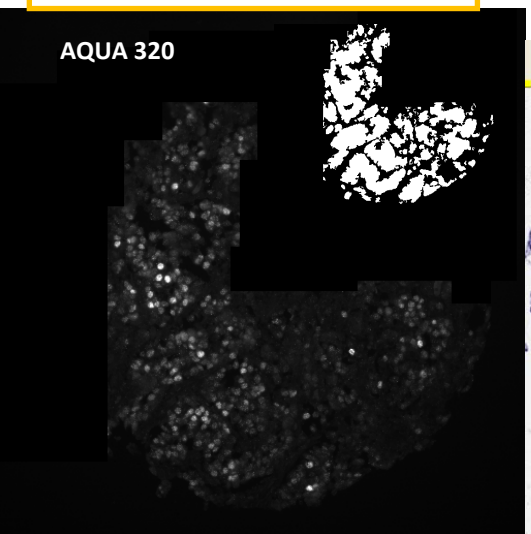

ER SP1 expression assessed with AQUA on 173-2-11 and with nucl algorithm on 173-1-8
ERSP1 AQUA scores, 173-2-11
16000
14000
12000
10000
8000
6000
4000
2000
0
0
1
2
3
nucl alg. ERSP1 173-1-8

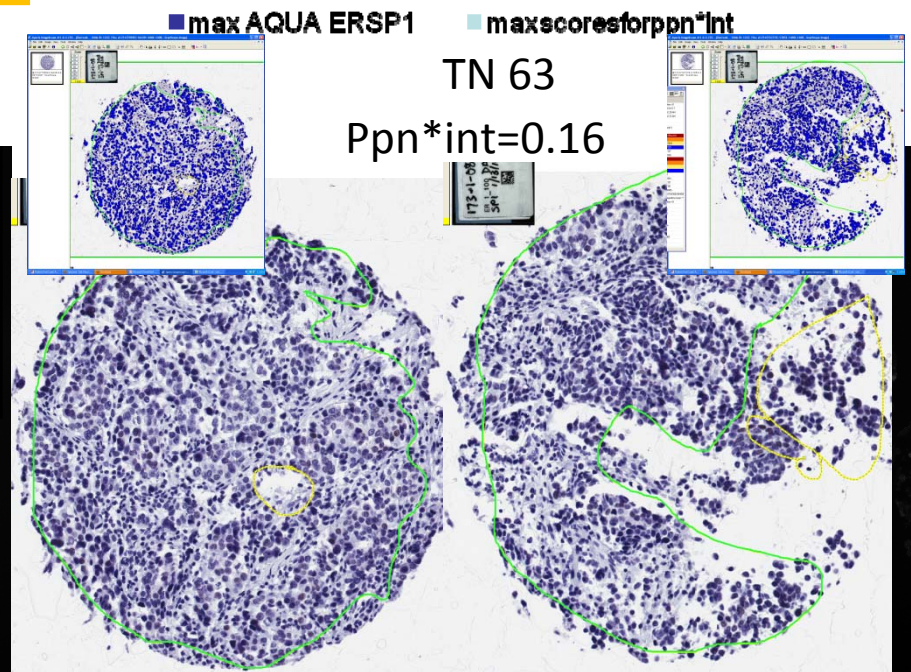

positive pixel count/AQUA scores for patients with nucl alg of 3
16000
14000
12000
10000
8000
6000
4000
2000
0
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
max AQUA ESR1
max perc pos pix ESR1

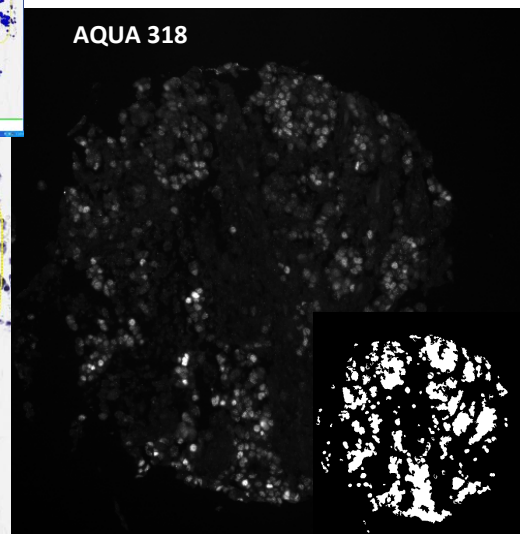
# Nuclear algorithm: from 1,2,3 to a scale from 0-300: intensity * percent positive nuclei



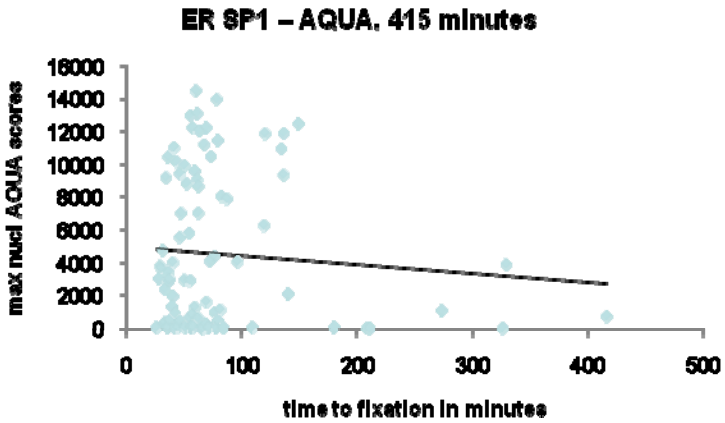

The yellow rectangle marks the patients who are positive for AQUA but have a score <1 when analyzed for %positive cells *intensity

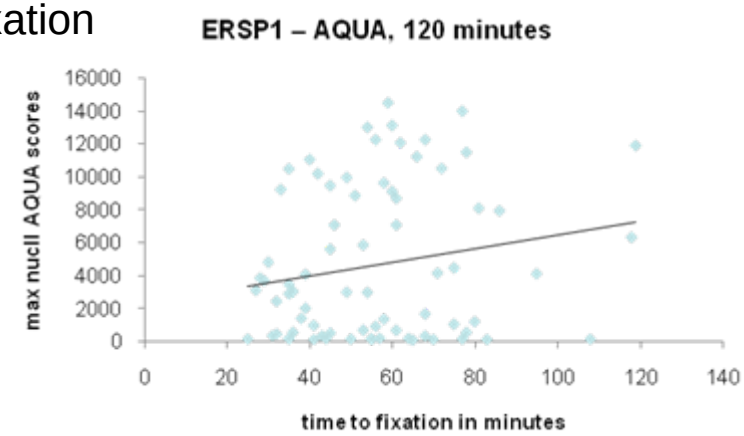


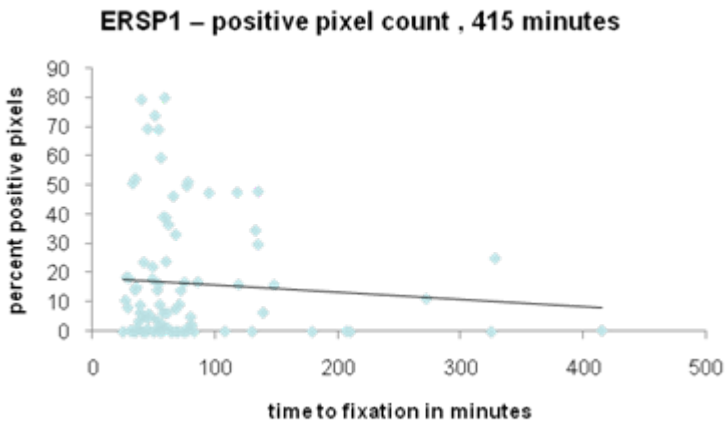



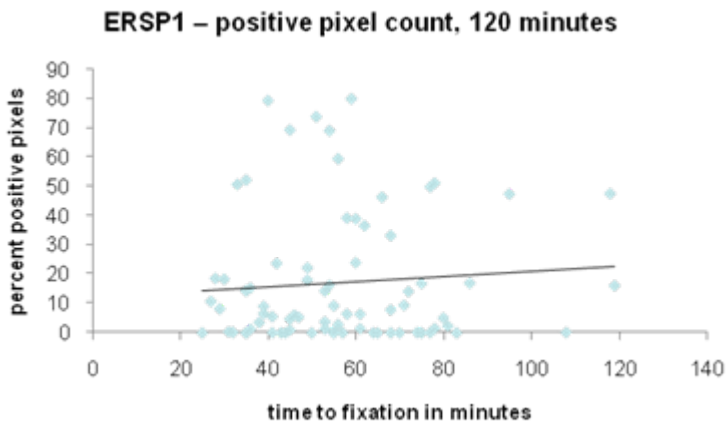



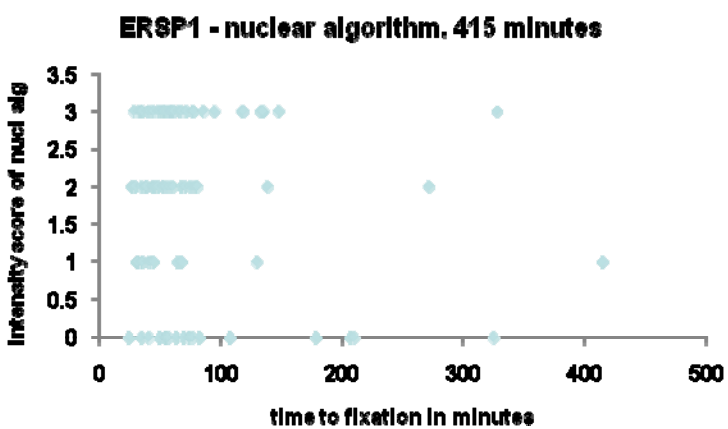
# Assessment of possible change of ER expression according to increasing time to formalin fixation

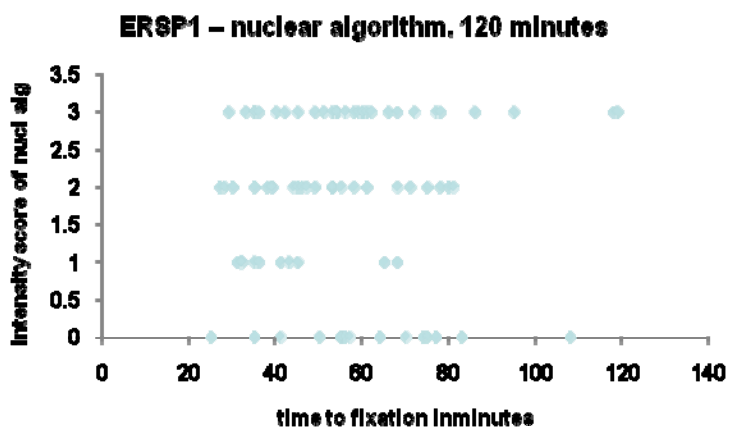


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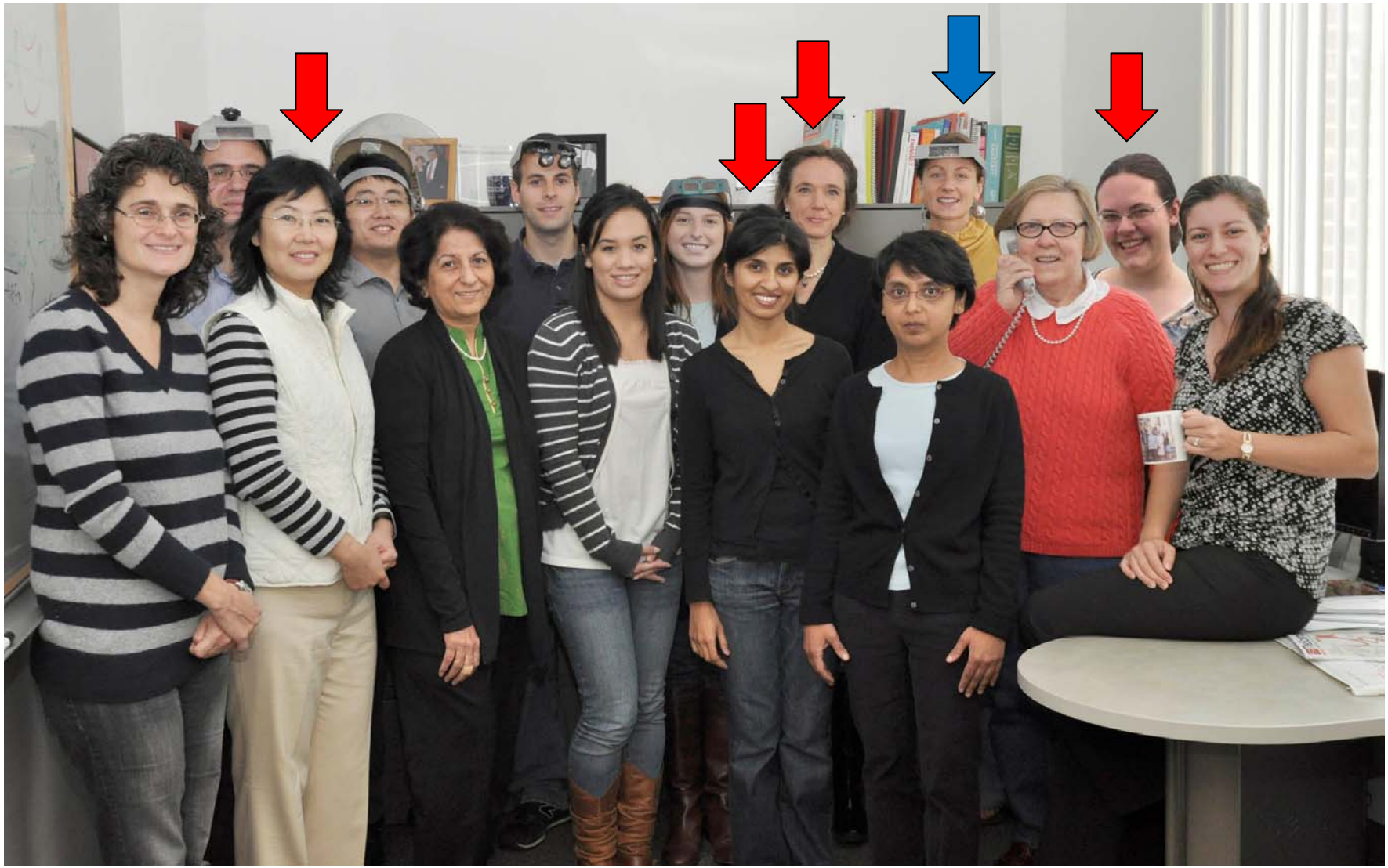

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