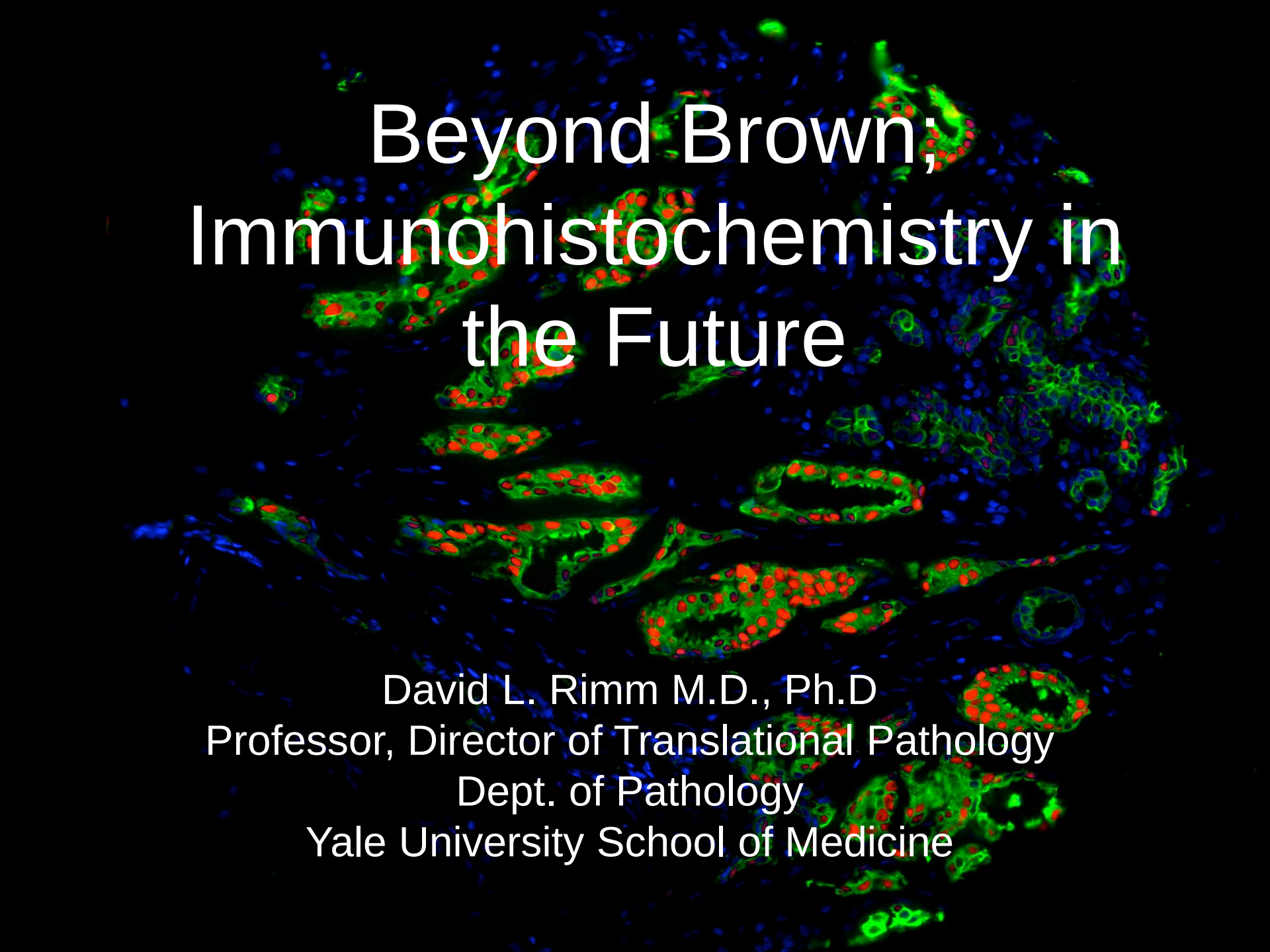


Beyond Brown; Immunohistochemistry in the Future

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

Disclosure

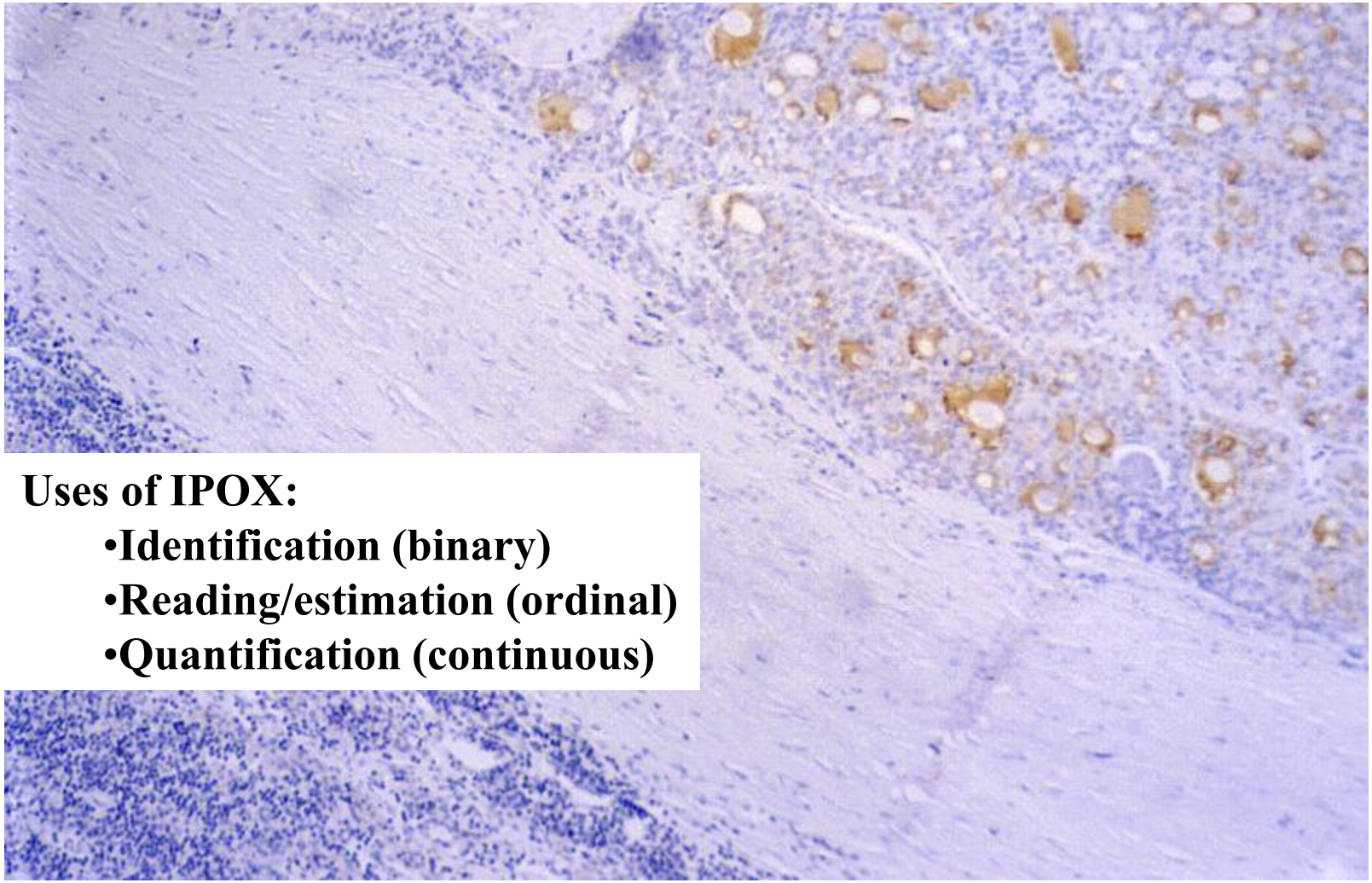
- I am a consultant to Genoptix/Novartis, Applied Cellular Diagnostics, BMS, Amgen, Optrascan, Biocept, Perkin Elmer, and Metamark Genetics
- I hold equity in Metamark Genetics
- Cepheid, Genoptix. Gilead Pharmaceuticals Kolltan and OncoplexDx fund research in my lab.

The value of two dimensional information: Morphology

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

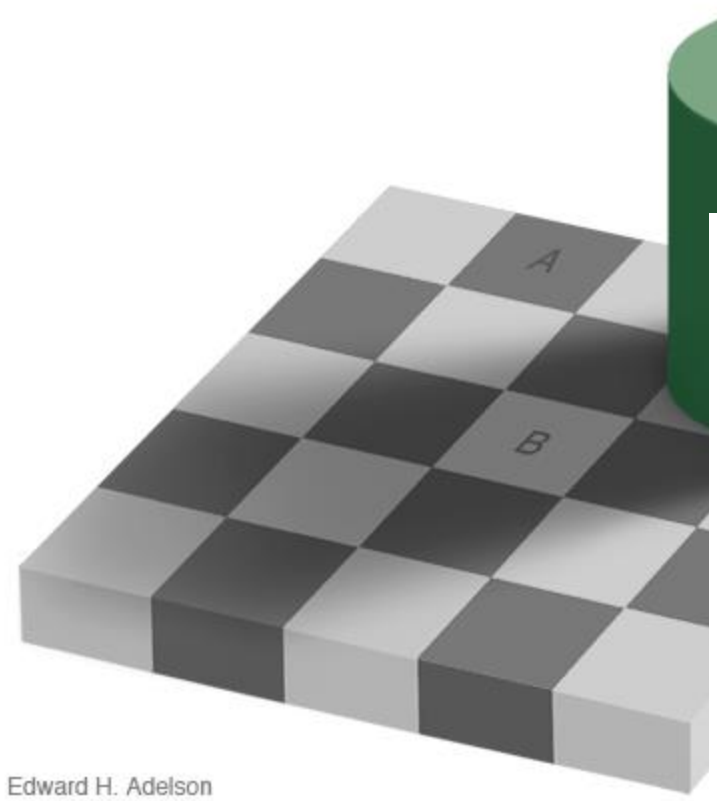
Immunoperoxidase stain



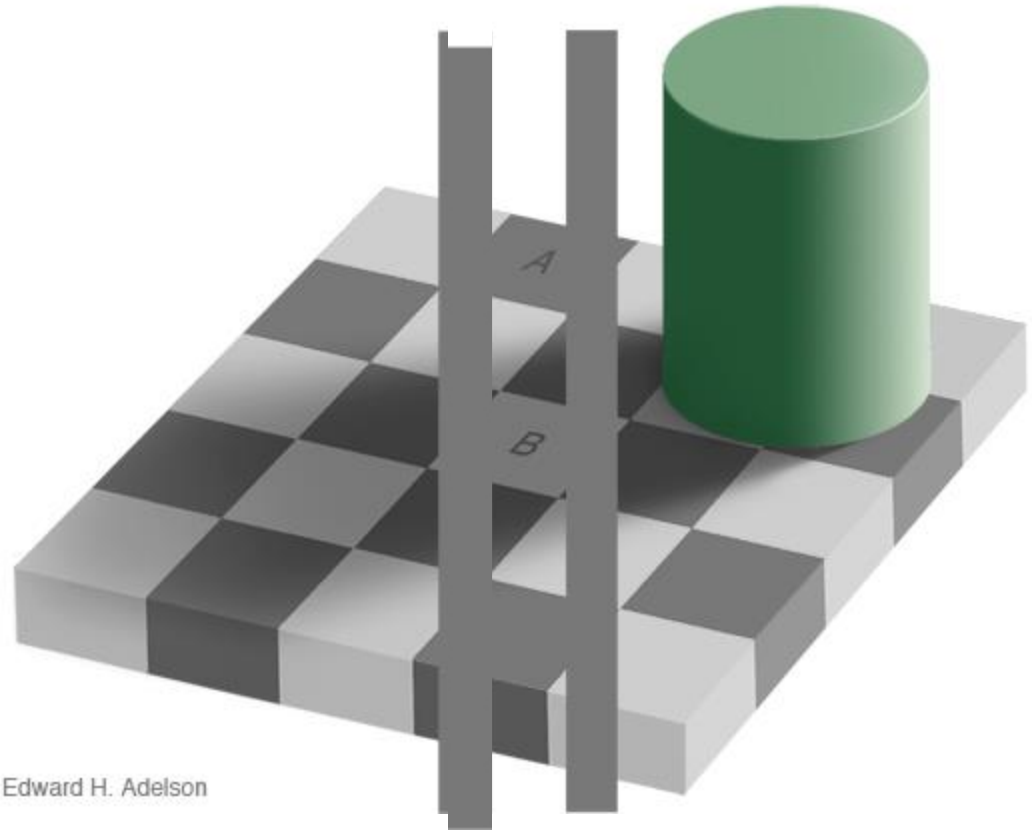
Uses of IPOX:

- **Identification (binary)**
- **Reading/estimation (ordinal)**
- **Quantification (continuous)**

The human eye is not a great tool for assessment of intensity



Edward H. Adelson



Edward H. Adelson

A Long History of Quantitative Microscopy

- Equipment for quantitative morphology dating back to the 1960's
- The CAS 200 (Jim and Sarah Bacus)
- Chromavision ACIS
- Bacus Labs, BLISS system
- Compucyte, Applied Imaging, Areol/Genetix, Definiens, CRI INform
- Aperio and Bioimagene

Commercially Available Pathology-Focused Image Analysis Platforms

- Definiens
- Visiopharm
- Perkin Elmer Vectra/Mantra/INform
- Hamamatsu Nanozoomer
- Leica/Aperio
- Tissuegnostics
- Ventana (formerly Bioimagene)
- Optra SCAN
- GE MultiOmyx
- Genoptix (formerly HistoRx) AQUA
- Many others sold as research platforms

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

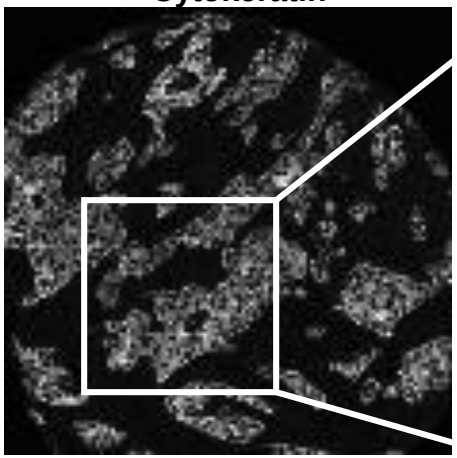
Step 2: Define the numerator (target) and denominator (compartment)

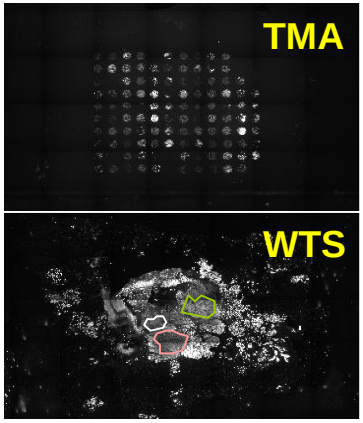
$$\text{Concentration} = \frac{\text{Numerator}}{\text{Denominator}} \Rightarrow \frac{\Sigma \text{ target intensity in compartment pixels}}{\Sigma \text{ compartment pixel area}} = \text{AQUA score}$$

Step 3: Calculate the AQUA score

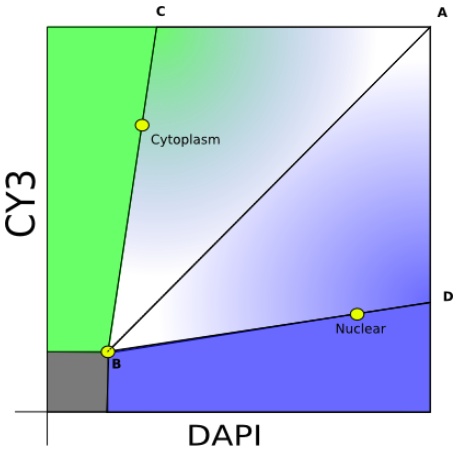
Step 4: Convert to absolute concentration or normalize to set of uniform standards

Generating the AQUA® score



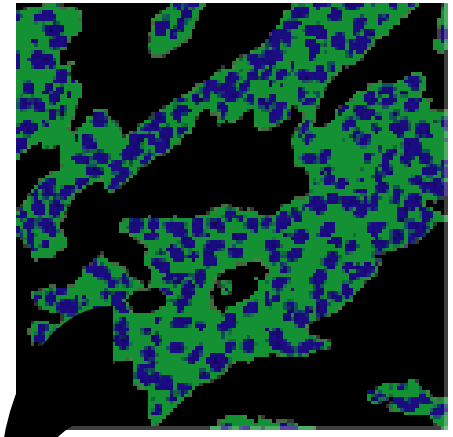


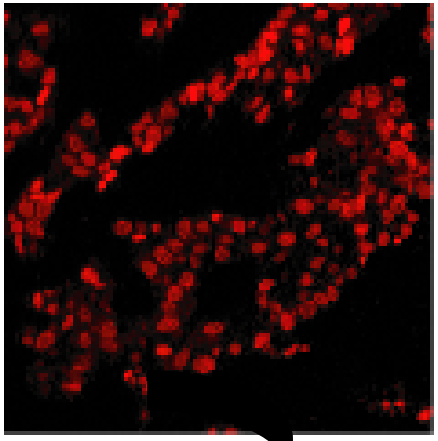
TMA-Tissue Microarray
WTS-Whole Tissue Section



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

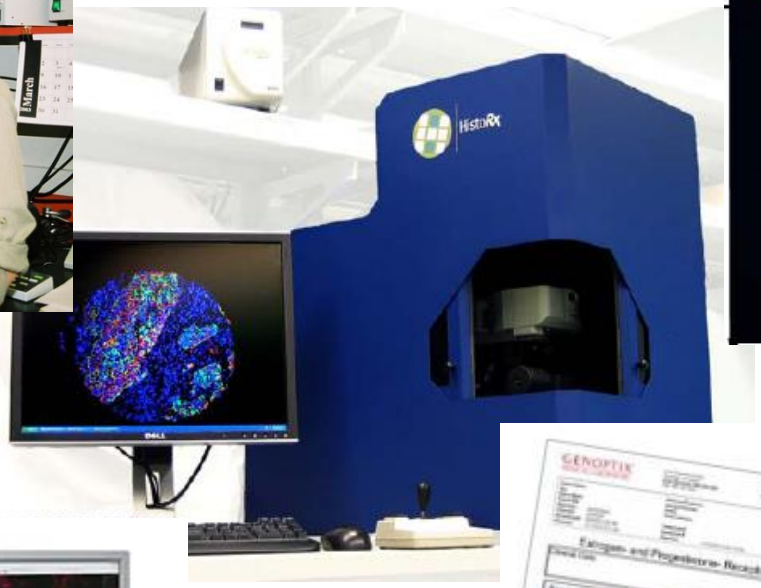
Estrogen Receptor

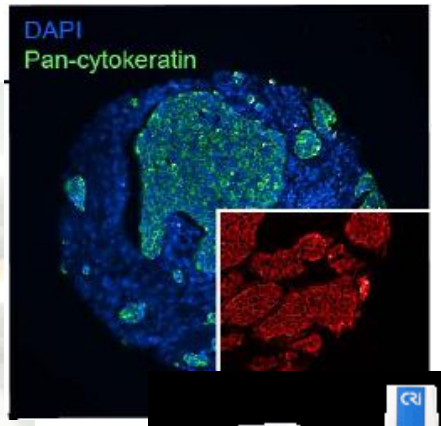


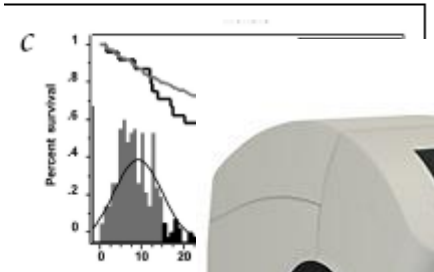


$$\frac{\Sigma \text{ target intensity in compartment pixels}}{\Sigma \text{ compartment pixel area}} = \text{AQUA score}$$

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







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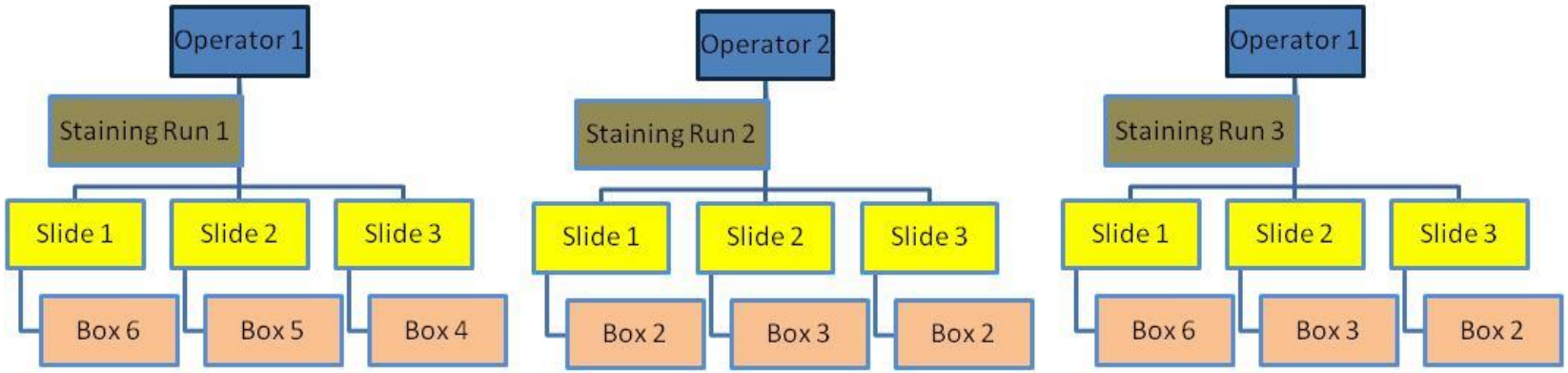
NEW TECHNOLOGY

NexCourse™ BCa

BREAST CANCER ASSAY BY AQUA® TECHNOLOGY

GENOPTIX®
MEDICAL LABORATORY

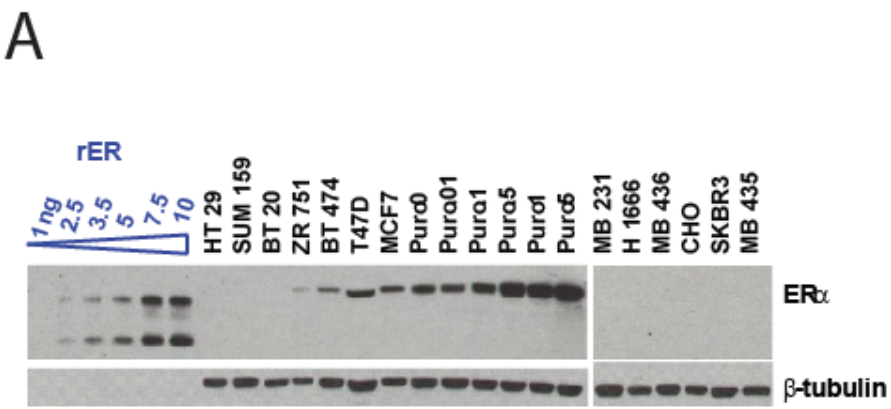
Precision Results (ER-alpha)

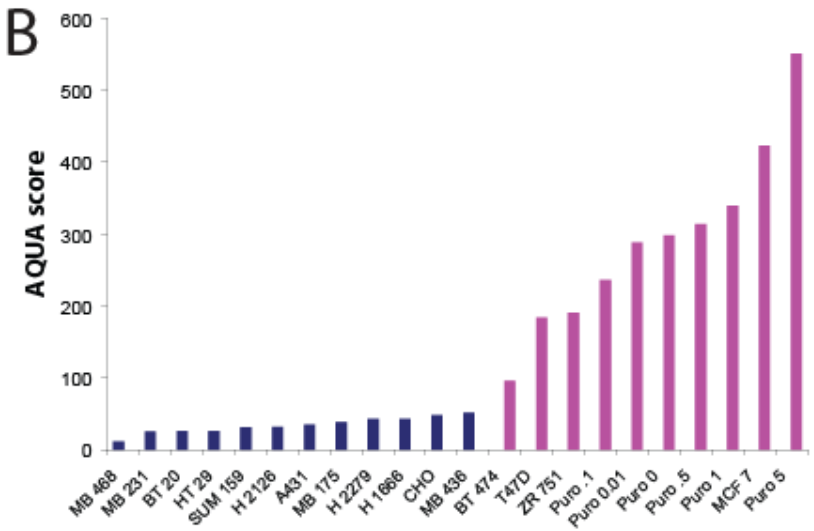


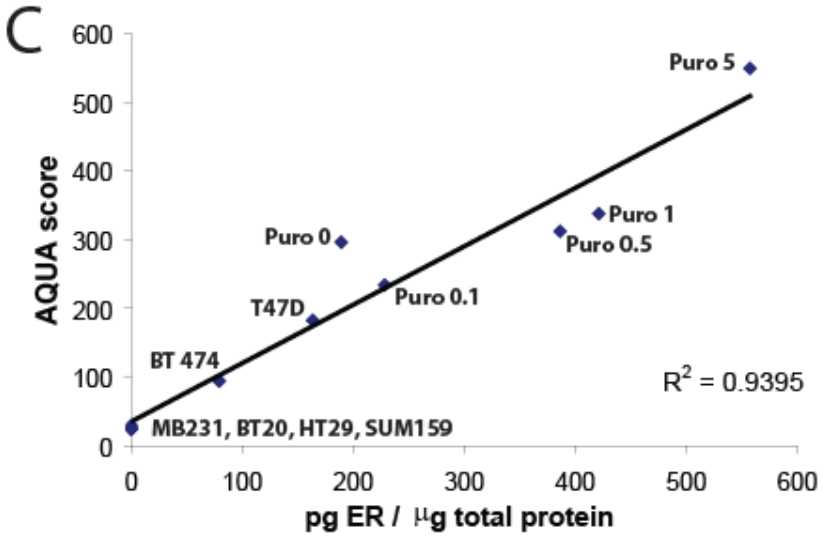
	Pearson R	Slope
Day 1 v. Day 2	**.97**	**.97**
Day 1 v. Day 3	**.97**	**1.01**
Day 2 v. Day 3	**.98**	**1.04**

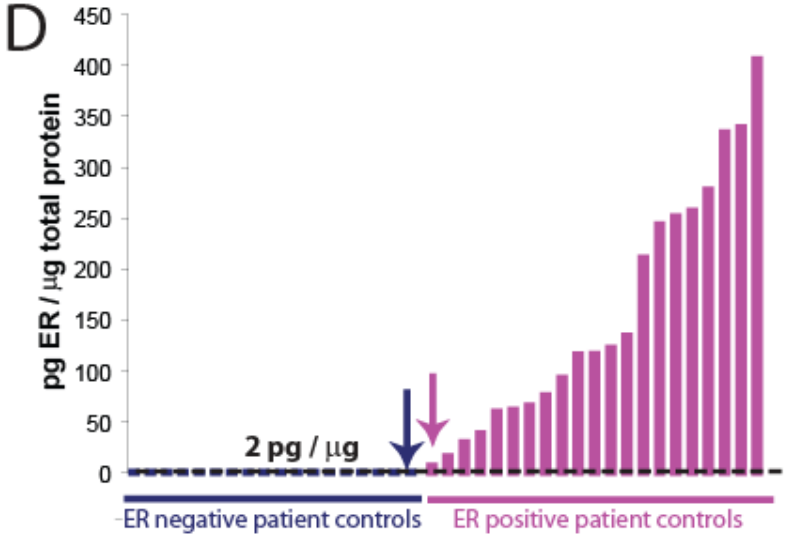
%CV = 4.2

Mark Gustavson and
Jason Christiansen







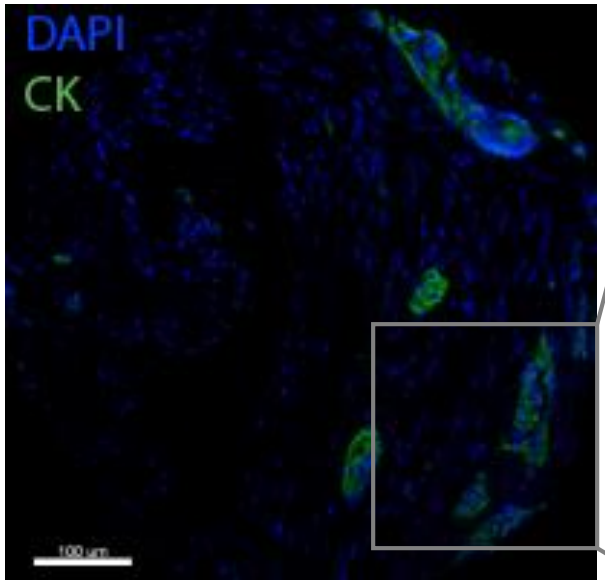


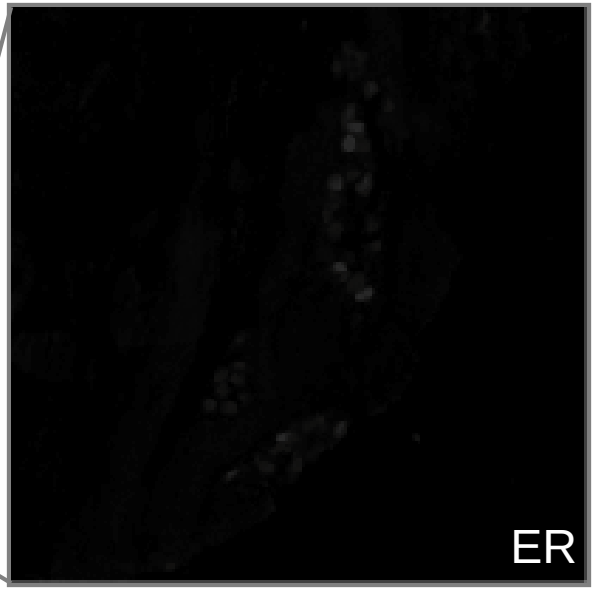
Standardized Index Array

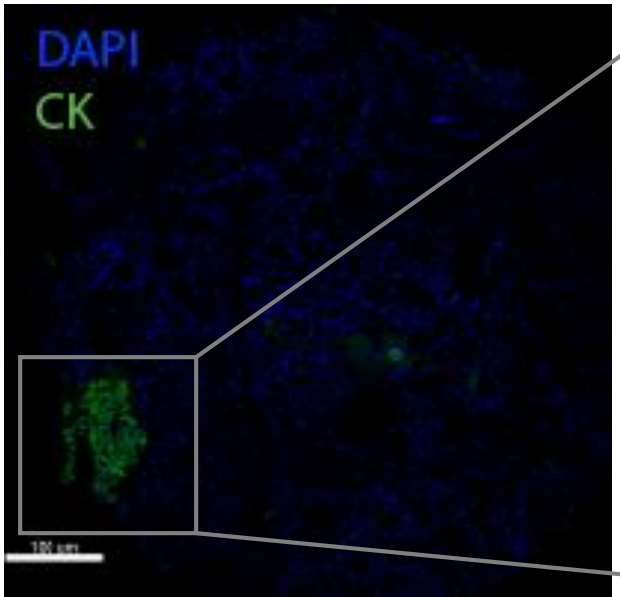
ER antibody used is 1D5

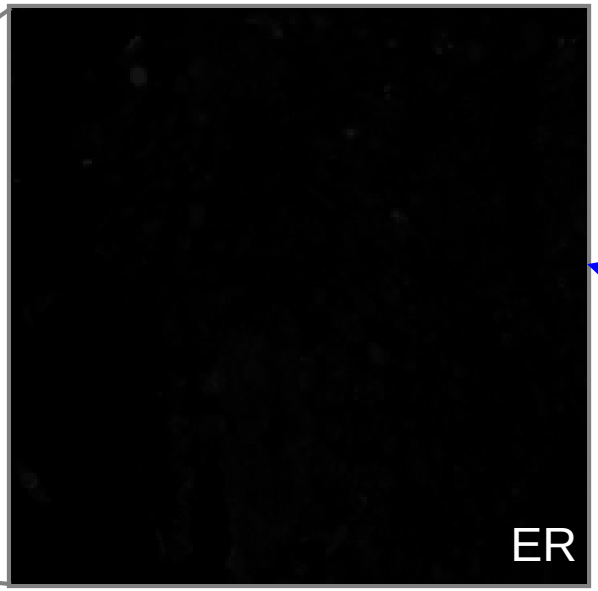
Alley Welsh

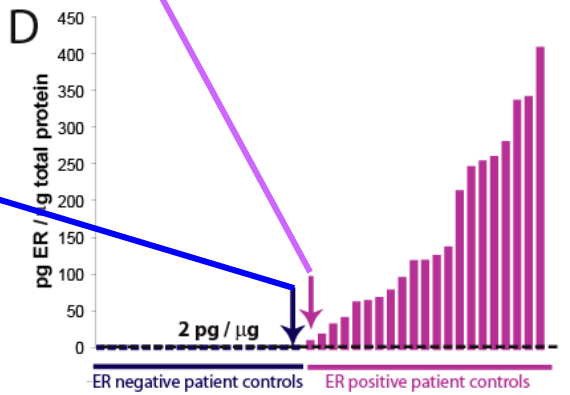
Lowest positive vs. highest negative







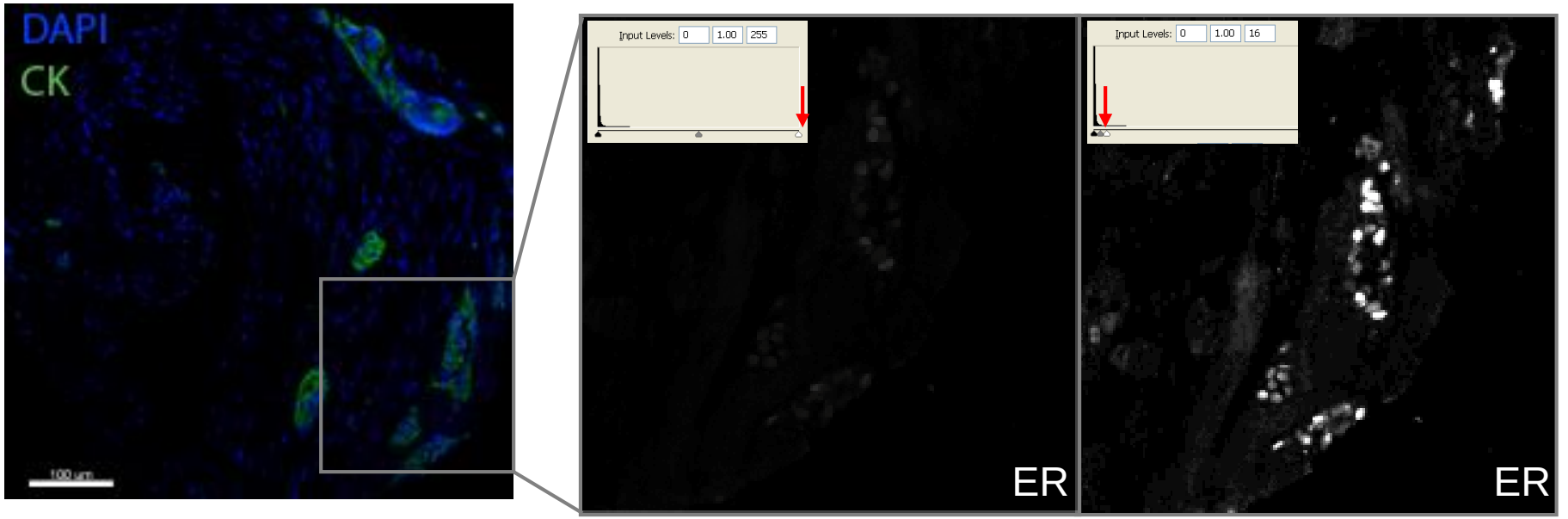


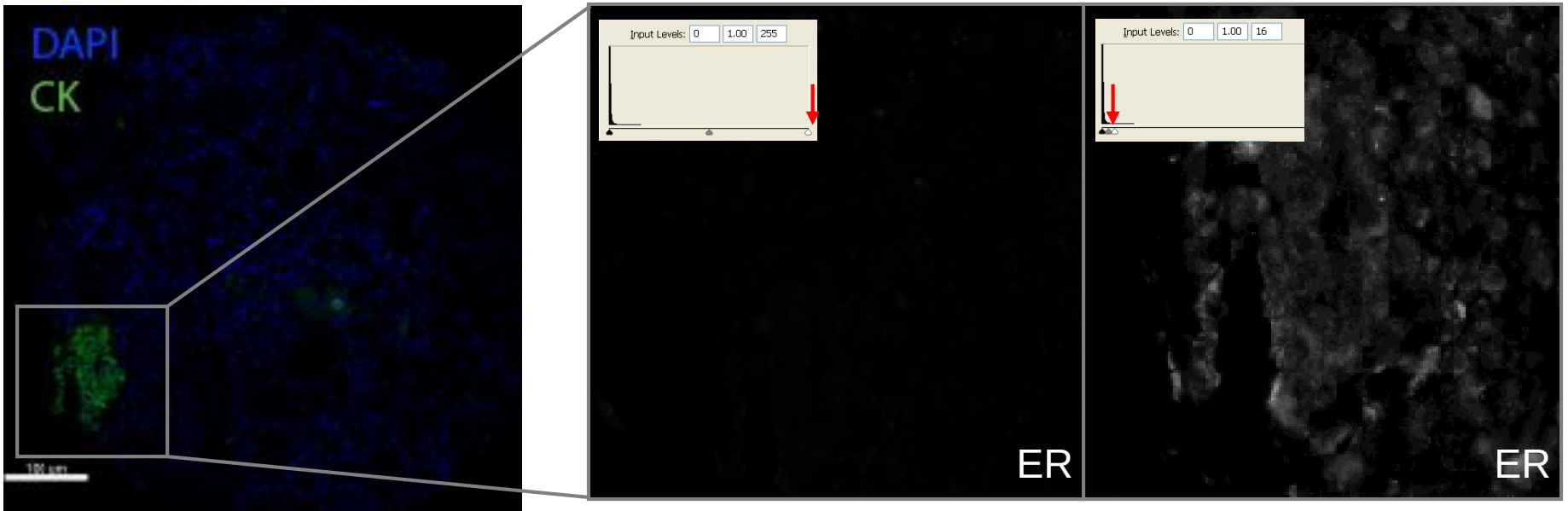


Alley Welsh

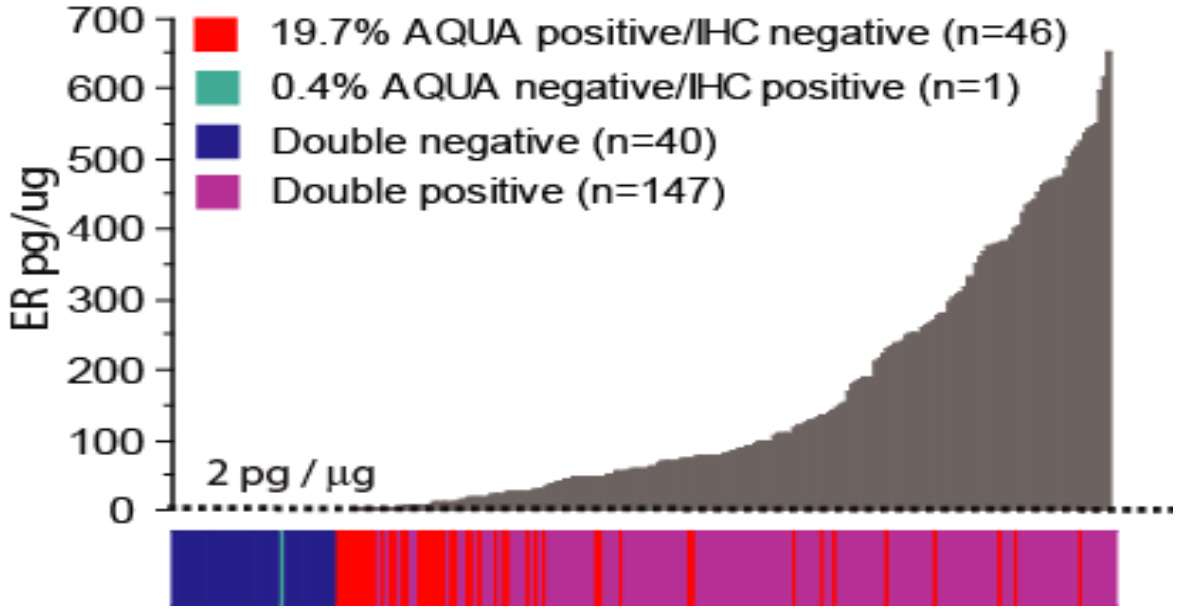
Expanded “levels” to visualize threshold

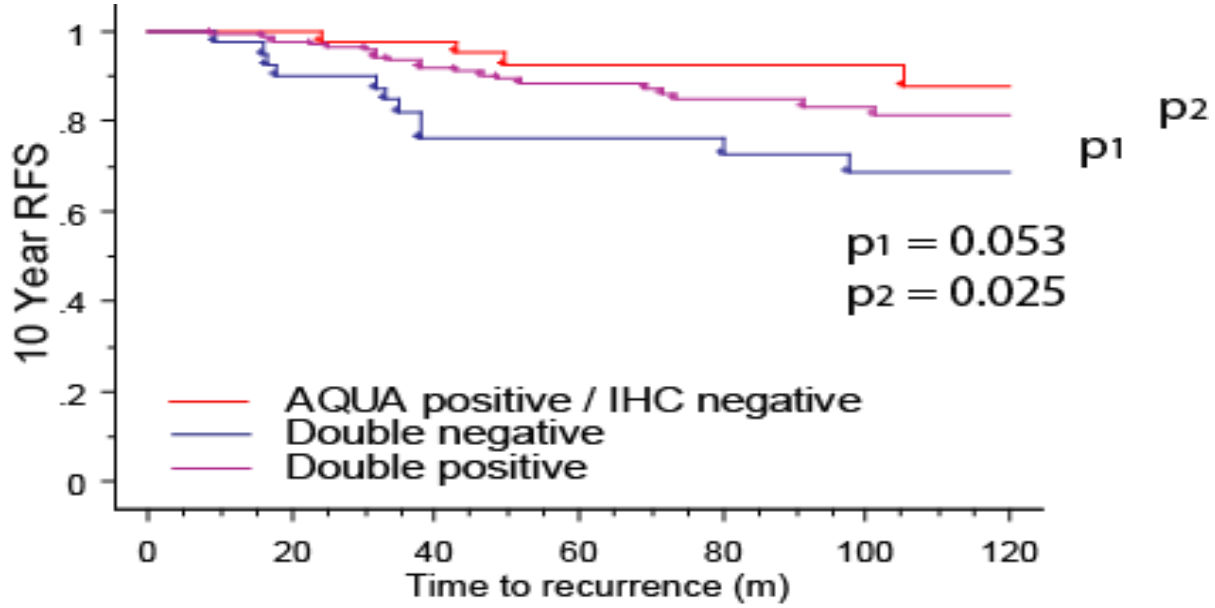
contracted dynamic range of grayscale (max RGB input level 255→16)





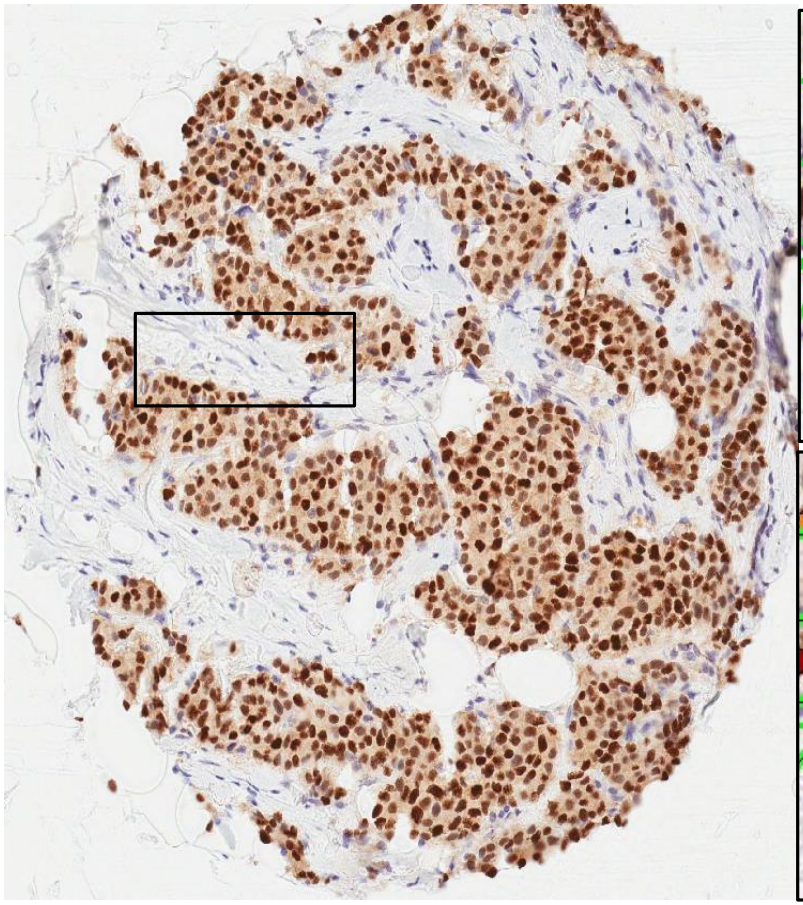
Discordant classification of ER status in YTMA 130 cohort

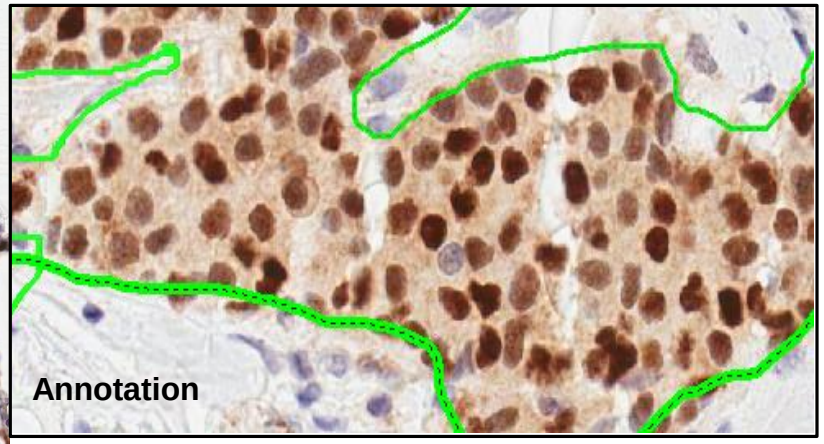


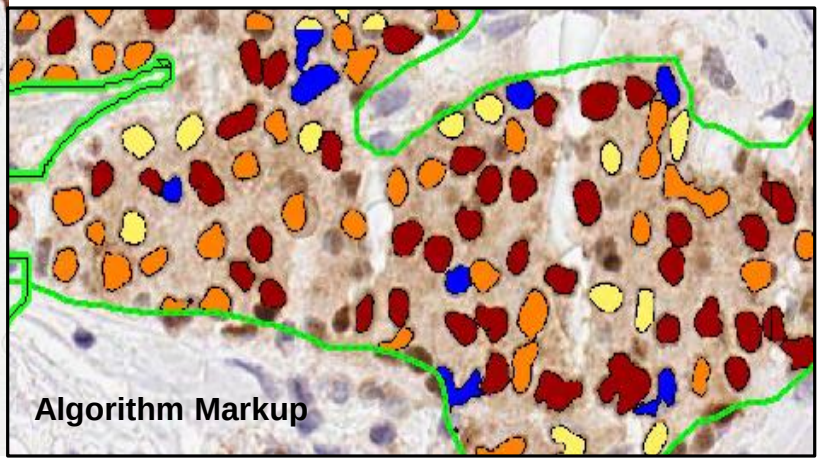


Welsh et al, JCO 2011

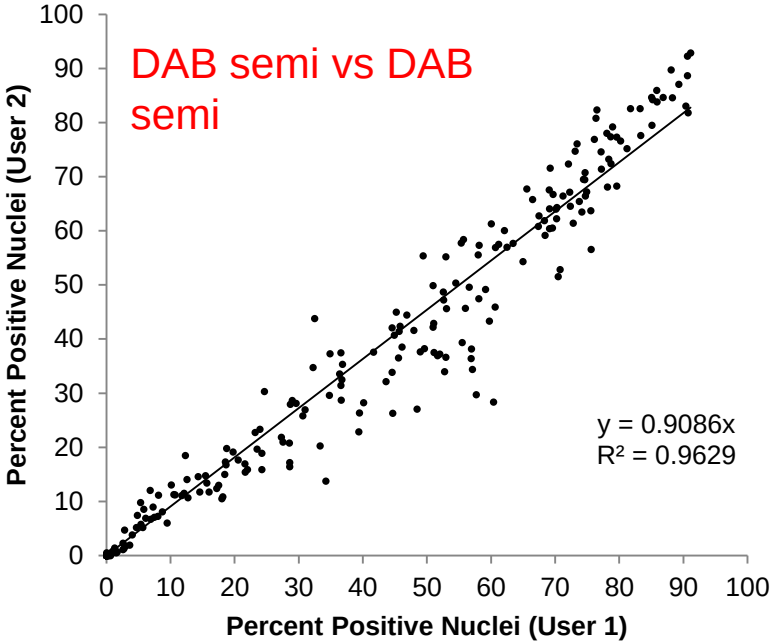
Quantification of DAB by Aperio Pixel Counter

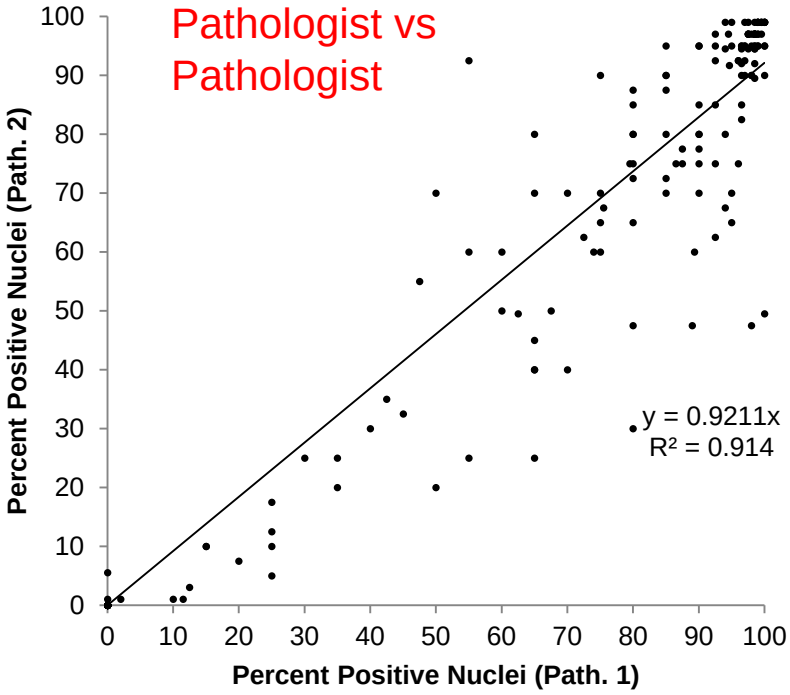


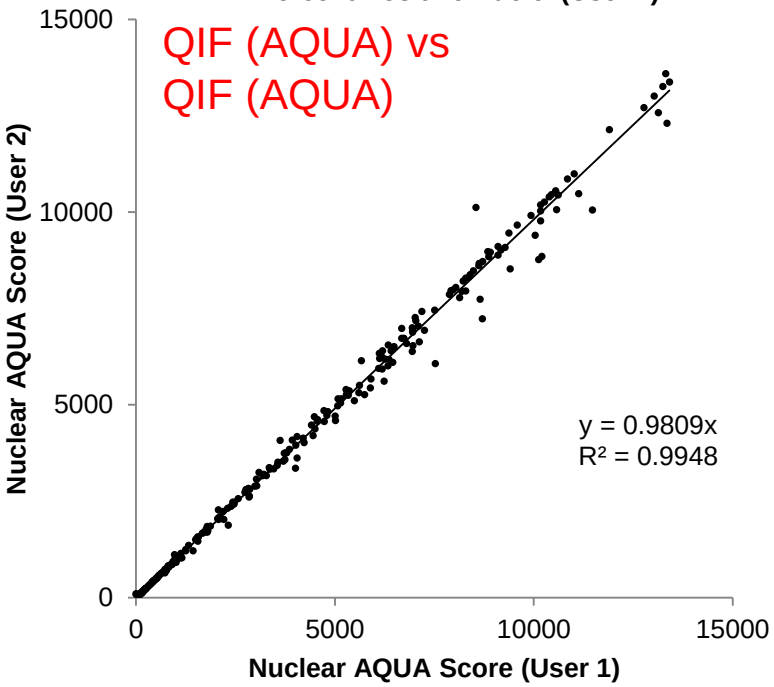




Comparison Between Methods (reproducibility)

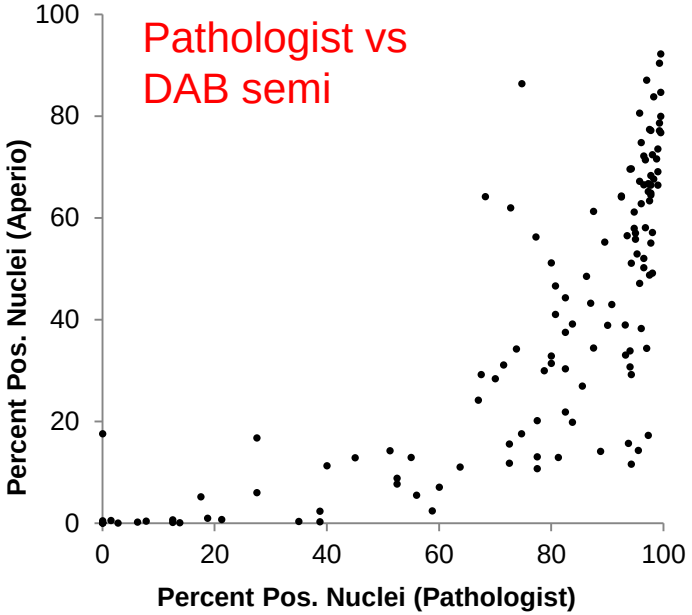


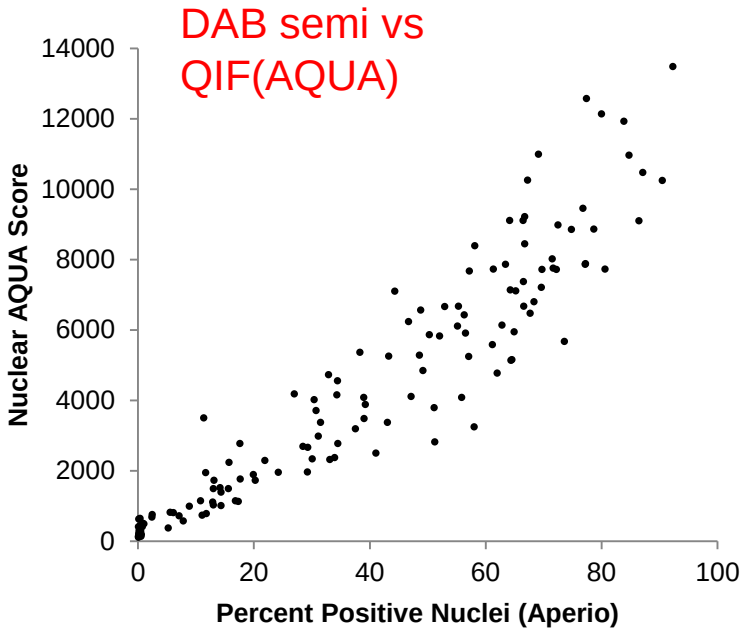


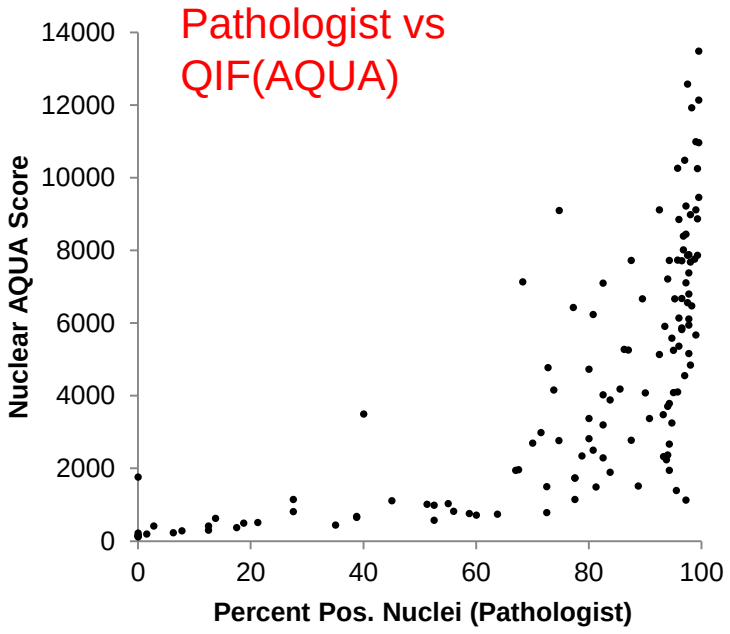


Elizabeth Zarrella and Veronique Neumeister

Comparison Between Methods



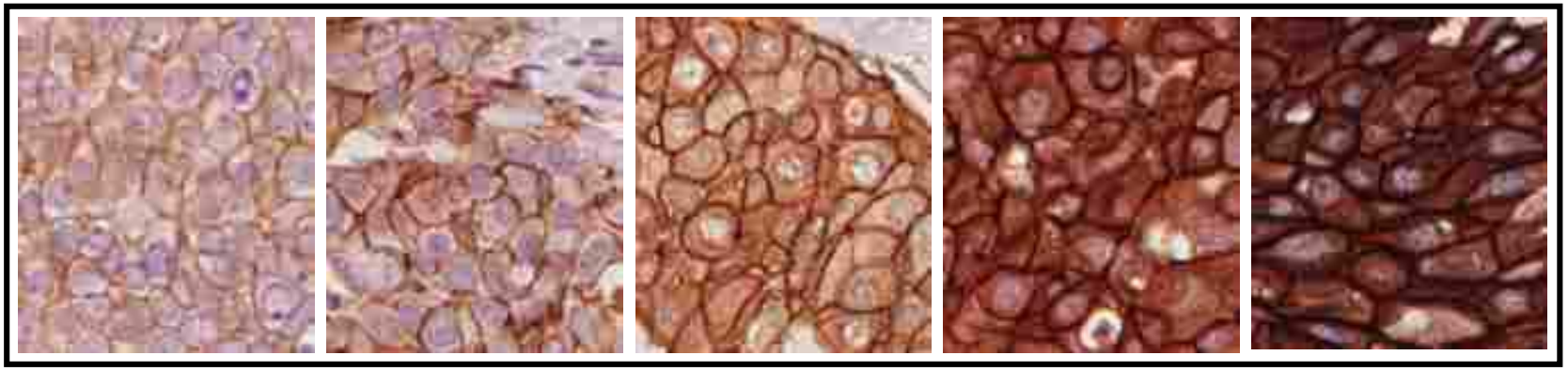




Elizabeth Zarrella and Veronique Neumeister

Different Intensities of HER2 IHC Staining Observed Within HER2(+) Patient Population

Intensities of HER2 IHC Staining Observed in HER2^{+} Patients From the HERA Trial



2+ **3+** **3+** **3+** **3+**

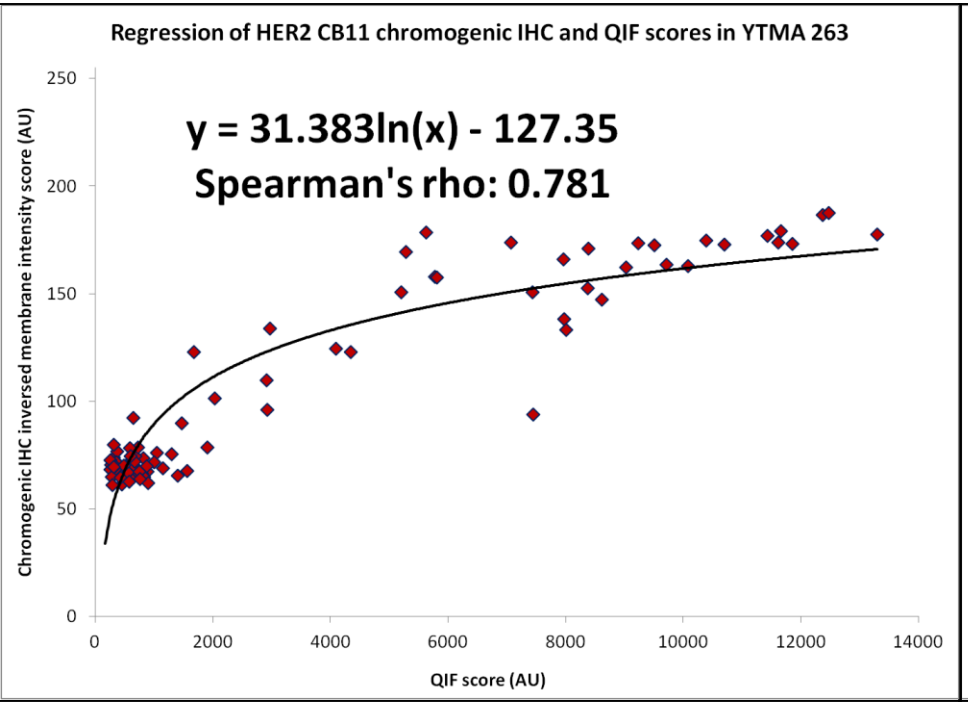
Although there was a high proportion of positive staining tumor cells in all of the 3+ samples the range of staining intensity varied.

Slide provided by Mitch Dowsett

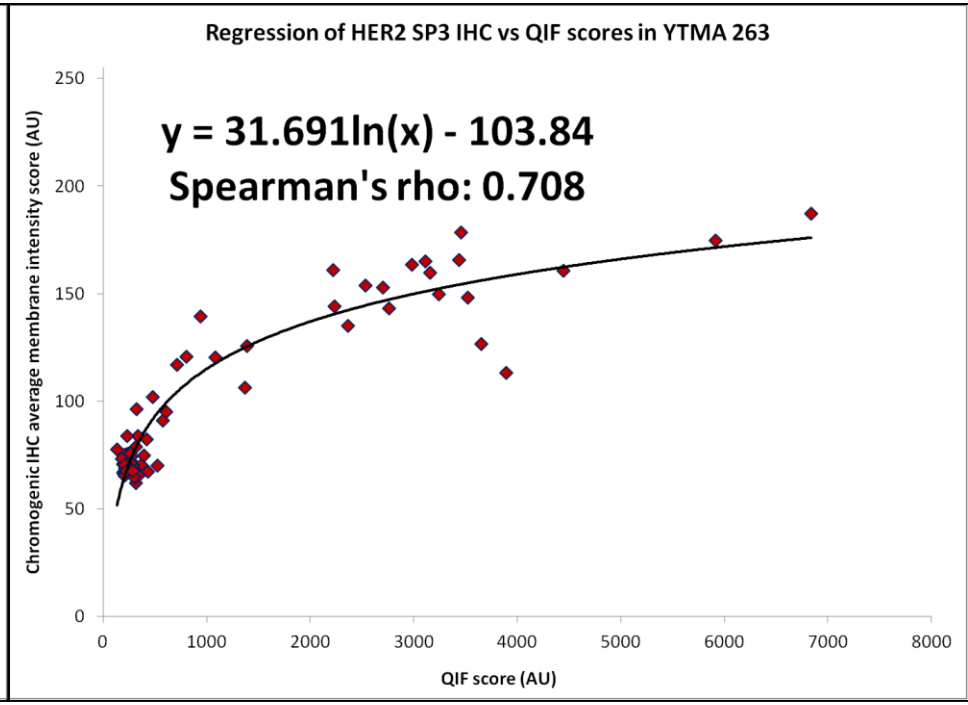
Zabaglo L, et al. Presented at 33rd SABCS; December 8-12, 2010; San Antonio, TX. Abstract PD10-01.

Regression of IHC vs QIF scores for CB11 and SP3 in YTMA 263

CB11

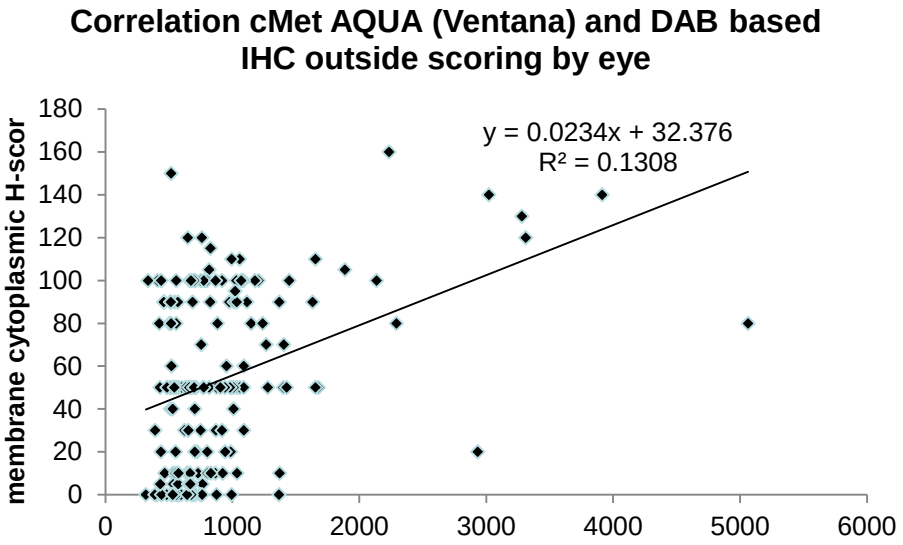


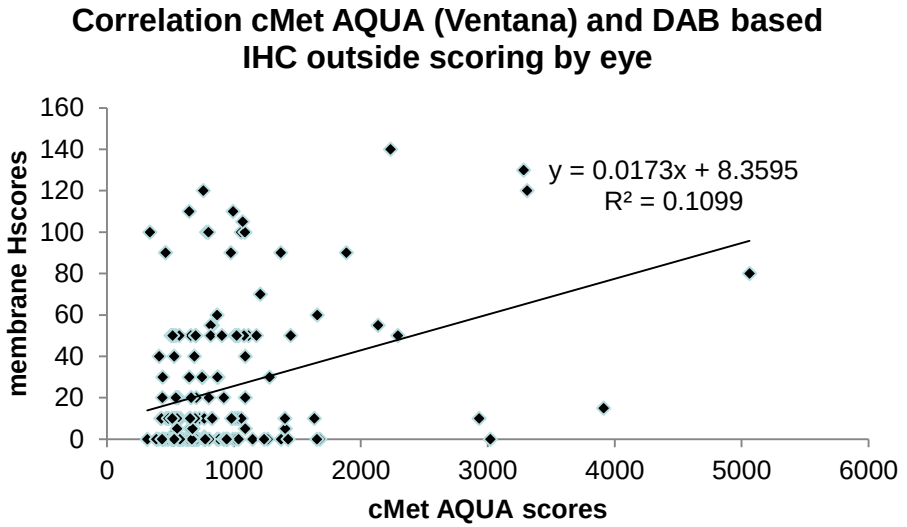
SP3

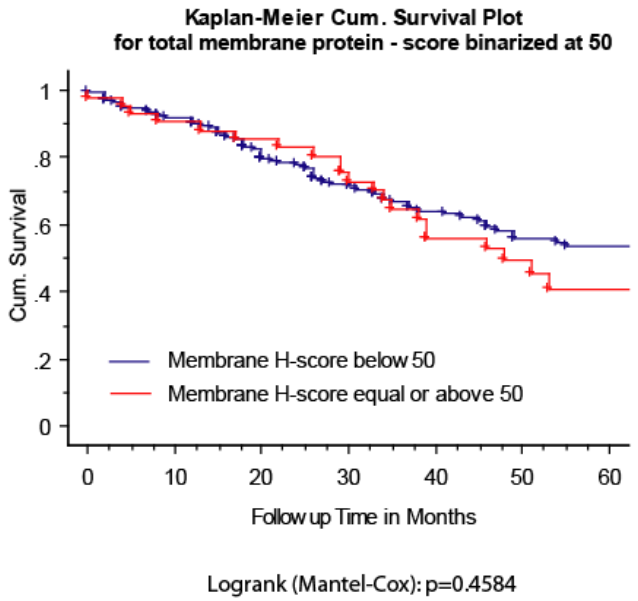


Daniel Carvajal

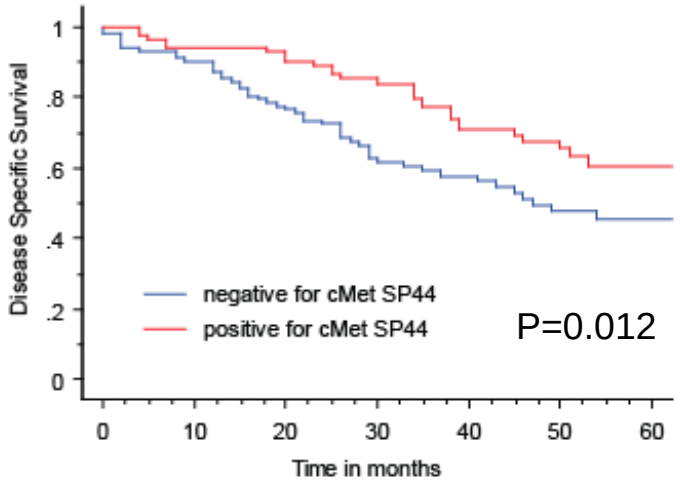
Scoring Affects Outcome Prediction



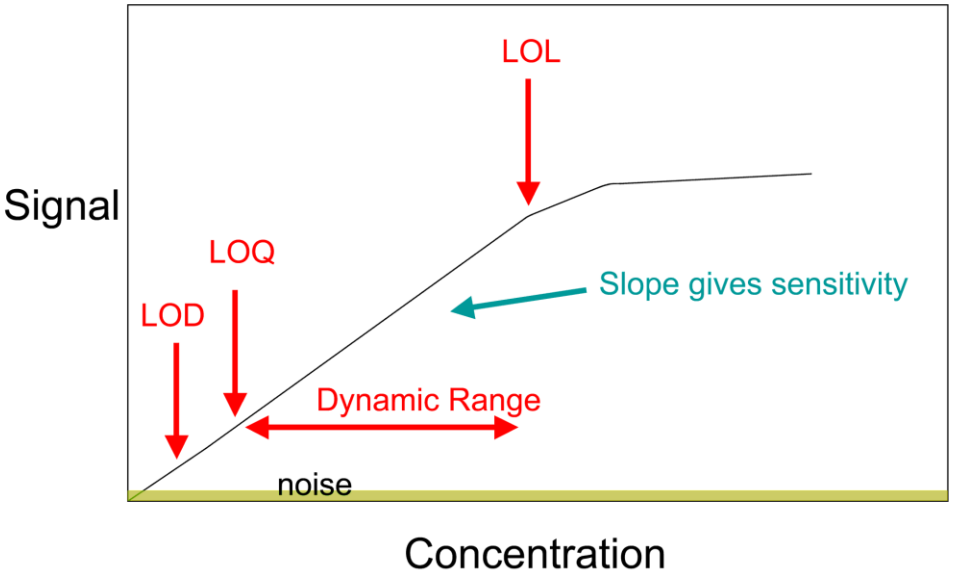


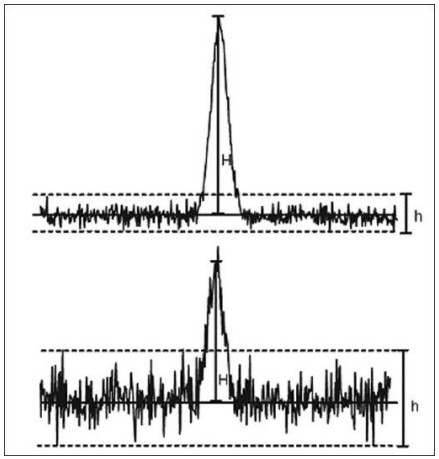


K-M plots of Met scored by AQUA

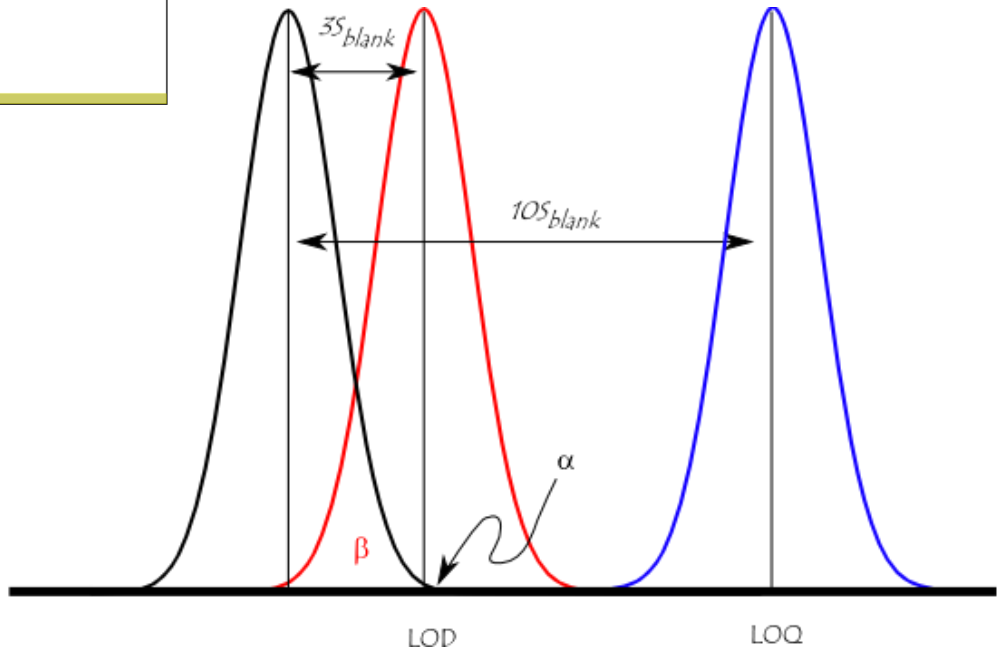


The Goal: To Measure Protein on a slide with the accuracy of Analytical Chemistry



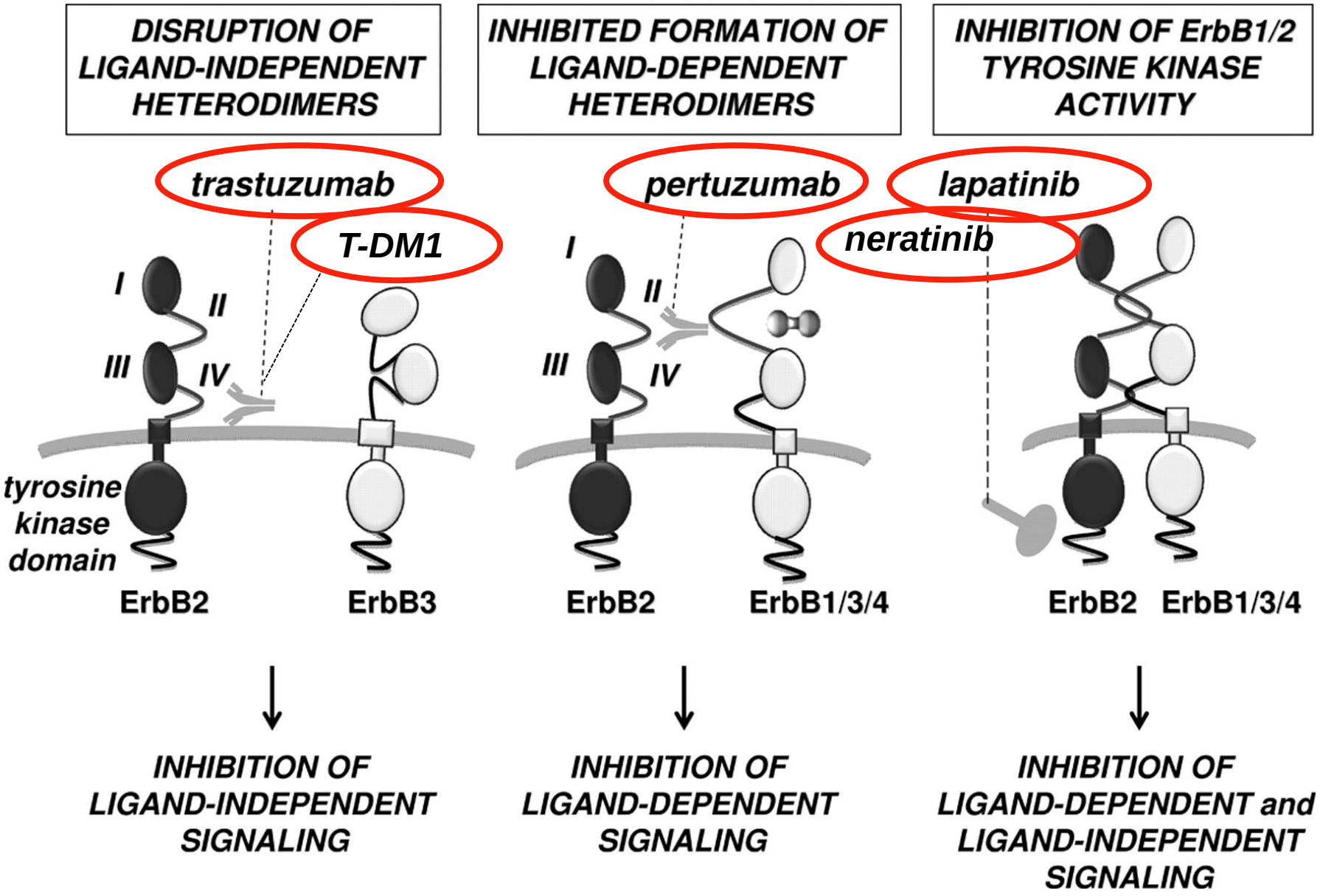


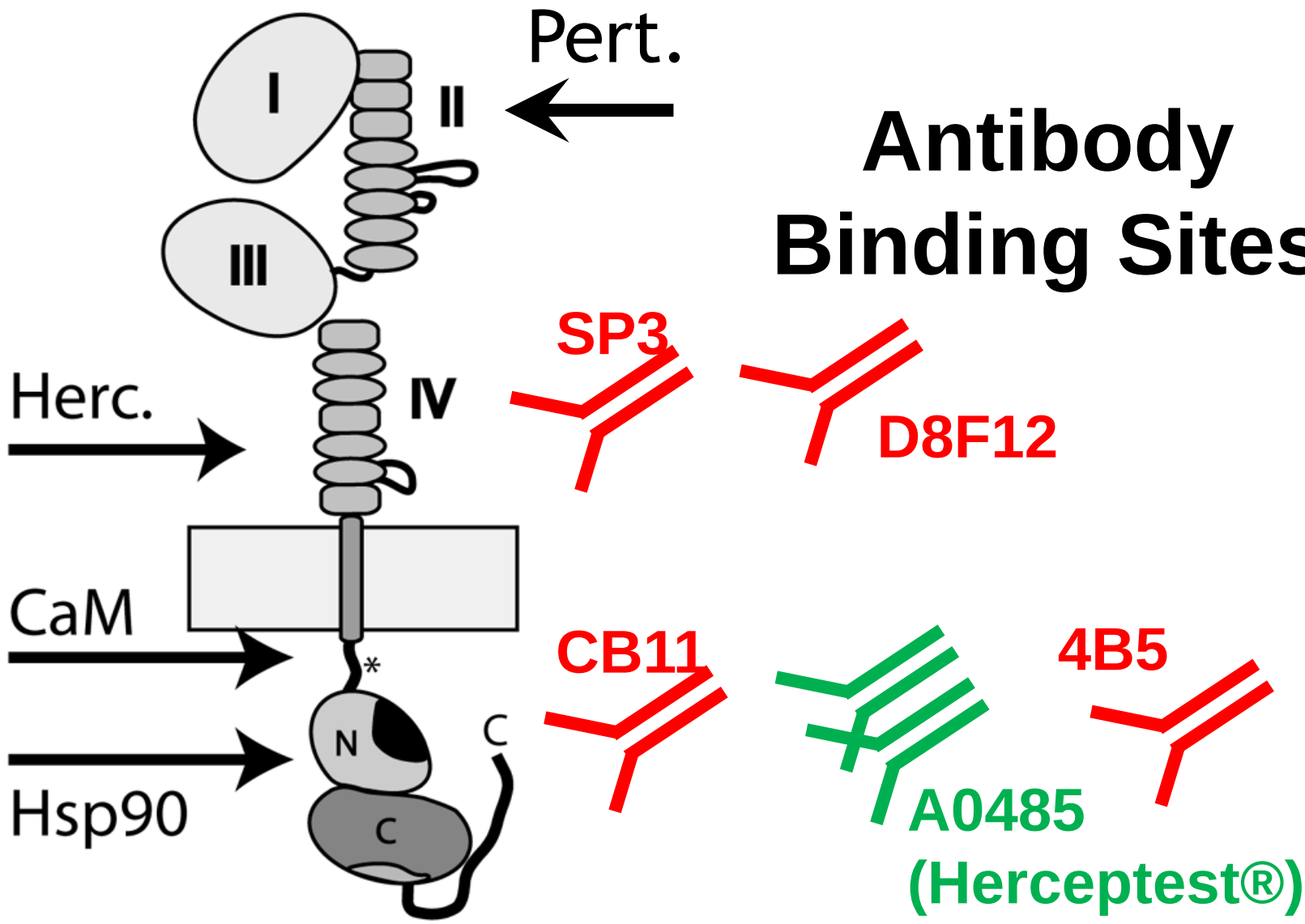
LOD= limit of detection
LOQ = limit of quantification
LOL = limit of linearity



Outline for Presentation

- Domain Specific Assessment of HER2 in Breast Cancer
- Quantitative Multiplexed assessment of TILs
- Beyond QIF


DISRUPTION OF LIGAND-INDEPENDENT HETERODIMERS
INHIBITED FORMATION OF LIGAND-DEPENDENT HETERODIMERS
INHIBITION OF ErbB1/2 TYROSINE KINASE ACTIVITY
trastuzumab
T-DM1
pertuzumab
lapatinib
neratinib
I
II
III
IV
tyrosine kinase domain
ErbB2
ErbB3
ErbB2
ErbB1/3/4
ErbB2
ErbB1/3/4
INHIBITION OF LIGAND-INDEPENDENT SIGNALING
INHIBITION OF LIGAND-DEPENDENT SIGNALING
INHIBITION OF LIGAND-DEPENDENT and LIGAND-INDEPENDENT SIGNALING


Pert.
I
II
III
IV
Herc.
CaM
Hsp90
N
C
C
*
Antibody
Binding Sites
SP3
D8F12
CB11
4B5
A0485
(Herceptest®)

What are we using for IHC?

Antibody	N	Percent
4B5	568	55%
A0485	54	5%
CB11	24	2%
Herceptest	296	29%
SP3	52	5%
other	37	4%
Total	1031	

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JOURNAL OF CLINICAL ONCOLOGY

ASCO SPECIAL ARTICLE

Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update

Antonio C. Wolff, M. Elizabeth H. Hammond,* David G. Hicks,* Mitch Dowsett,* Lisa M. McShane,* Kimberly H. Allison, Donald C. Allred, John M.S. Bartlett, Michael Bilous, Patrick Fitzgibbons, Wedad Hanna, Robert B. Jenkins, Pamela B. Mangu, Soonmyung Paik, Edith A. Perez, Michael F. Press, Patricia A. Spears, Gail H. Vance, Giuseppe Viale, and Daniel F. Hayes**

Dekker *et al. Breast Cancer Research* 2012, **14**:R93
http://breast-cancer-research.com/content/14/3/R93

Breast Cancer RESEARCH

RESEARCH ARTICLE Open Access

Determining sensitivity and specificity of HER2 testing in breast cancer using a tissue micro-array approach

Tim JA Dekker[1,2], Susan Ter Borg[3], Gerrit KJ Hooijer[3], Sybren L Meijer[3], Jelle Wesseling[4], James E Boers[5], Ed Schuuring[6], Jos Bart[6], Joost van Gorp[7], Wilma E Mesker[2], Judith R Kroep[1], Vincent THBM Smit[8] and Marc J van de Vijver[3*]

Studies done "by eye" cannot see discordance

Table 3 SP3, 4B5 and Herceptest.

		SP3					4B5					Herceptest		
		0,1+	2+	3+			0,1+	2+	3+			0,1+	2+	3+
4B5	0,1+	911	15	0	4B5	0,1+	920	7	0	SP3	0,1+	930	9	0
	2+	30	26	1		2+	42	15	0		2+	27	18	2
	3+	1	7	72		3+	7	8	68		3+	6	3	66
Total		942	48	73	Total		969	30	68	Total		963	30	68

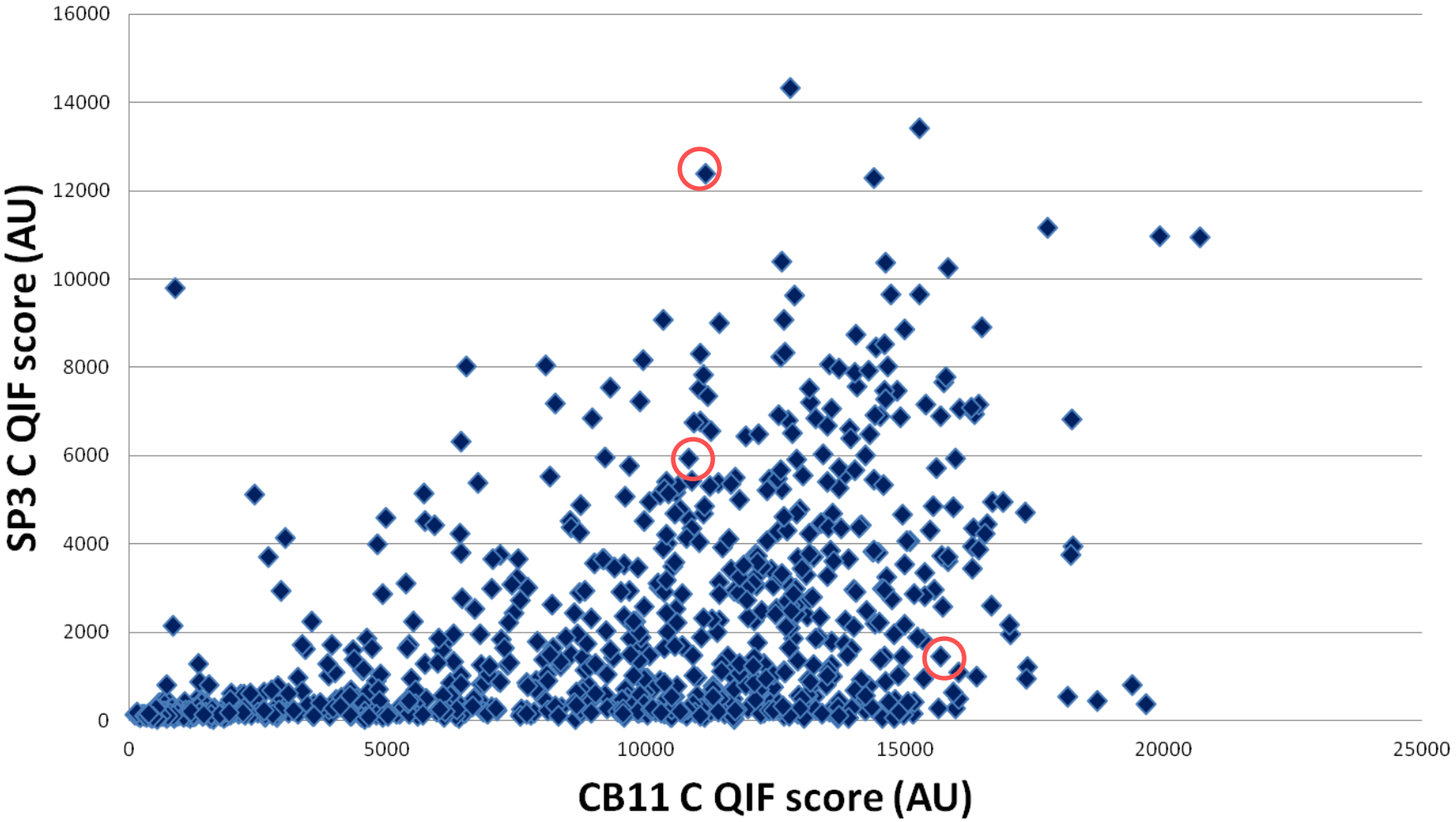
Table 4 HER2 status determined on TMA (SP3, 4B5 and mono color SISH)

Total complete results	1,020 (84.3%)
Negative	932 (91.4%)
Positive	80 (7.8%)
Discordant results	8 (0.8%)
Incomplete results	190 (15.7%)
Total number of cases	1210 (100%)

HER2, human epidermal growth factor receptor 2; SISH, silver *in situ* hybridization; TMA, tissue micro-array.

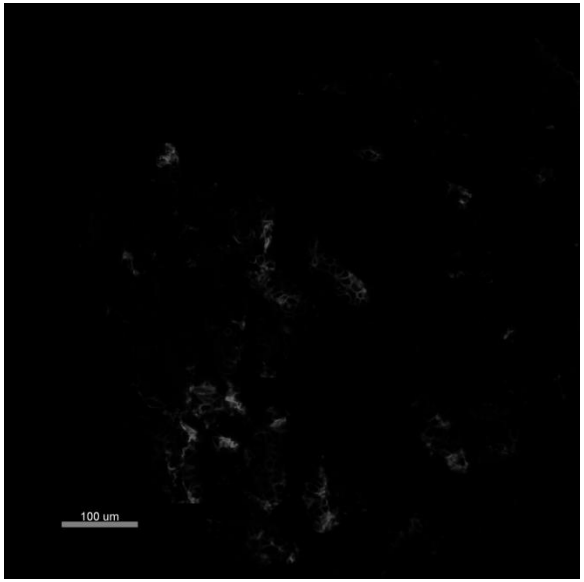
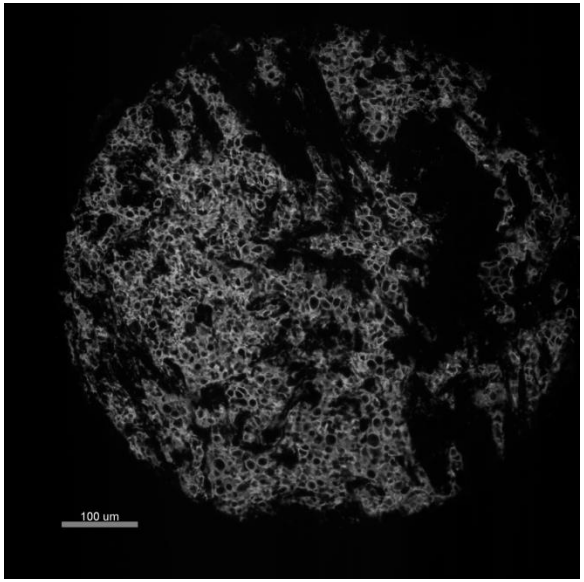
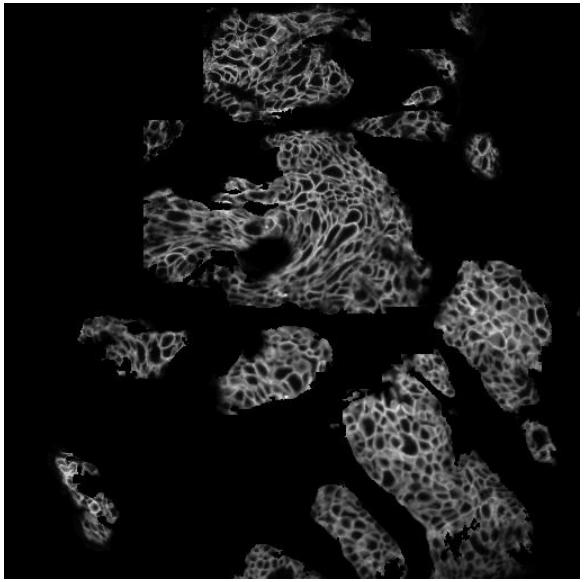
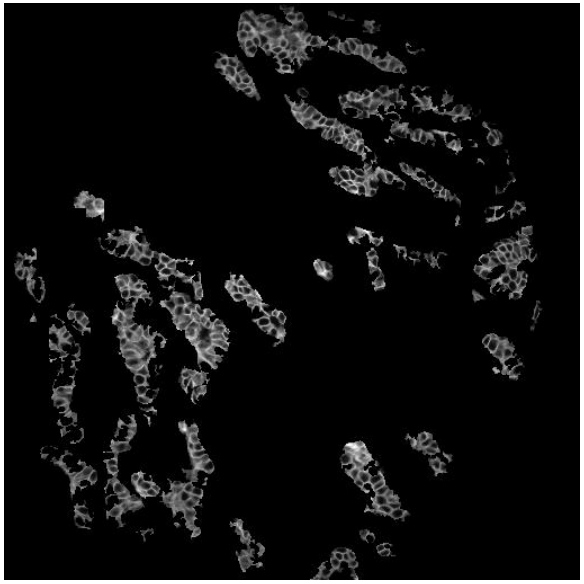
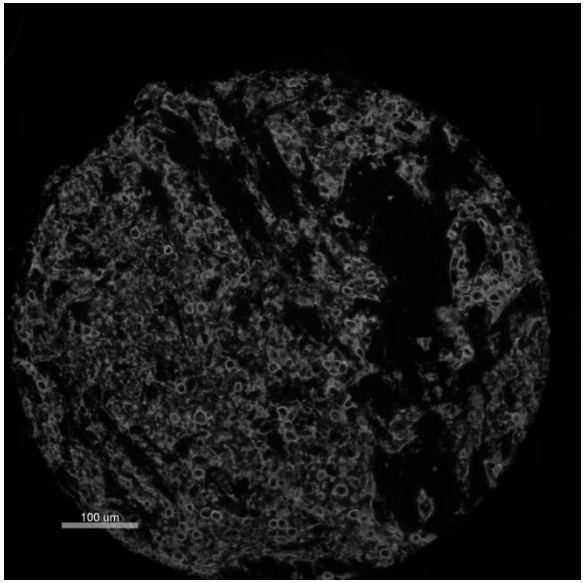
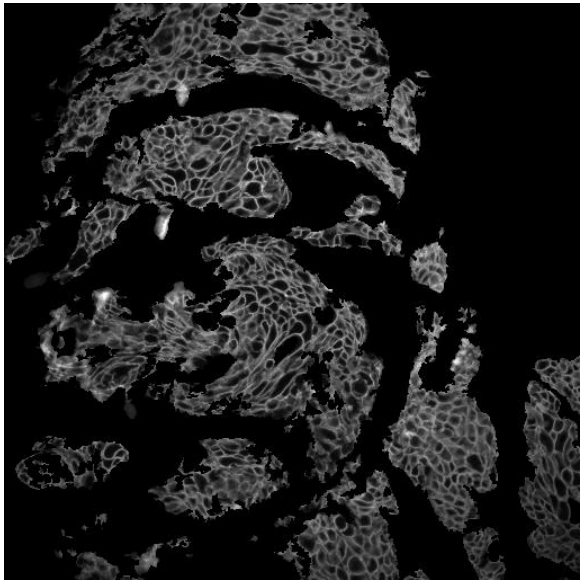
Less than 1% discordance between cytoplasmic and extracellular domain antibodies

SP3 C vs CB11 C (NCCTG 9831 Arm C)



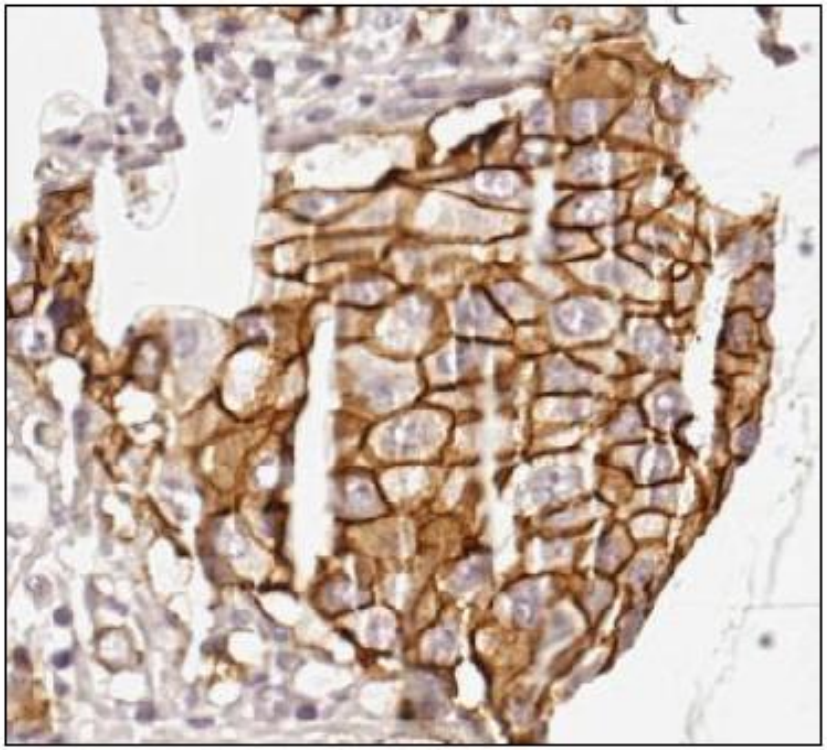
Data from NCCTG9831 from ongoing collaboration with Edith Perez and Karla Ballman

Images

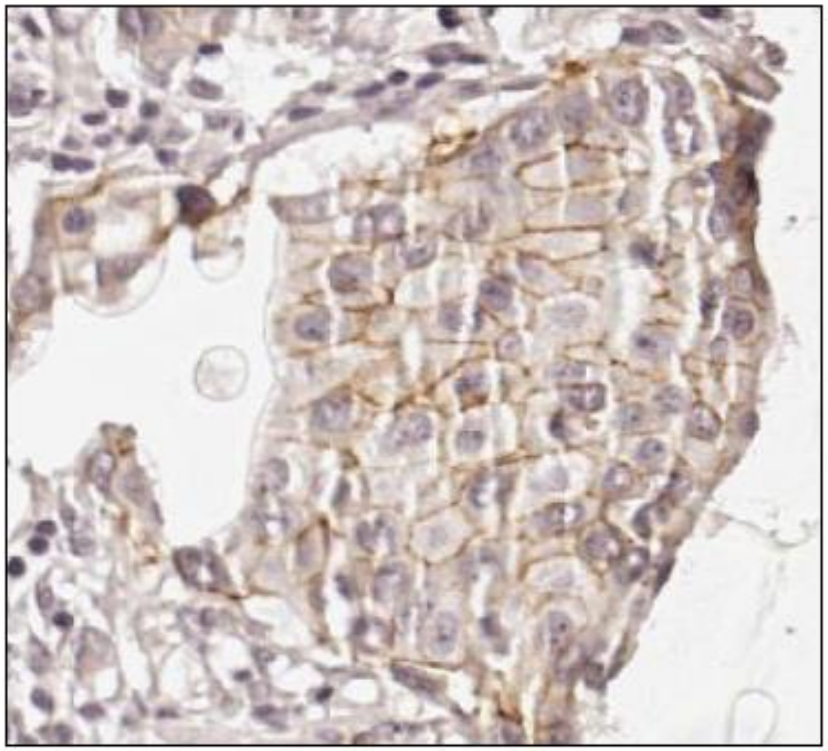
	21	262	78
SP3			
CB11			

DAB shows same effect, but more subtle

CB11 (ICD)

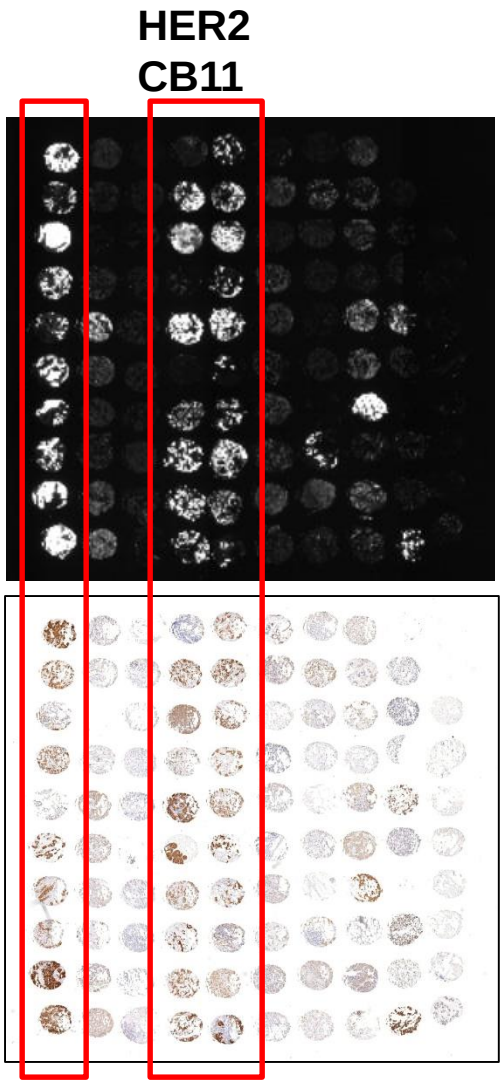


SP3 (ECD)

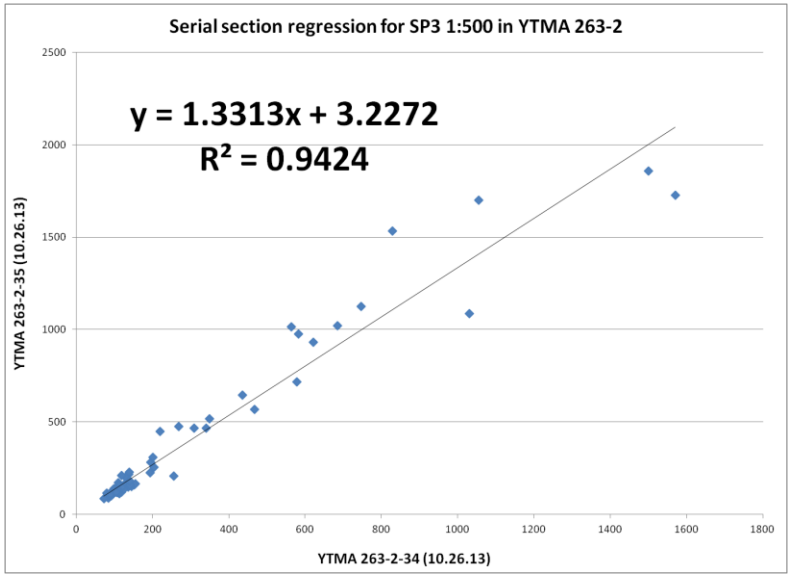


HER2 Standardization Array (YTMA 263)

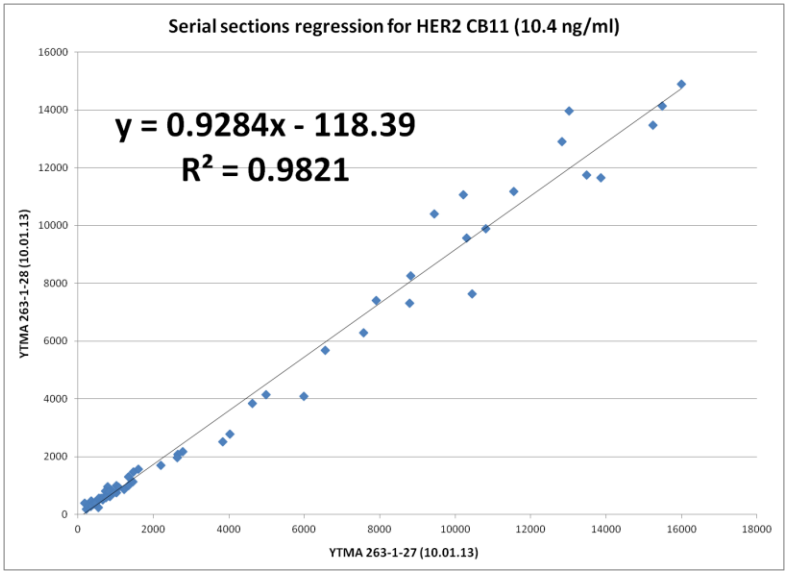
3+, AMP	1+, NOT AMP	0, NOT AMP	2+, AMP	3+, AMP	1+, NOT AMP	0, NOT AMP	2+, NOT AMP	CELL LINES	NORMAL BREAST TISSUE (NBT)
1	2	3	4	5	6	7	8	9	10
2008	2011	2011	2010	2005	2009	2004	2011	MB453	NBT
2007	2011	2011	2007	2004	2009	2004	2011	BT20	NBT
2011	2011	2011	2006	2004	2008	2009	2011	MCF7	NBT
2011	2011	2011	1998	2003	2007	2009	2011	MB468	NBT
2011	2011	2010	2004	2003	2007	2009	2011	ZR751	NBT
2010	2011	2010	2004	2002	2006	2007	2011	MB361	NBT
2009	2011	2007	2003	2002	2011	2007	2011	SKBR3	NBT
2009	2011	2007	2003	2002	2011	2011	2011	UACC812	NBT
2008	2010	2006	2011	2002	2011	2011	2007	MDA231	NBT
2008	2010	2006	2010	2002	2011	2011	2007	BT474	NBT



Serial section Reproducibility for Domain Specific antibodies



Extra-Cellular Domain (SP3)



Intra-Cellular Domain (CB11)

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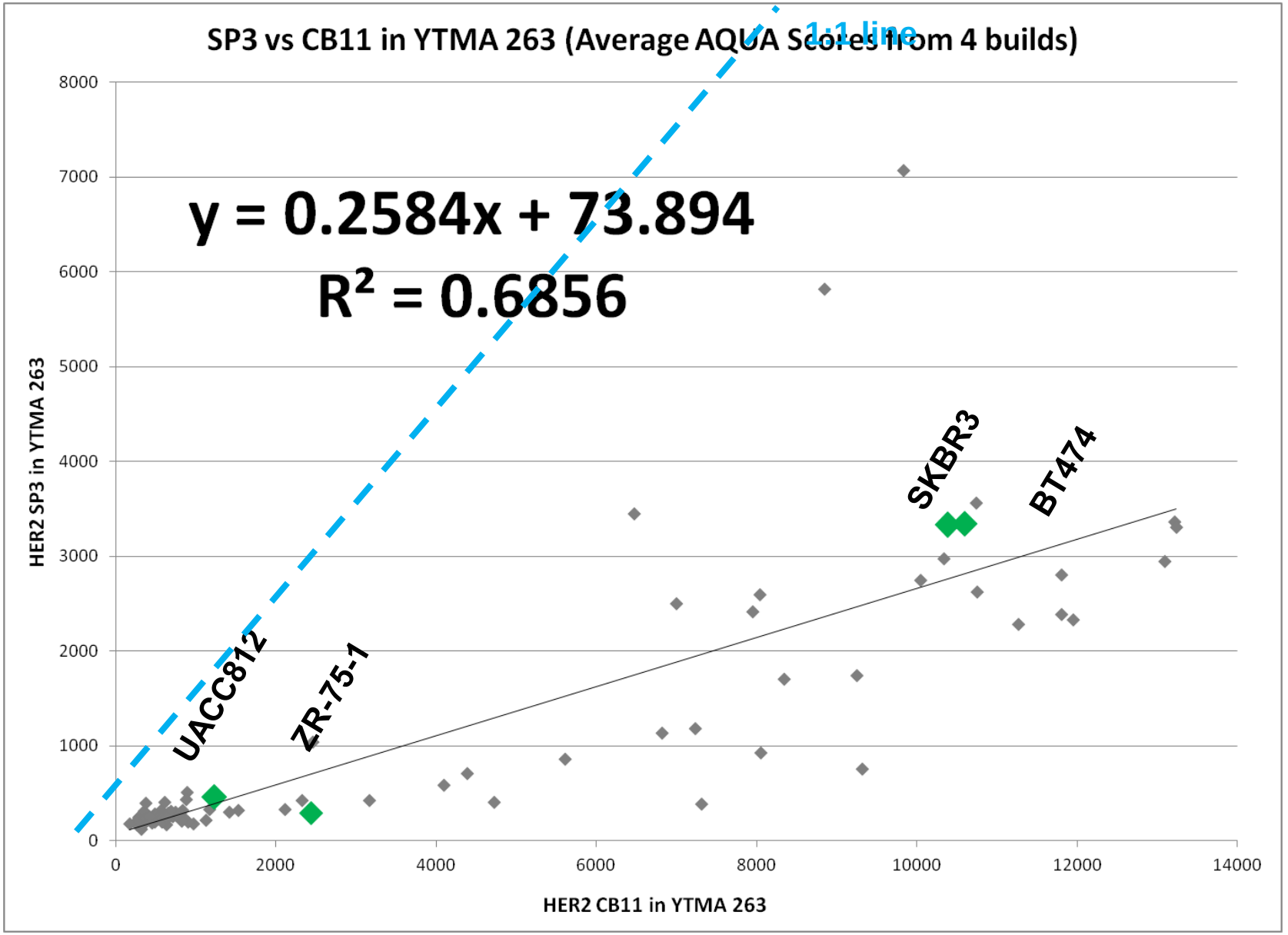
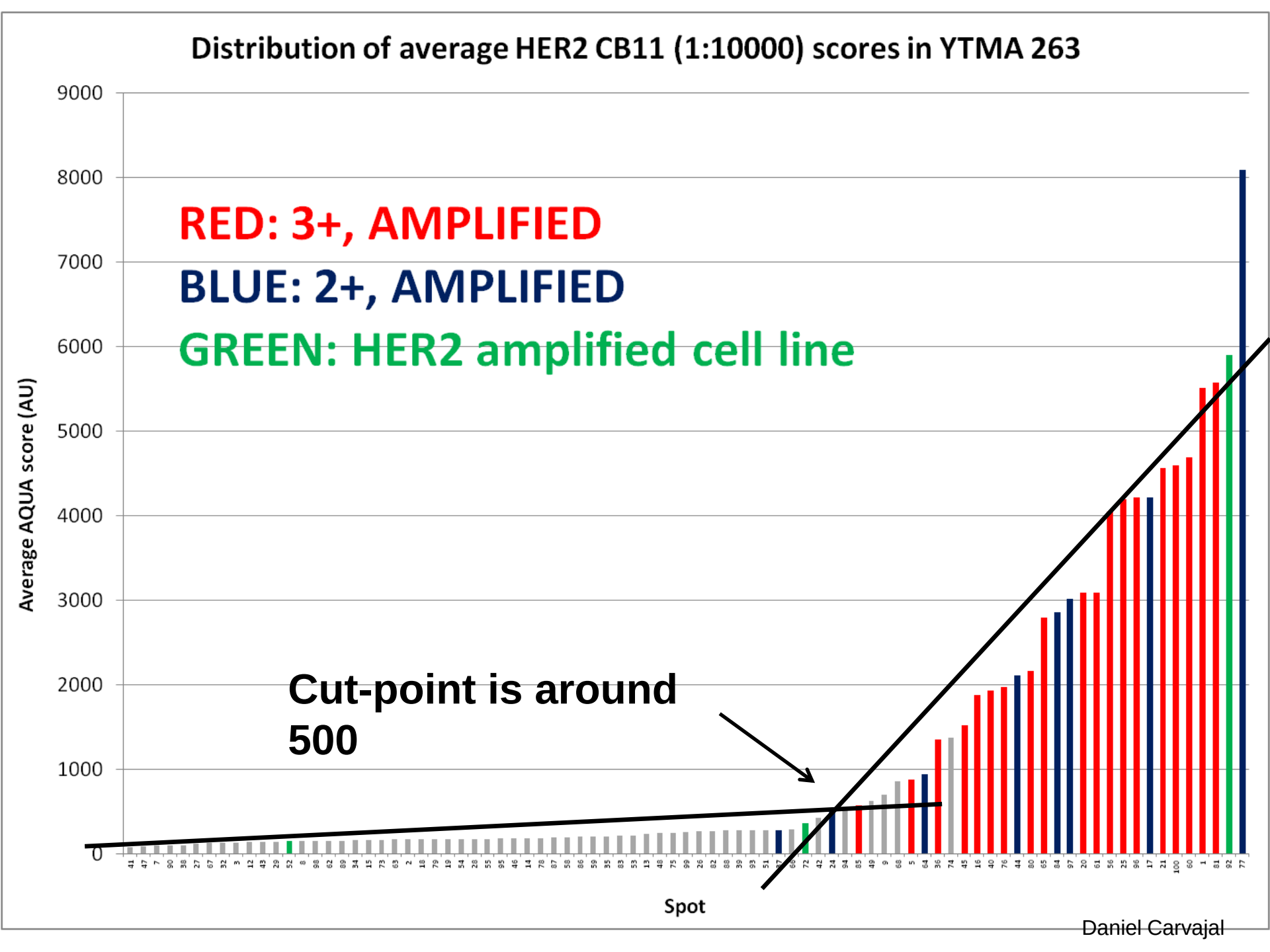
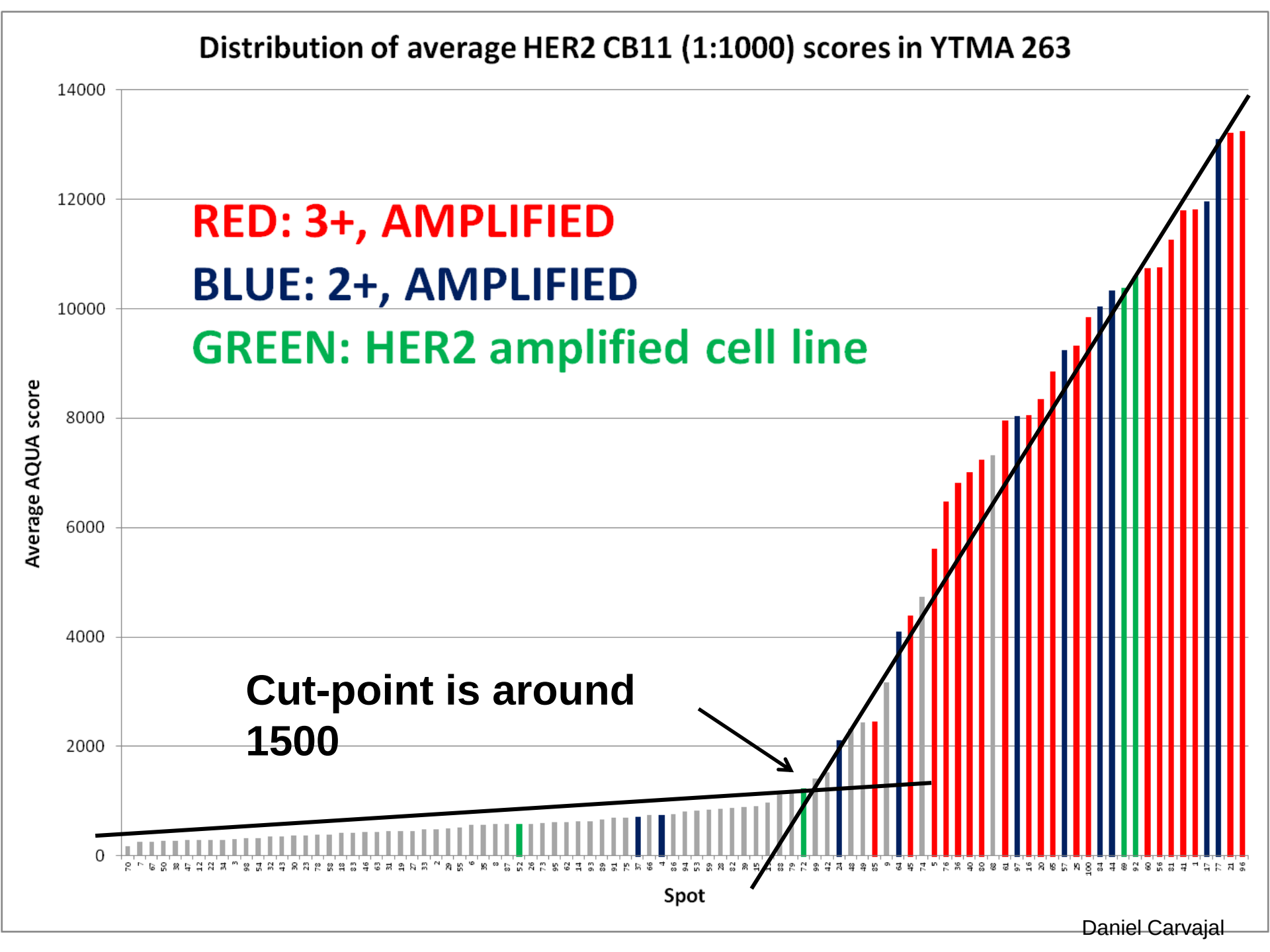


Daniel Carvajal


Distribution of average HER2 CB11 (1:10000) scores in YTMA 263
RED: 3+, AMPLIFIED
BLUE: 2+, AMPLIFIED
GREEN: HER2 amplified cell line
Cut-point is around 500
Average AQUA score (AU)
Spot
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
Daniel Carvajal



Daniel Carvajal

JOINPOINT
REGRESSION PROGRAM

surveillance.cancer.gov/joinpoint
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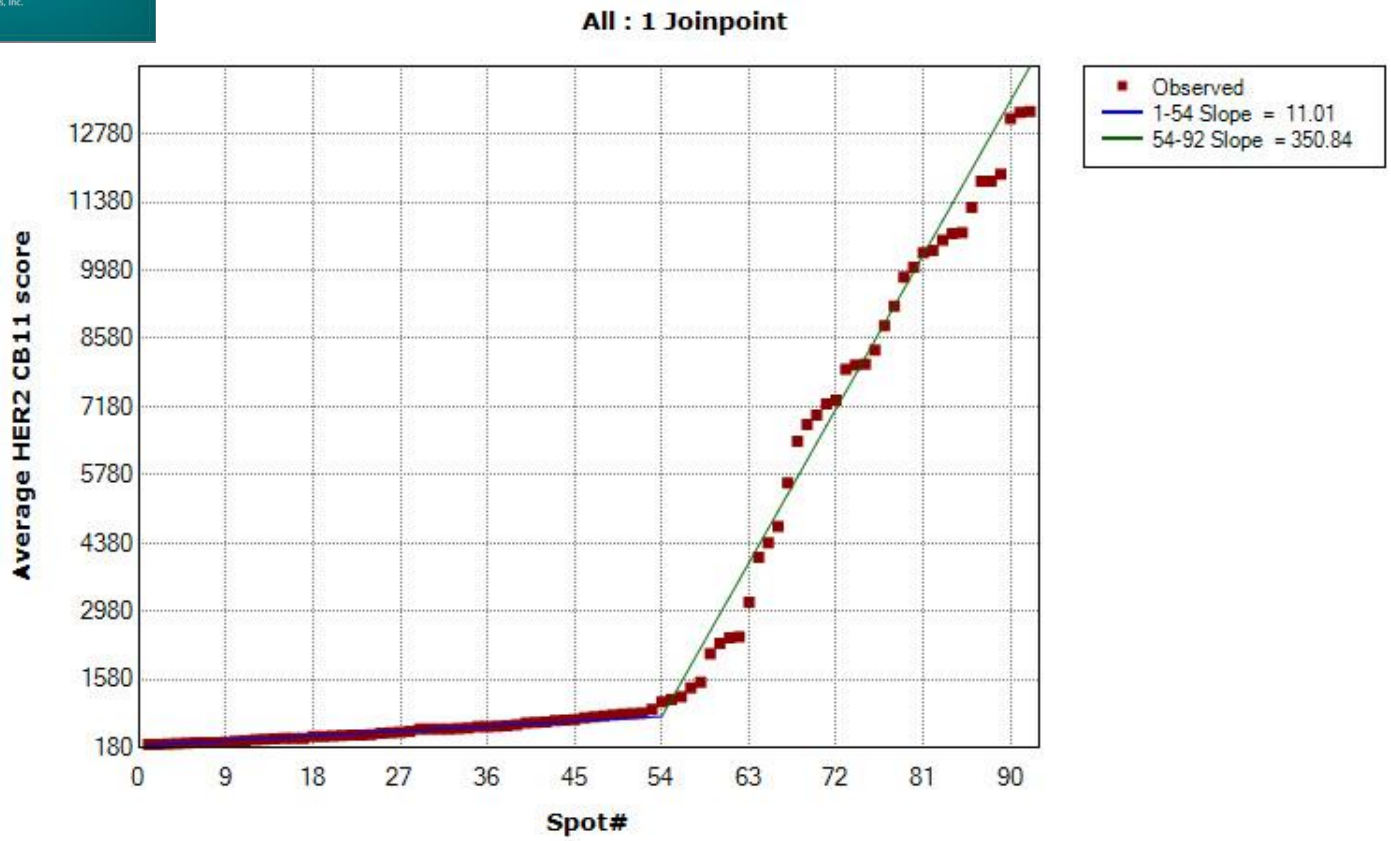
PRODUCED BY
Statistical Methodology and Applications Branch
Surveillance Research Program, Division of Cancer Control and Population Sciences
National Cancer Institute

Information Management Services, Inc.
Calverton, MD

HER2 CB11 analysis



Test For Number of Joinpoints

Test Number	Null Hypothesis	Alternate Hypothesis	Numerator Degrees of Freedom	Denominator Degrees of Freedom	Number of Permutations	P-Value	Significance Level~
#1	0 Joinpoint(s)	1 Joinpoint(s) *	2	88	4500	0.0002222	0.0500000

Final Selected Model 1 Joinpoint(s)

Daniel Carvajal

Using Joinpoint to Assess Ab Sensitivity and Specificity to Predict FISH amplification in YTMA 263

HER2 antibody	Joinpoint cut-point (AU)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
CB11	1299.3	94.12	85.48	78.05	96.36
A0485	1495.9	90.62	85.71	78.38	94.12
SP3	525.8	88.24	**98.41**	96.77	93.94
D8F12	767.6	90.91	**93.88**	90.91	93.88

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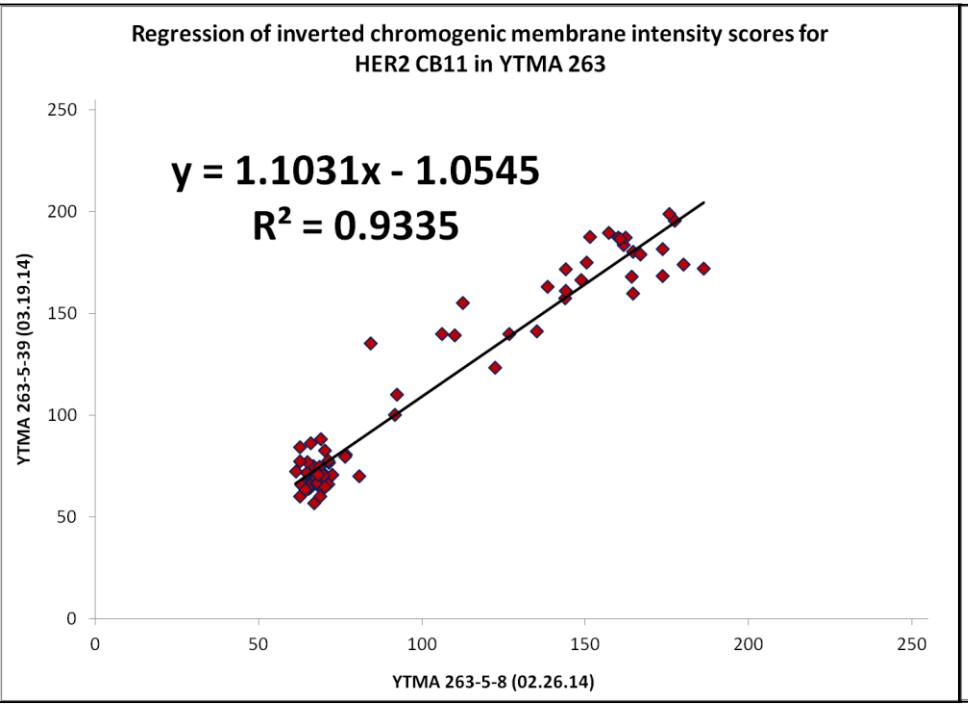
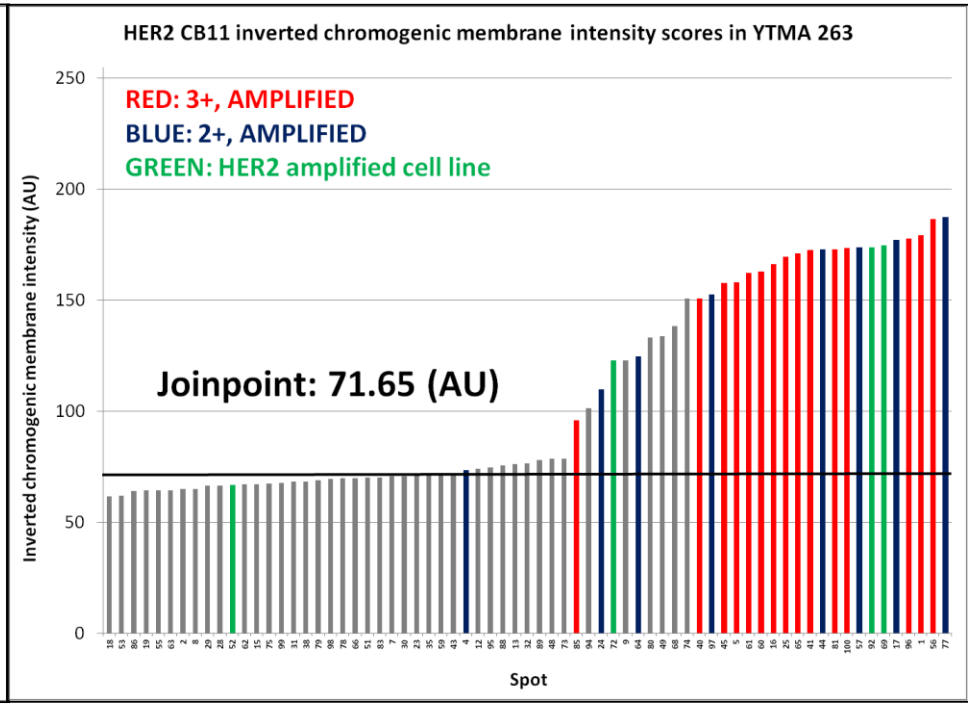
Using Spectrum/Aperio scores from Membrane/Total pixel count for HER2 CB11





Daniel Carvajal

Using Joinpoint to Assess Ab Sensitivity and Specificity to Predict FISH amplification in YTMA 263 (Chromogenic IHC)

HER2 antibody	Joinpoint cut-point (AU)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
CB11	71.65	96.3	64.29	63.41	96.43
SP3	75.75	89.66	82.61	76.47	92.68

Daniel Carvajal

Conclusions

- The binding site of the HER2 antibody effects the results of the HER2 assay
- Compared to FISH, the cytoplasmic domain is more sensitive for prediction of amplification, while the extracellular domain is more specific.
- This result is independent of detection method (QIF vs DAB)
- Prediction of response to therapy is more important than prediction of FISH amplification

HeCOG 10/05 Trial arms

- **Group A** epirubicin (110 mg/m^2) followed by paclitaxel (200 mg/m^2) CMF (cyclophosphamide; 840 mg/m^2, methotrexate; 57 mg/m^2 and fluorouracil; 840 mg/m^2) every 2 weeks.
- **Group B** epirubicin (110 mg/m^2) followed CMF every 2 weeks followed later by Docetaxel; 35 mg/m^2.
- **Group C** epirubicin (110 mg/m^2) followed by CMF followed later by Paclitaxel 80 mg/m^2.

- HER2 positive patients treated with **trastuzumab every 3 weeks for 1 year after the completion of chemotherapy. In these patients hormonal treatment with tamoxifen or anastrazole will be started following termination of treatment with trastuzumab.**

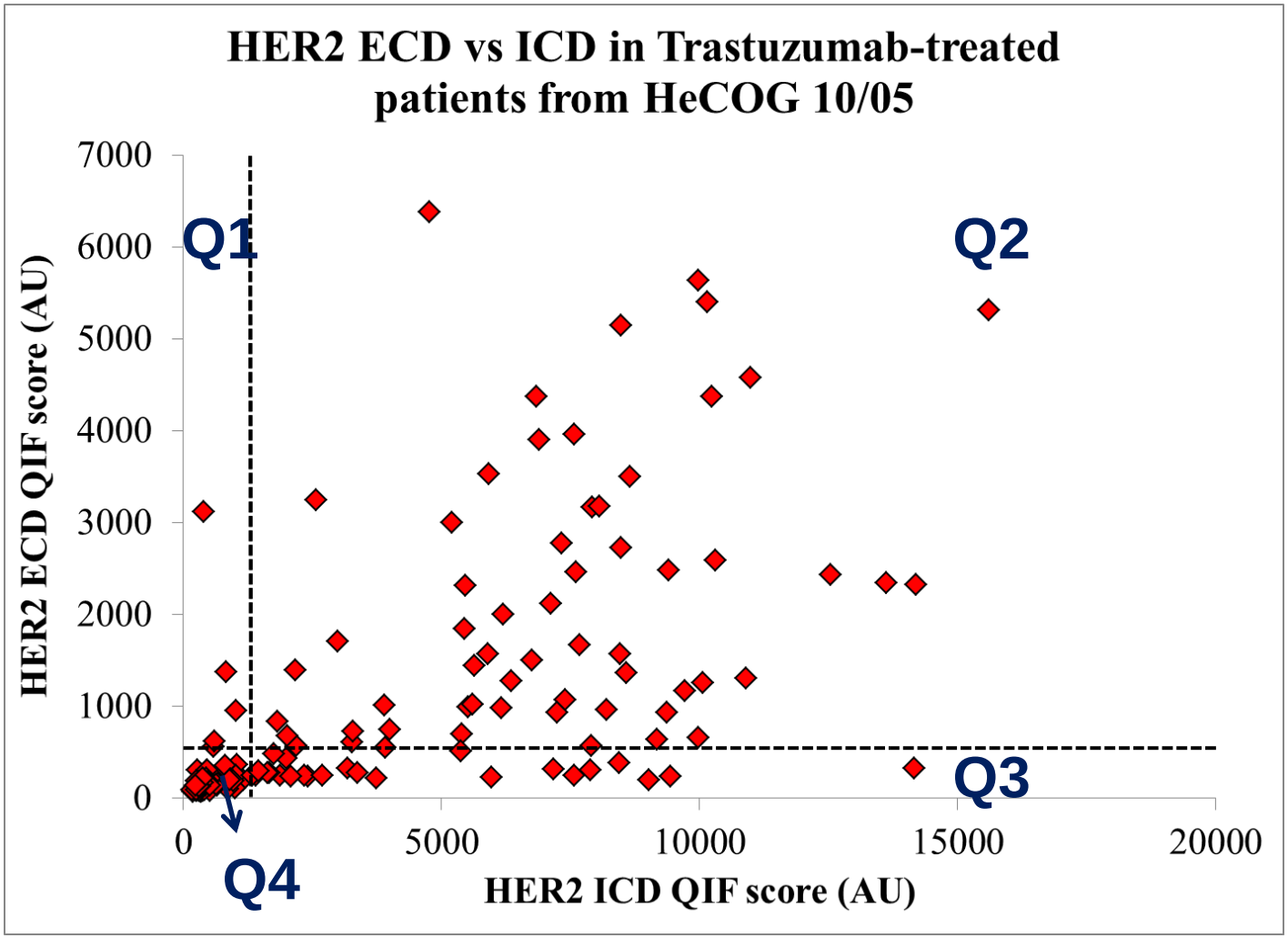
In summary, all patients received chemotherapy and the HER2 positive were further treated with sequential trastuzumab (like arm B of N9831).

HeCOG 10/05 patient subgroups

Table 2. Biological subgroups	
Subgroup	**N (%)**
ER(+)/PgR(+)/HER2(-)	366 (51.9)
ER(+)/PgR(+)/HER2(+)	90 (12.8)
ER(+)/PgR(-)/HER2(-)	66 (9.4)
ER(+)/PgR(-)/HER2(+)	23 (3.3)
ER(-)/PgR(-)/HER2(-) (TNBC)	68 (9.6)
ER(-)/PgR(-)/HER2(+)	69 (9.8)
ER(-)/PgR(+)/HER2(-)	15 (2.1)
ER(-)/PgR(+)/HER2(+)	7 (0.9)
Not determined	1 (0.1)

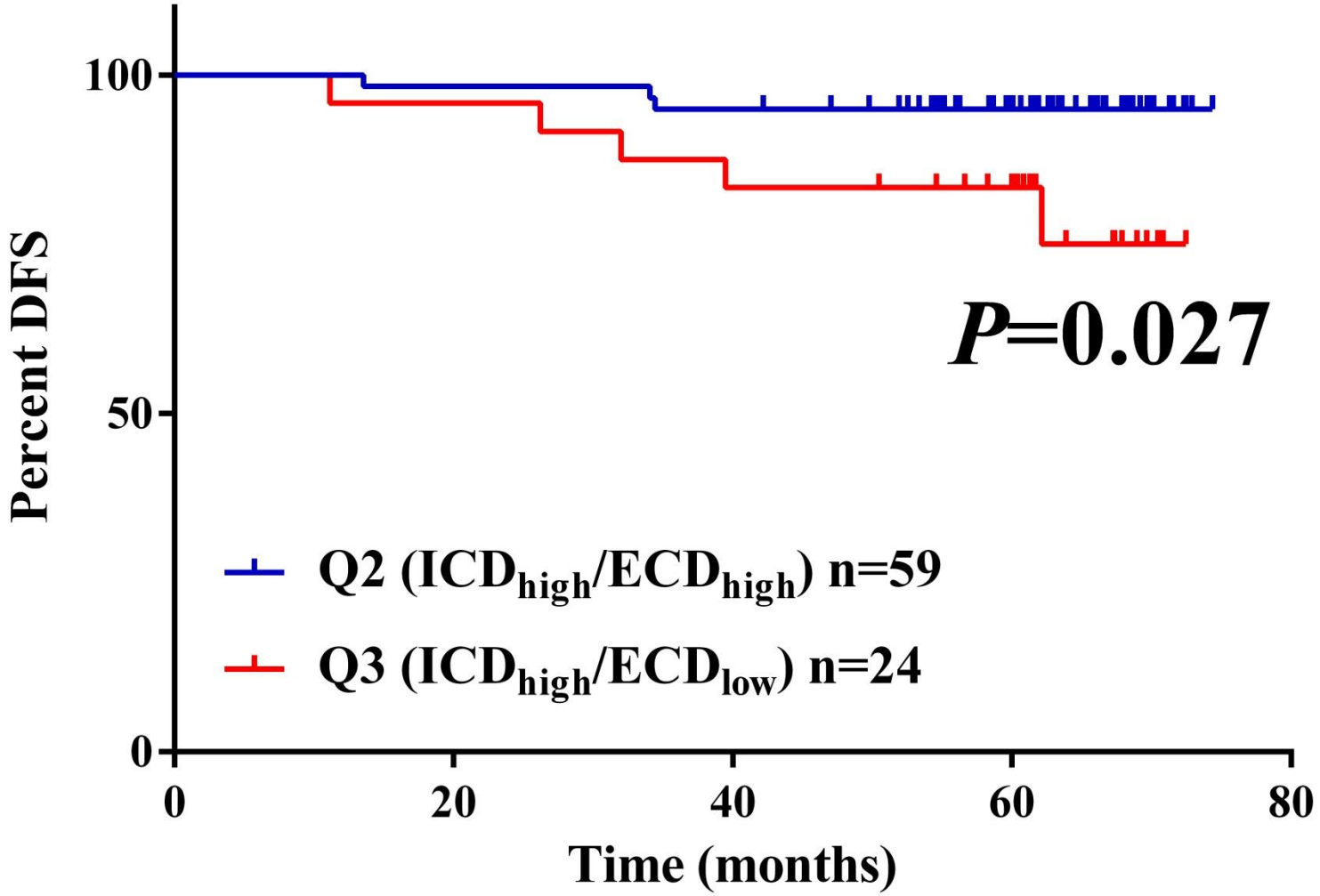
Table 3. Treatment subgroups	
Treatment group	**N (%)**
ER(+) with hormonotherapy	527 (96.7 of ER[+])
ER(+) without hormonotherapy	18 (3.3 of ER[+])
HER2(+) with trastuzumab	186 (98.4 of HER2[+])
HER2(+) without trastuzumab	3 (1.6 of HER2[+])
Hormonotherapy and trastuzumab	111 (15.7 of total)
ER(-) with hormonotherapy	0 (0)
HER2 (-) with trastuzumab	0 (0)

HER2 ICD/ECD protein expression by quadrants



- Cut-points obtained using YTMA 263 slides stained alongside HeCOG and analyzed using Joinpoint software.
- **Quadrants:**
- **Q1 (ICD_{low}/ECD_{high}):** 5/159 patients **(3.1%)**.
- **Q2 (ICD_{high}/ECD_{high}):** 59/159 patients **(37.1%)**.
- **Q3 (ICD_{high}/ECD_{low}):** 24/159 patients **(15.1%)**.
- **Q4 (ICD_{low}/ECD_{low}):** 71/159 patients **(44.7%)**.

Disease-free survival in HER2-positive, Trastuzumab-treated patients from HeCOG 10/05: Q2 vs Q3



Log rank *P*=0.027; HR=0.23 (95% C.I.: 0.037 to 0.815)

OXFORD

JNCI J Natl Cancer Inst (2015) 107(8): djv136

doi:10.1093/jnci/djv136
First published online May 19, 2015
Article

ARTICLE

Measurement of Domain-Specific HER2 (ERBB2) Expression May Classify Benefit From Trastuzumab in Breast Cancer

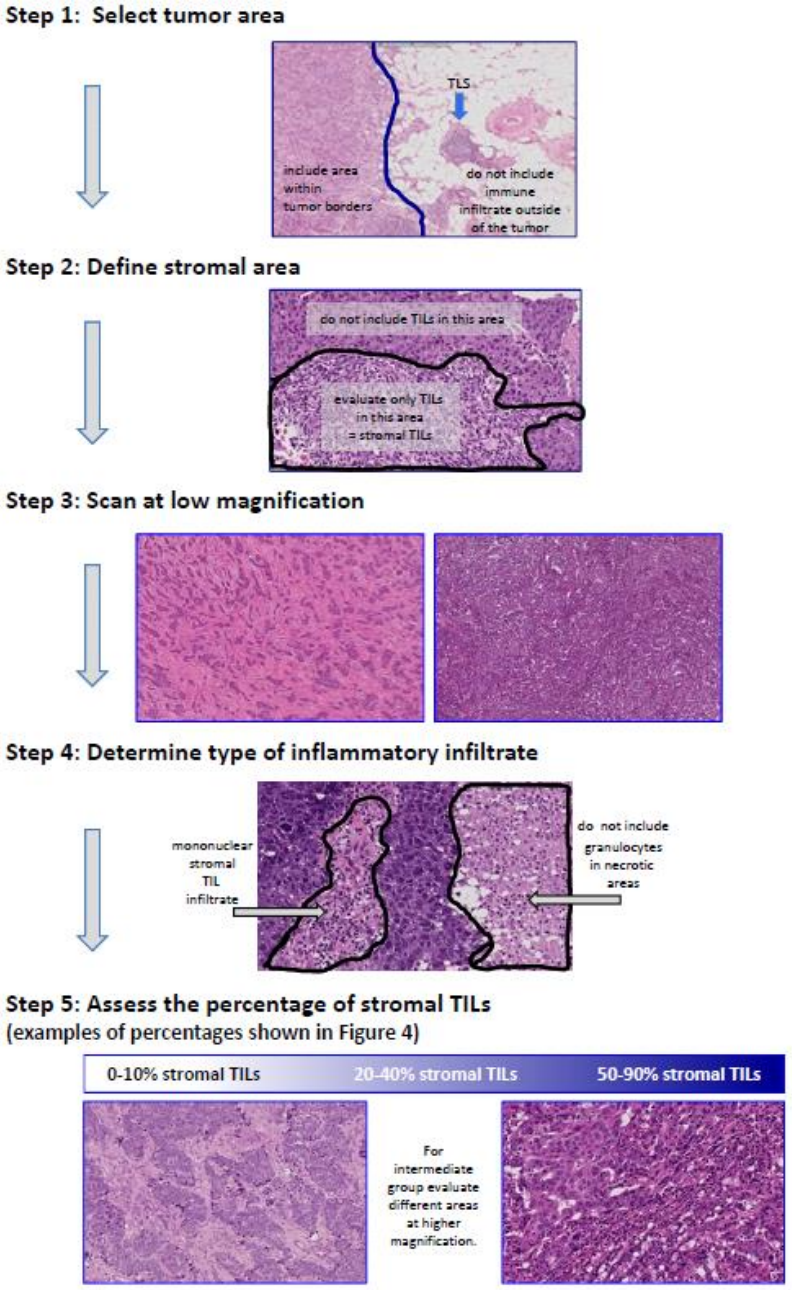
Daniel E. Carvajal-Hausdorf, Kurt A. Schalper, Lajos Pusztai, Amanda Psyrri, Konstantine T. Kalogeras, Vasiliki Kotoula, George Fountzilas, David L. Rimm

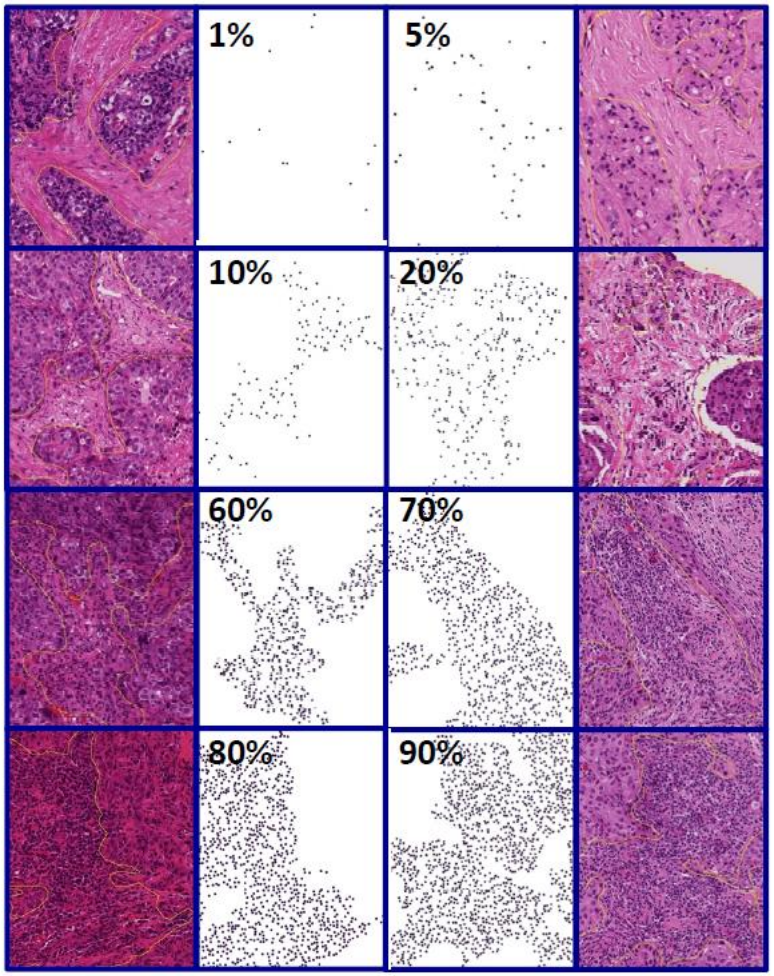
Outline for Presentation

- Domain Specific Assessment of HER2 in Breast Cancer
- Quantitative Multiplexed assessment of TILs
- Beyond QIF

TILs in Breast Cancer

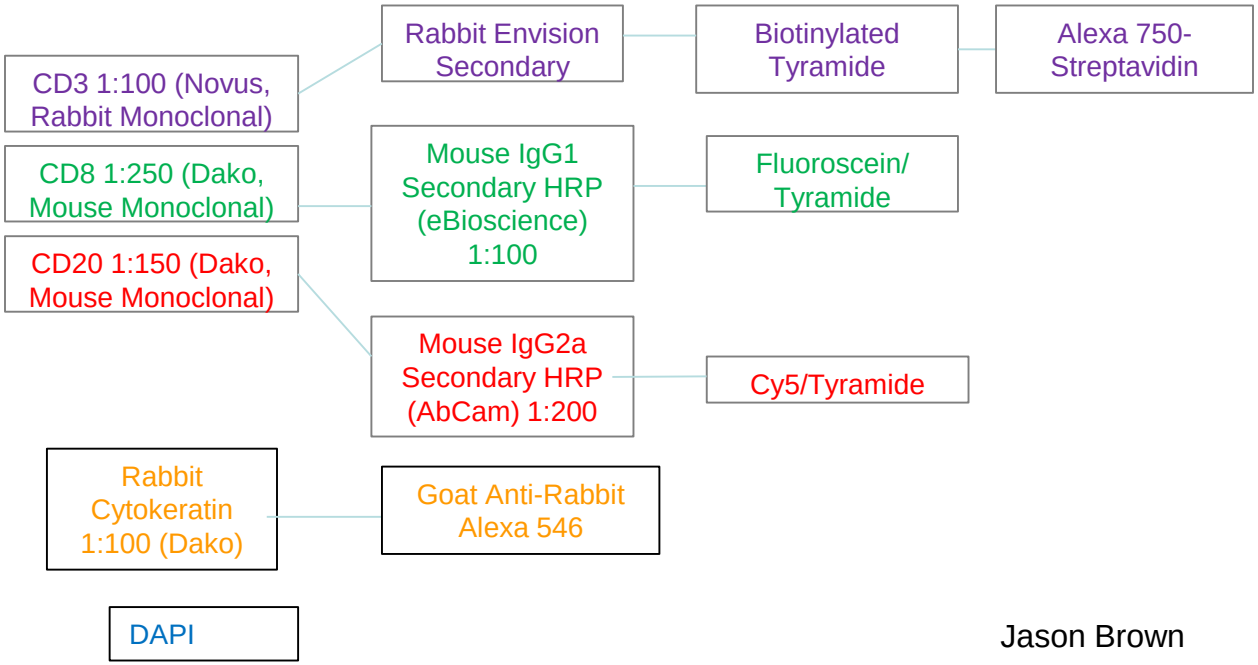
Salgado et al – Annals of Oncology





The Yale Immunoscore: Triple Multiplexing to measure CD3, 8 and 20 with quantitative immunofluorescence (AQUA)

- Quantitatively assess each marker in the stroma
- Validate on Tonsil compared to flow cytometry and then validate on breast, lung and colon cohorts
- Measure CD8 (or in the future CD4 or other markers) in the CD 3 compartment
- Could be done on whole slides or TMAs
- Schematic:



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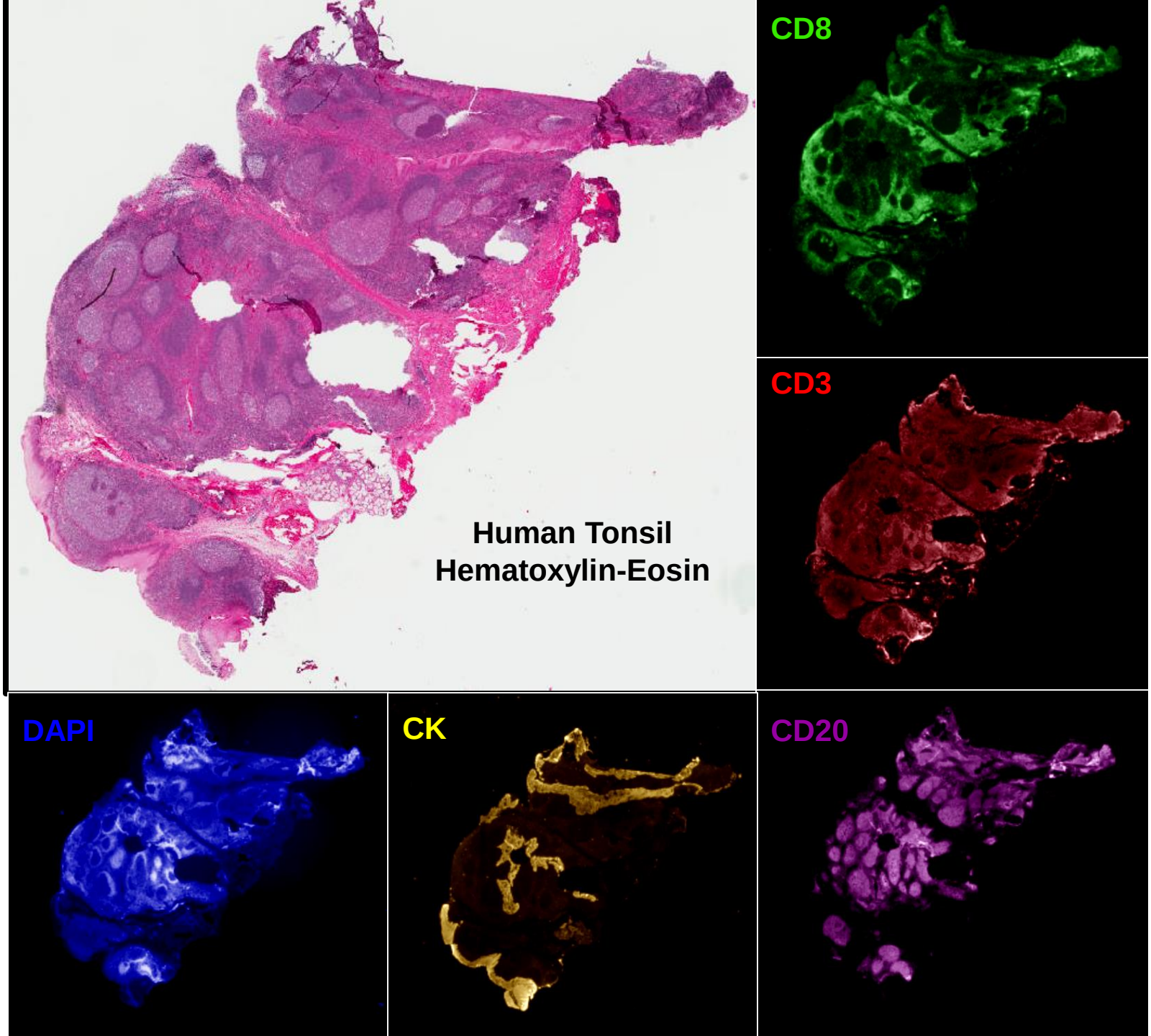
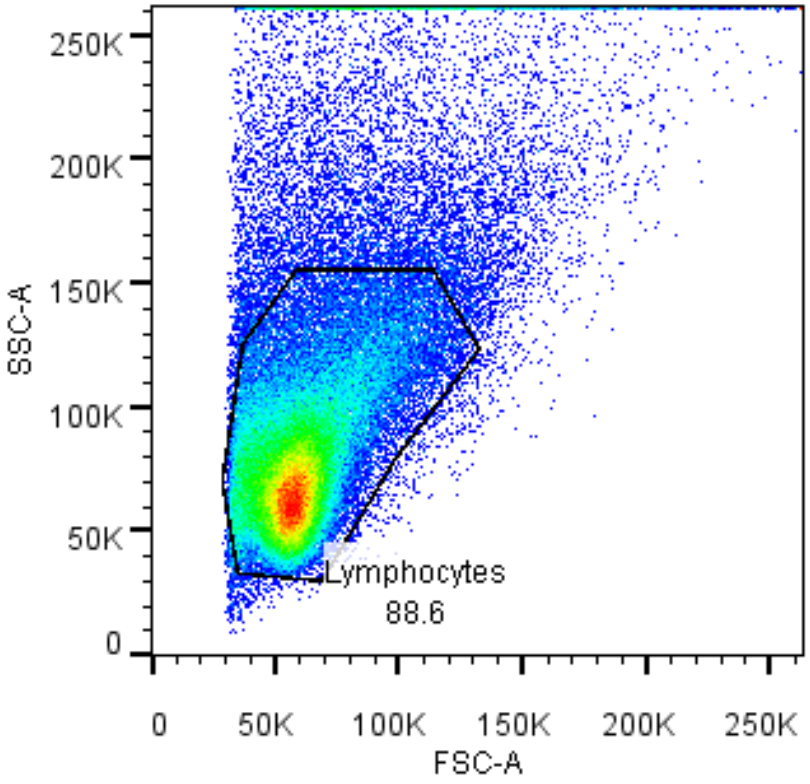
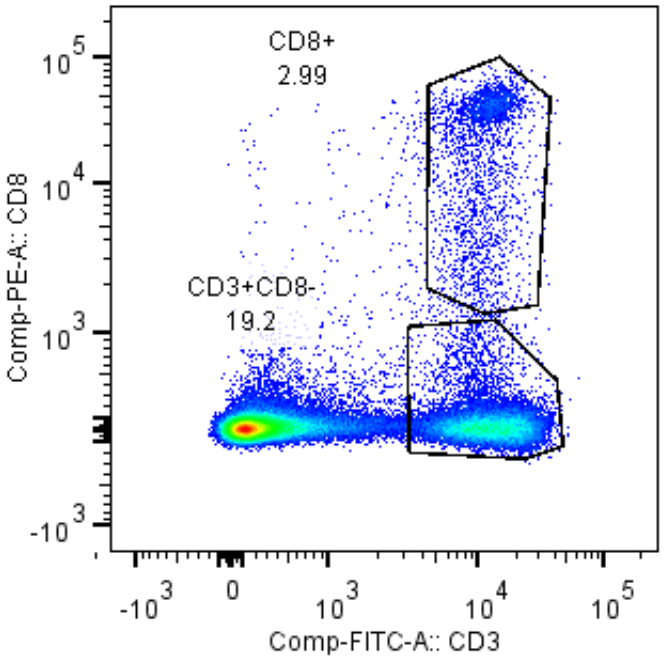
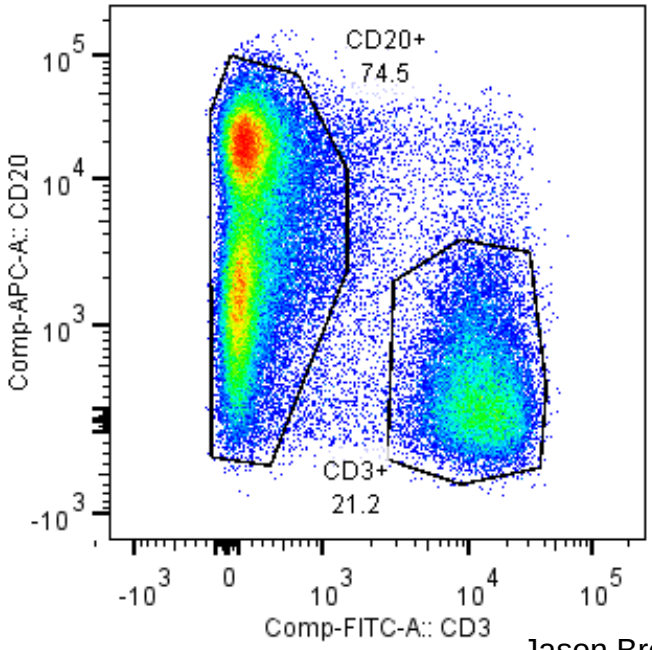

Human Tonsil
Hematoxylin-Eosin
CD8
CD3
DAPI
CK
CD20

Flow Cytometry Analysis on Tonsil Specimens







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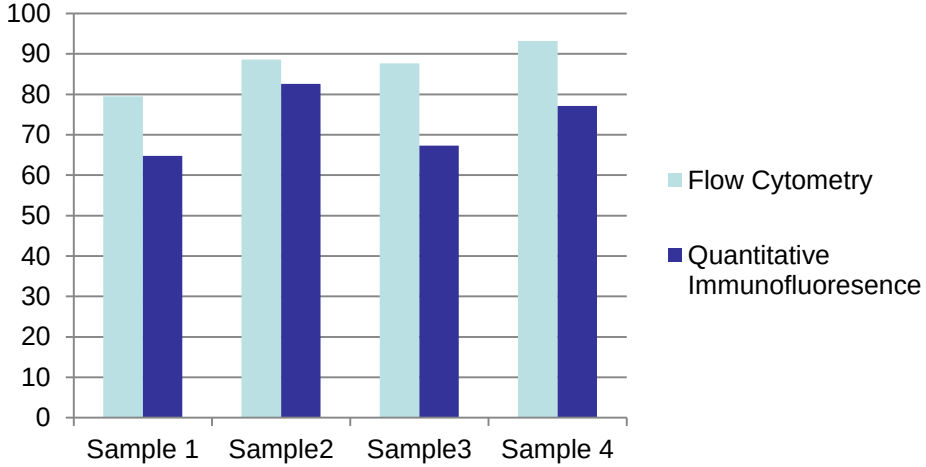
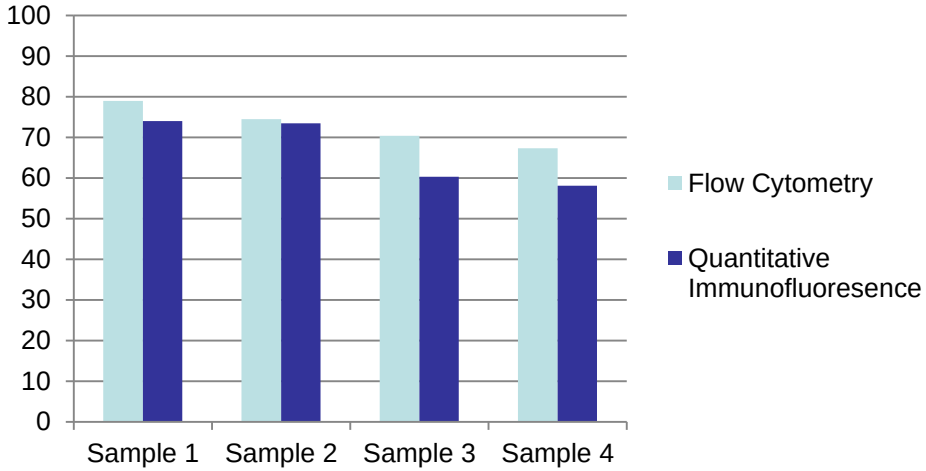
Measurement by QIF is very similar to measurement by flow cytometry of tonsil control tissue

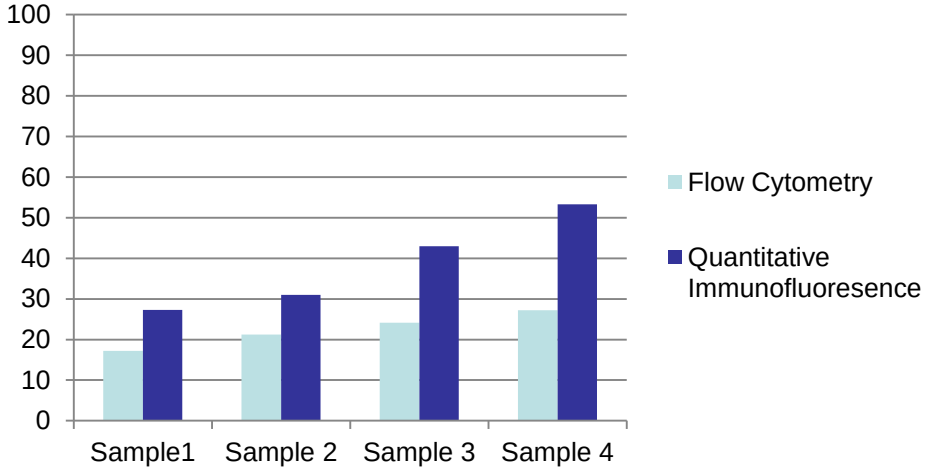
% Lymphocyte Comparison



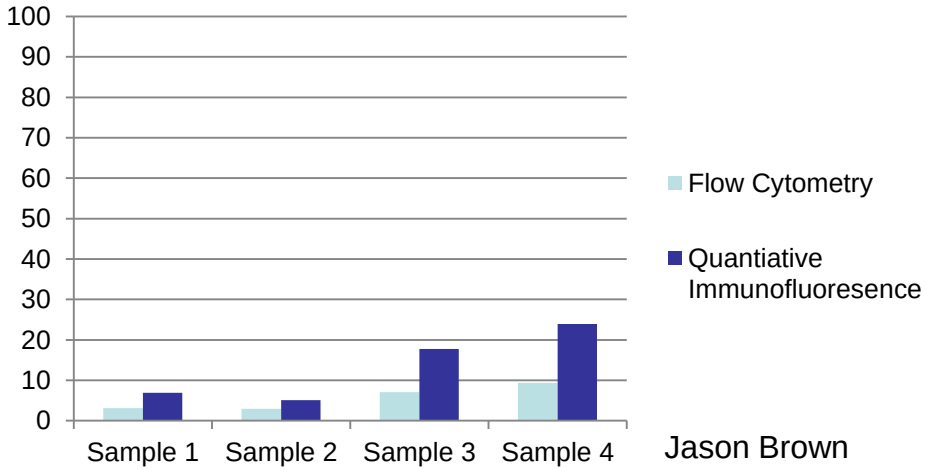
% CD20 Comparison



% CD3 Comparison



% CD8 Comparison



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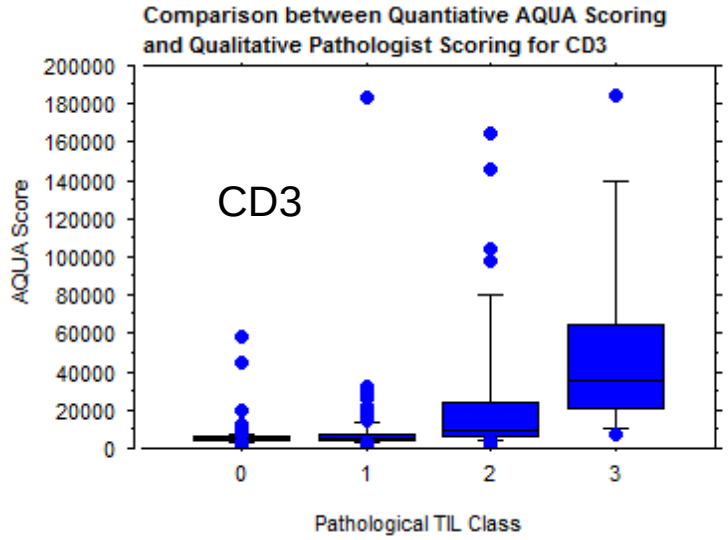
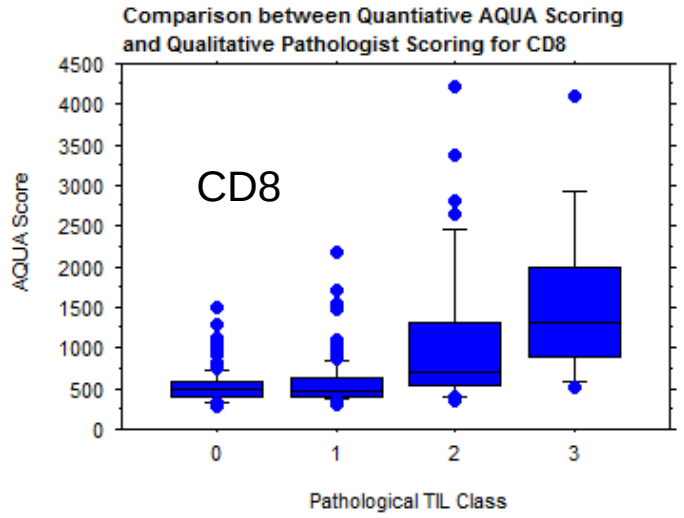
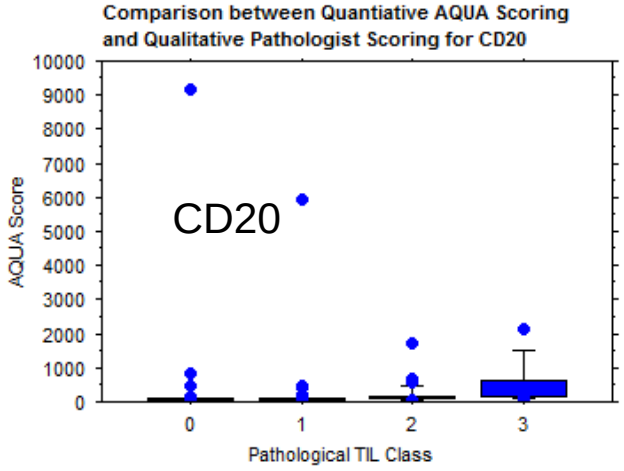
Comparison between AQUA and Pathologist on Yale Breast Cancer Cohort 2

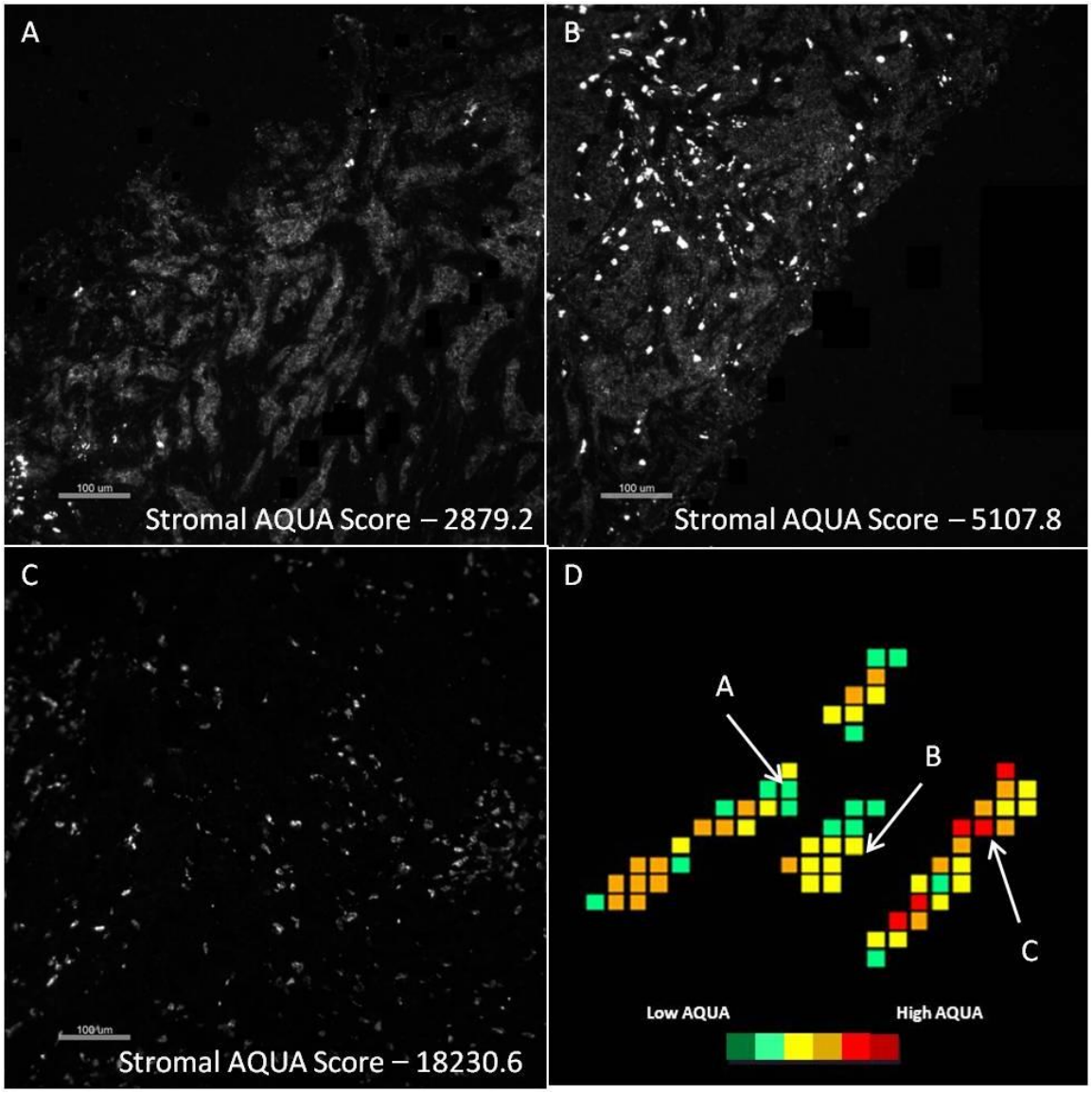






Jason Brown and Kurt Schalper

Measuring FOVs in whole sections of breast biopsies



Jason Brown and Kurt Schalper

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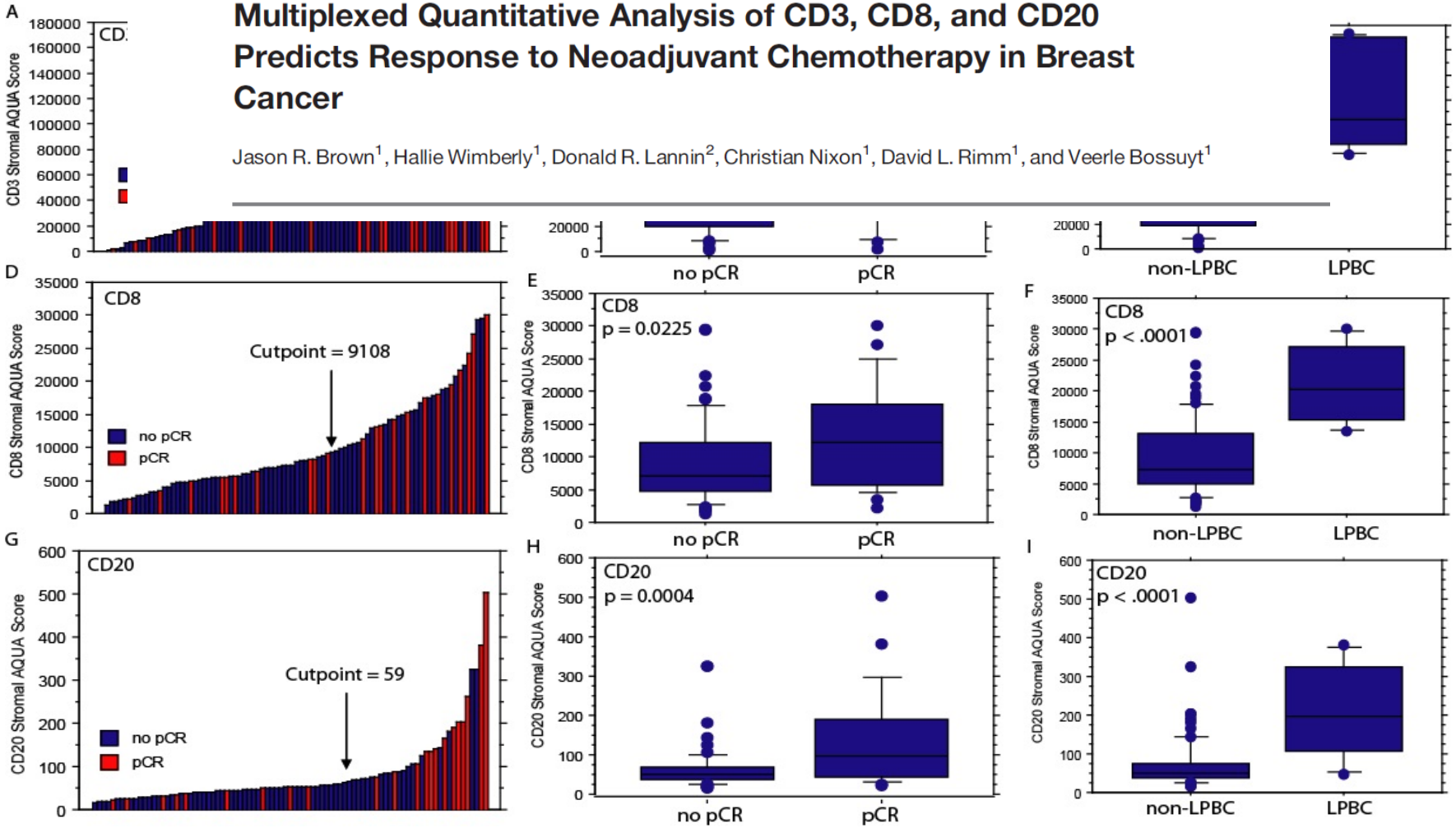
Prt

Personalized Medicine and Imaging

Clinical Cancer Research

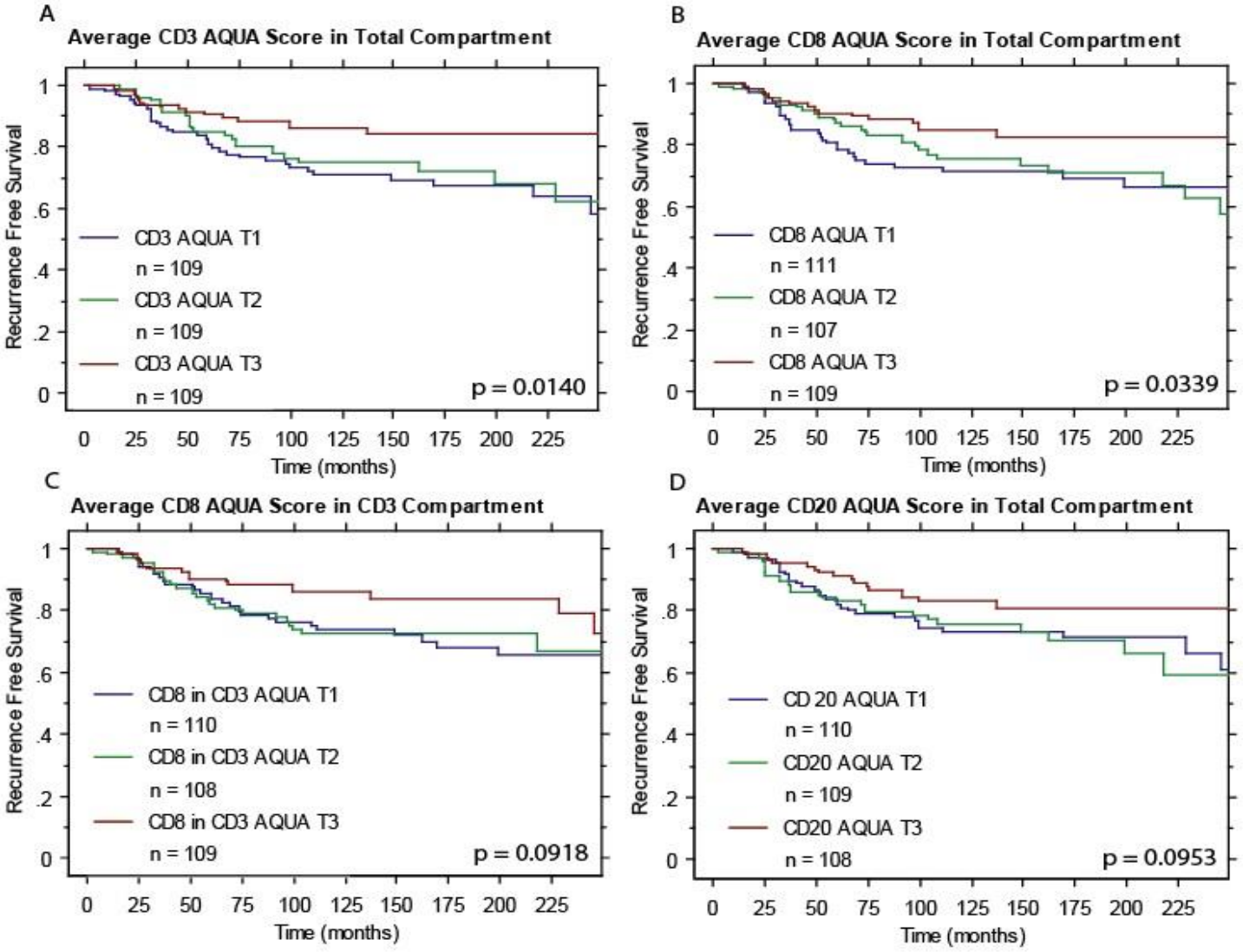
Multiplexed Quantitative Analysis of CD3, CD8, and CD20 Predicts Response to Neoadjuvant Chemotherapy in Breast Cancer

Jason R. Brown[1], Hallie Wimberly[1], Donald R. Lannin[2], Christian Nixon[1], David L. Rimm[1], and Veerle Bossuyt[1]

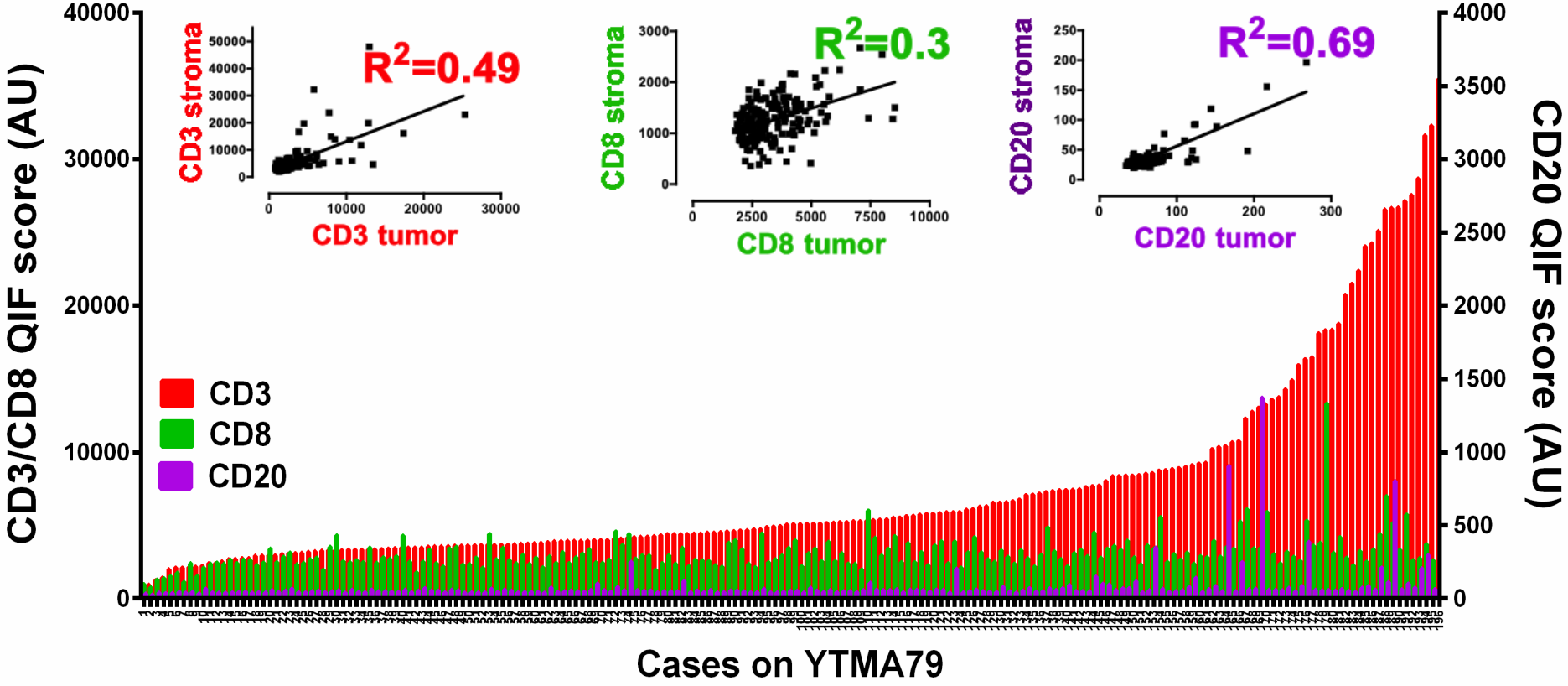


Jason Brown and Kurt Schalper

TILs show Prognostic Value in Breast Cancer



Distribution of TILs signal in NSCLC samples on YTMA79



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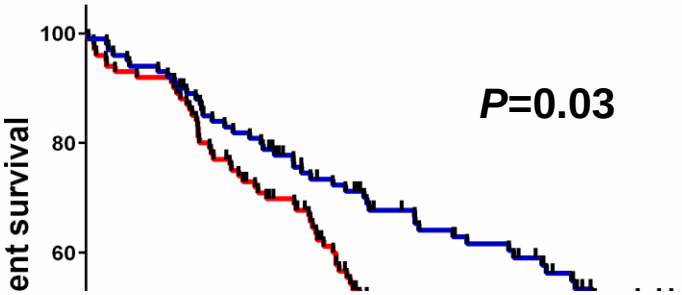
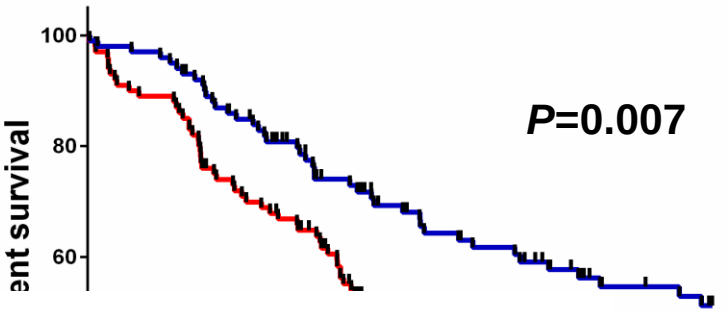
OS by CD3 Signal on YTMA79



OS by CD8 Signal on YTMA79



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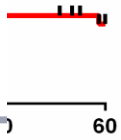
Article



4 27
8 23

TMA79

2

ARTICLE

Objective Measurement and Clinical Significance of TILs in Non–Small Cell Lung Cancer

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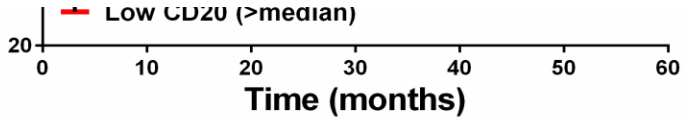

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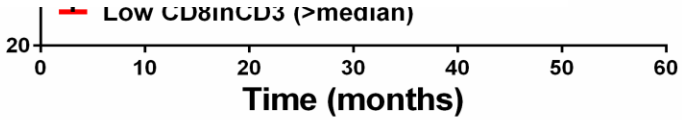
100 85 67 44 39 30 25



100 91 72 52 46 32 25
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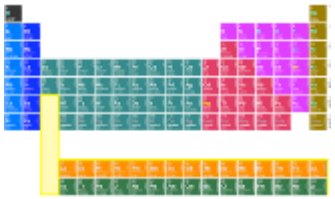
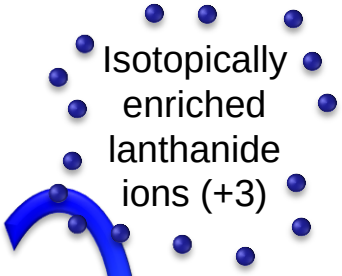
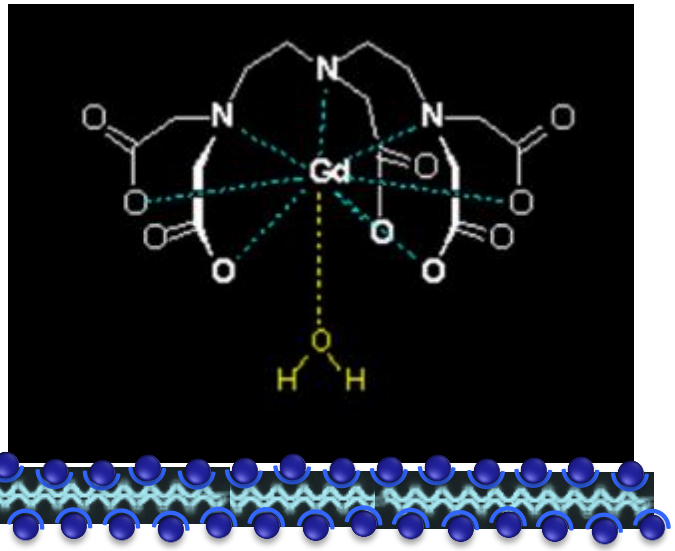
Outline for Presentation

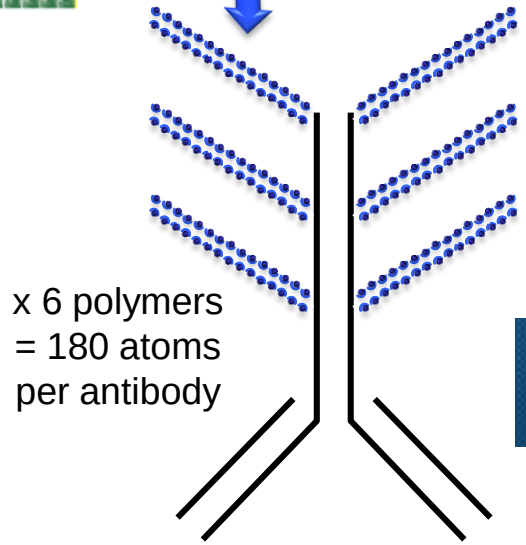
- Domain Specific Assessment of HER2 in Breast Cancer
- Quantitative Multiplexed assessment of TILs
- Beyond QIF

Labelling antibodies for CyTOF or MSIHC





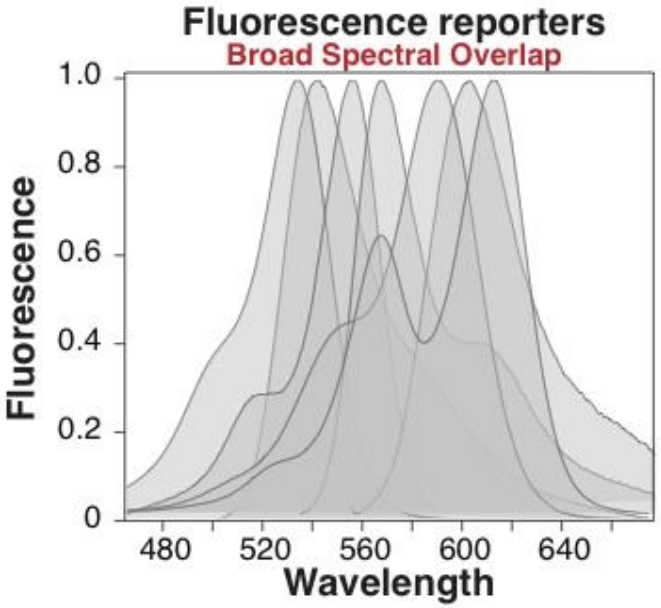


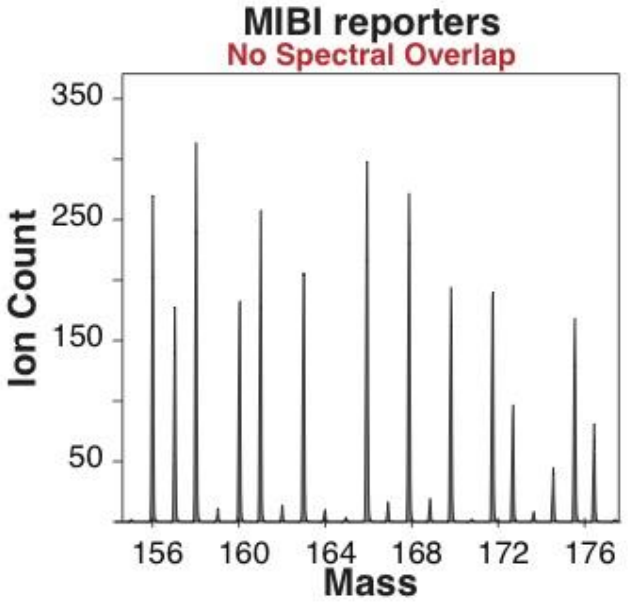


fluidigm

Secondary Ion Mass Spec compared to Fluorescence

Why is mass spectrometry better suited for multiplexing compared to fluorescence?





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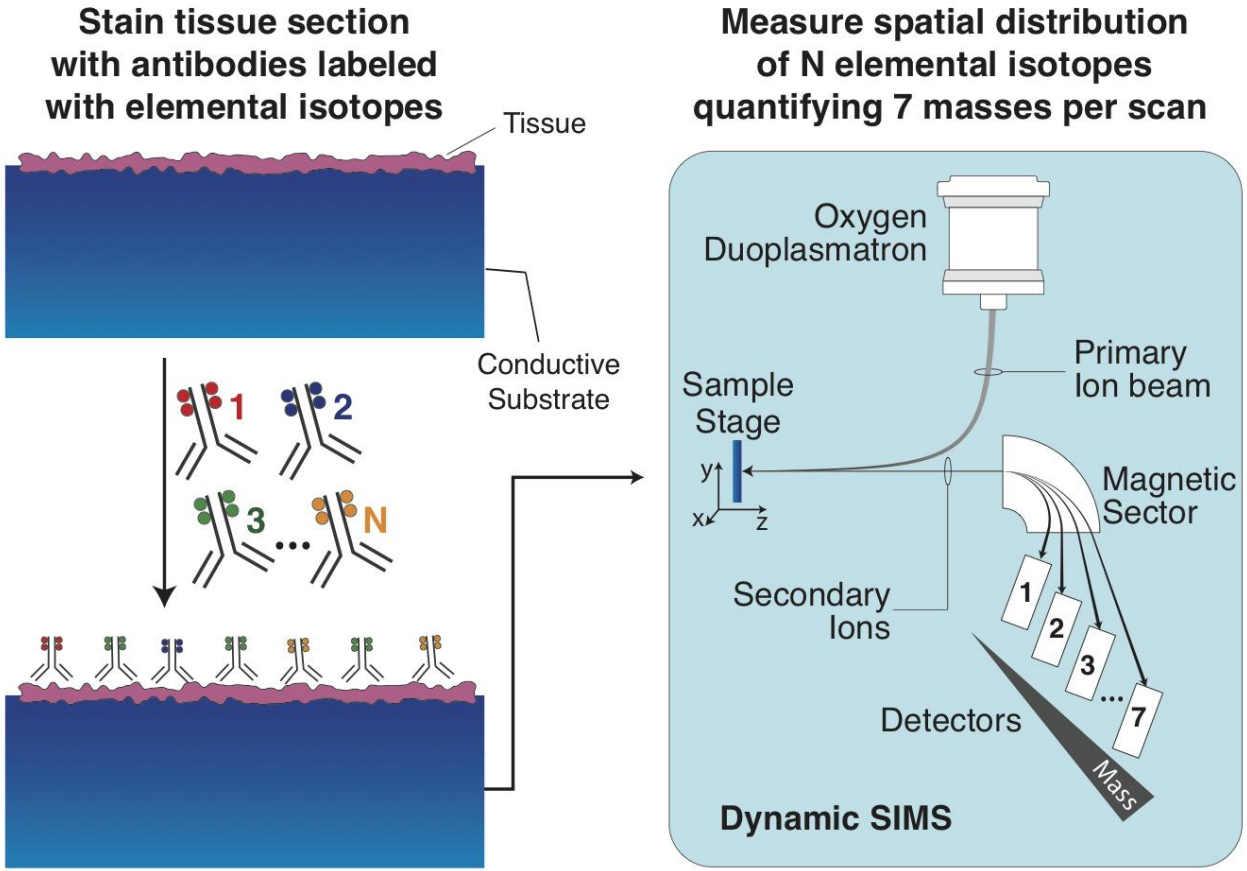
Multiplexed Ion Beam Imaging



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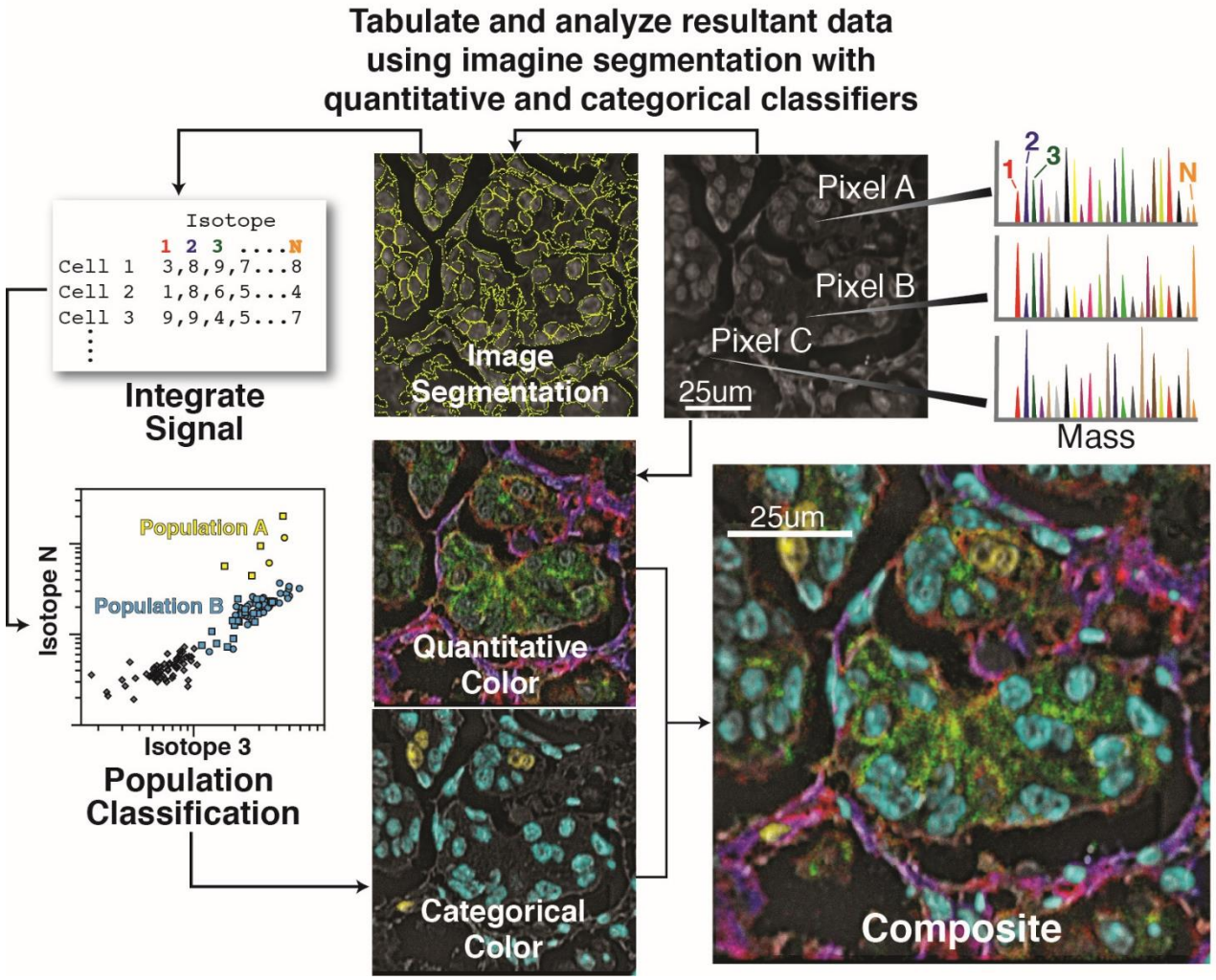
Multiplexed Ion Beam Imaging



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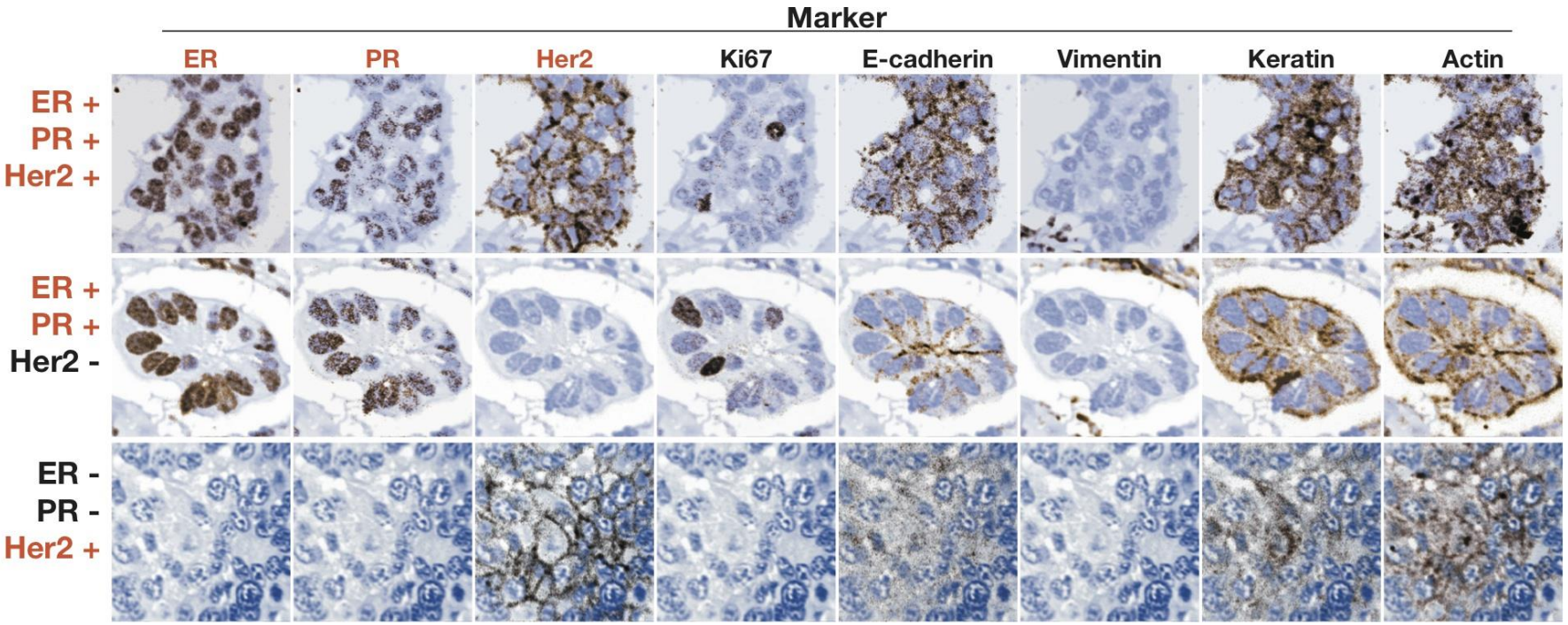
Multiplexed Ion Beam Imaging

10-color imaging of human breast tumors using MIBI



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TECHNICAL REPORTS

nature medicine

Multiplexed ion beam imaging of human breast tumors

Michael Angelo[1,2], Sean C Bendall[1], Rachel Finck[1], Matthew B Hale[1], Chuck Hitzman[3], Alexander D Borowsky[4], Richard M Levenson[4], John B Lowe[5], Scot D Liu[5], Shuchun Zhao[6], Yasodha Natkunam[6] & Garry P Nolan[1]

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NATURE METHODS | ADVANCE ONLINE PUBLICATION |

Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry

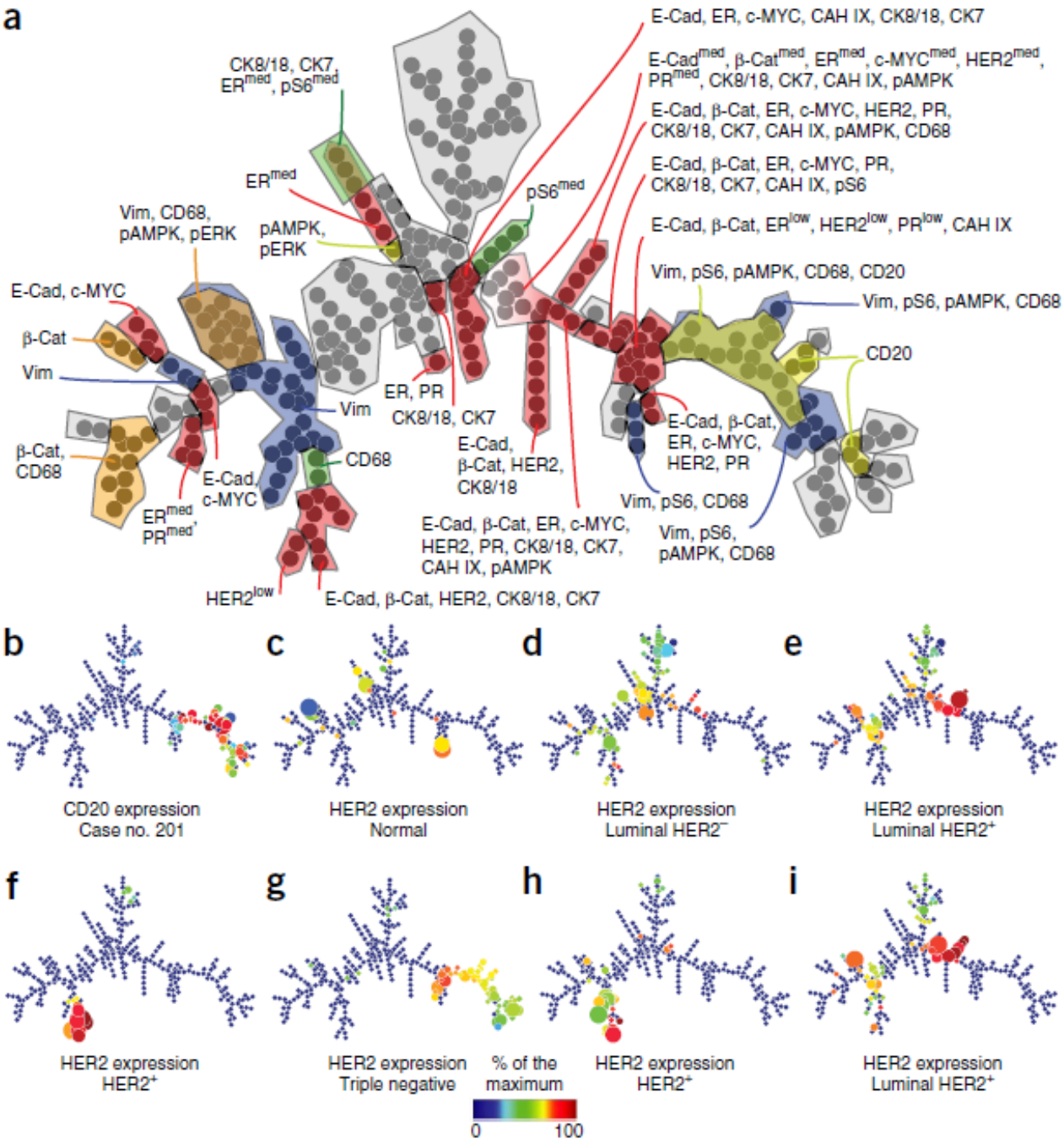
Charlotte Giesen[1,8], Hao A O Wang[2,3,8], Denis Schapiro[1,4], Nevena Zivanovic[1,5], Andrea Jacobs[1], Bodo Hattendorf[2], Peter J Schüffler[6], Daniel Grolimund[3], Joachim M Buhmann[6], Simone Brandt[7], Zsuzsanna Varga[7], Peter J Wild[7], Detlef Günther[2] & Bernd Bodenmiller[1]

March 2014

Highly multi
subcellular re

Charlotte Giesen[1,8], Hao A
Bodo Hattendorf[2], Peter J S
Zsuzsanna Varga[7], Peter J V

NATURE METHODS | ADVAN(



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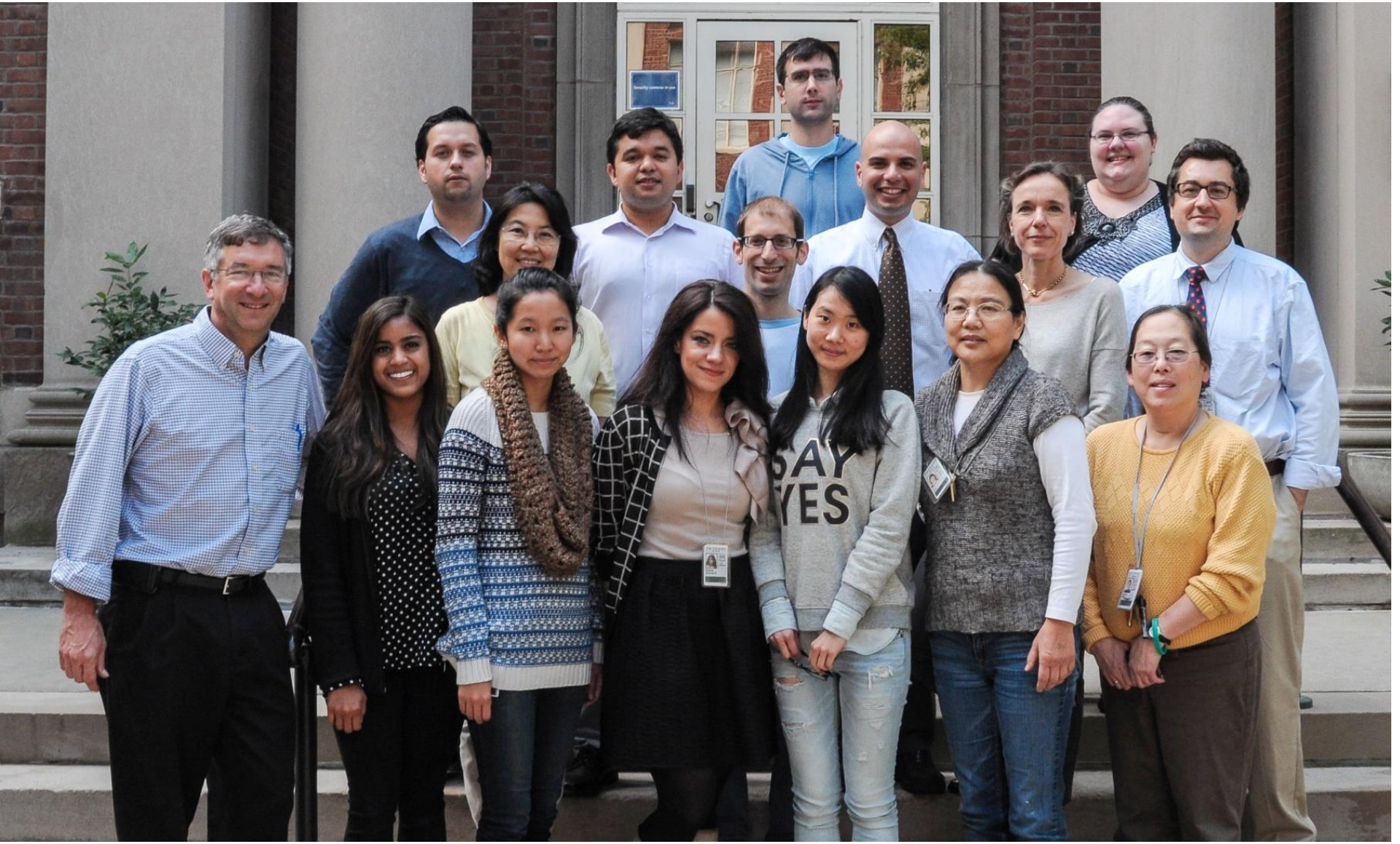

Rimm Lab, Fall 2014

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