WISCONSIN
UNIVERSITY OF WISCONSIN–MADISON

Pathology TRIP Laboratory
Wei Huang

VECTRA™ (AND INFORM) AND ITS APPLICATION

Background

- Traditionally, assessment of biomarker expression is done by manual estimation of staining intensity, and reported as
  - Binary: positive versus negative
  - 4-tiered: 0, 1+, 2+ and 3+
  - Semi-quantitatively: H-score
    - H-Score = (% at 0) x 0 + (% at 1+) x 1 + (% at 2+) x 2 + (% at 3+) x 3. Thus, this score produces a continuous variable that ranges from 0 to 300.

- New molecular techniques, such as serial analysis of gene expression, cDNA microarrays have enabled measurement of thousands of genes in a single experiment revealing hundreds of potential important candidate cancer genes.
- To validate the diagnostic, prognostic and therapeutic importance of the emerging potential cancer genes, hundreds of tissue samples from cancer patients at different stages need to be analyzed at protein level.

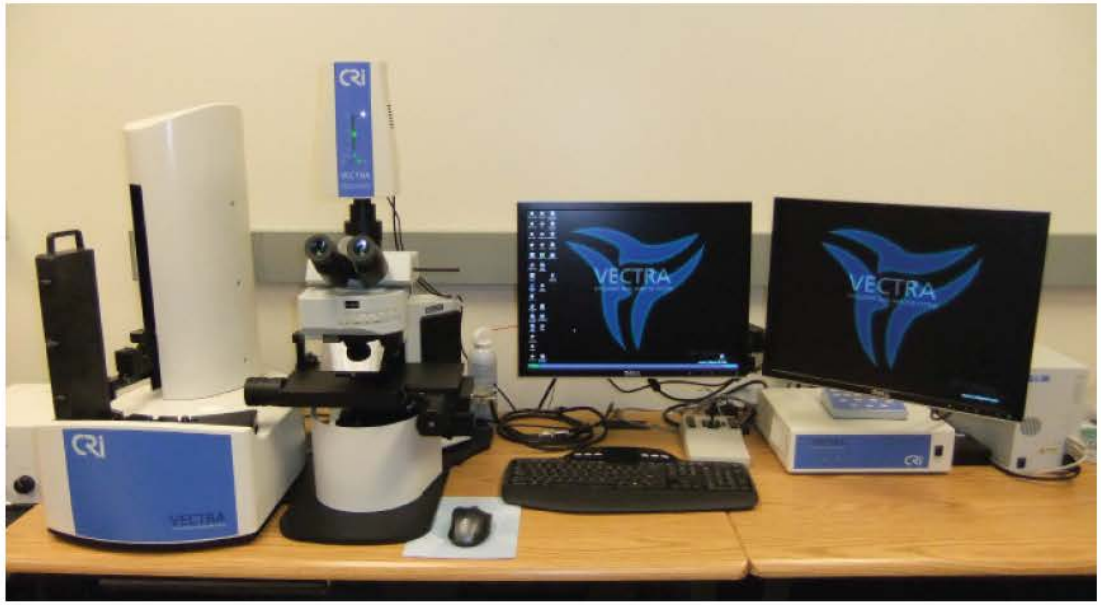
- The analysis is limited by
  - Speed of traditional (manual) pathology evaluation, creating a biomarker bottleneck on the microscope stage of academic pathologists.
  - Inherent shortcomings of manual quantitation.

The shortcomings of manual quantitation

- While the eye can be very sensitive to low light levels and can also “see” in very bright conditions, it does not discriminate light levels very well.
- The eye also has a limited ability to discriminate levels of illumination ranging from the lowest level to the highest level at any given sensitivity.
- With the inherent limitation of human perception of light comes intra- and interobserver variability (subjectivity) and limited precision.

- One recent advance is the development of Vectra™ technology (PerkinElmer).
- Vectra™ merges automated slide-scanner, multispectral imaging technology (Nuance Imaging Module and software) and unique pattern-recognition-based image analysis (inForm software) into a powerful system for biomarker discovery and clinical studies.

- Vectra™ is designed to measure protein expressions and morphometric characteristics in distinct tissue regions of interest on TMA or on whole section slides.
- Sections can be labeled with either immunofluorescent (IF) or IHC stains, or with stains such as H&E and trichrome.
- Up to 200 whole section slides can be scanned in a single run.



CONTACT US

Email: trip@pathology.wisc.edu

http://www.pathology.wisc.edu/research/trip

- With IF or IHC, the expression levels of single or multiple proteins or molecular markers (mRNA or DNA) can be measured on a per-tissue, per-cell, and per-cellullar compartment (eg. nuclear, cytoplasmic or membraneous) basis, even if those signals are spectrally similar, spatially overlapping or obscured by autofluorescence.

- The number or sizes of tissue structures of interest (ducts, glomeruli, etc.) can be counted or measured.
- Areas of different tissue types of interest (epithelium, stroma, etc.) can be measured.
- Cells of interest (CD4+, CD8+, etc.) can be counted.

- A scanning protocol will be created based on the TMA core number and size or tissue morphology.
- Nuance multispectral image cubes (8-bit or 12-bit) can be acquired with a 20x objective lens (0.5 micron/pixel) and using a full CCD frame for analysis.
  - As the number of bits of resolution increases, the number of gray levels also increases.
  - With a digital electronic sensor system, any sensor that has 8-bit resolution can detect 256 levels of gray. A12-bits resolution yields 4096 levels of gray.
- Digital electronic sensors are linear in response to light levels.

- Nuance FX software is used to build the spectral libraries.
- Each chromogen/dye has its unique spectral characteristics (curve), which is the basis for building the spectral libraries.

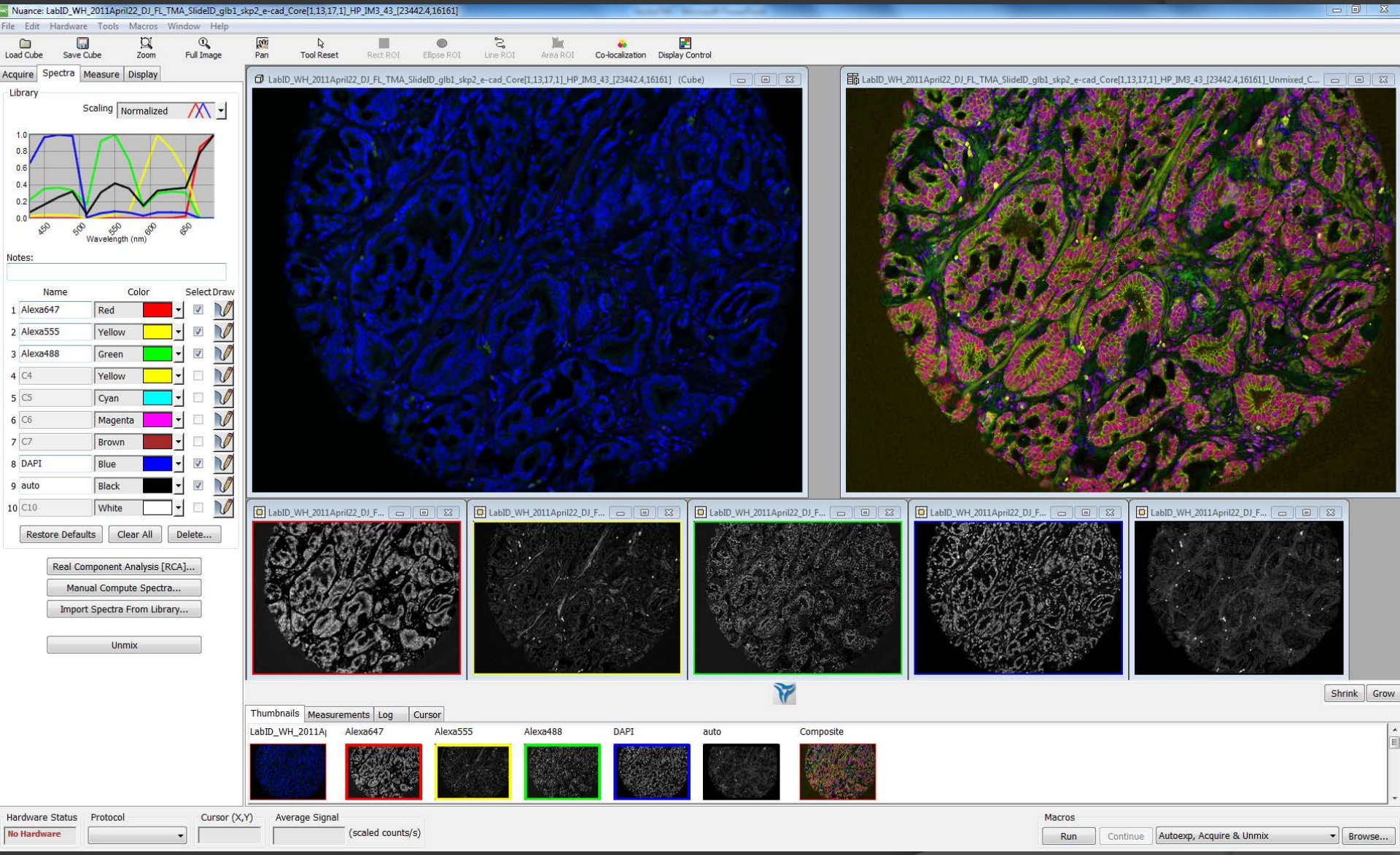
- Nuance FX operates on or off the microscope in the 420-720 nm range.
- In theory: 14 ideal chromogens/dyes with peak spectral difference ≥ 20 nm can be stained on a single slide and unmixed for quantitation.
- In reality: 10 is the maximum number may be achieved with the current software.

Bright Field IHC vs. Dark Field IF

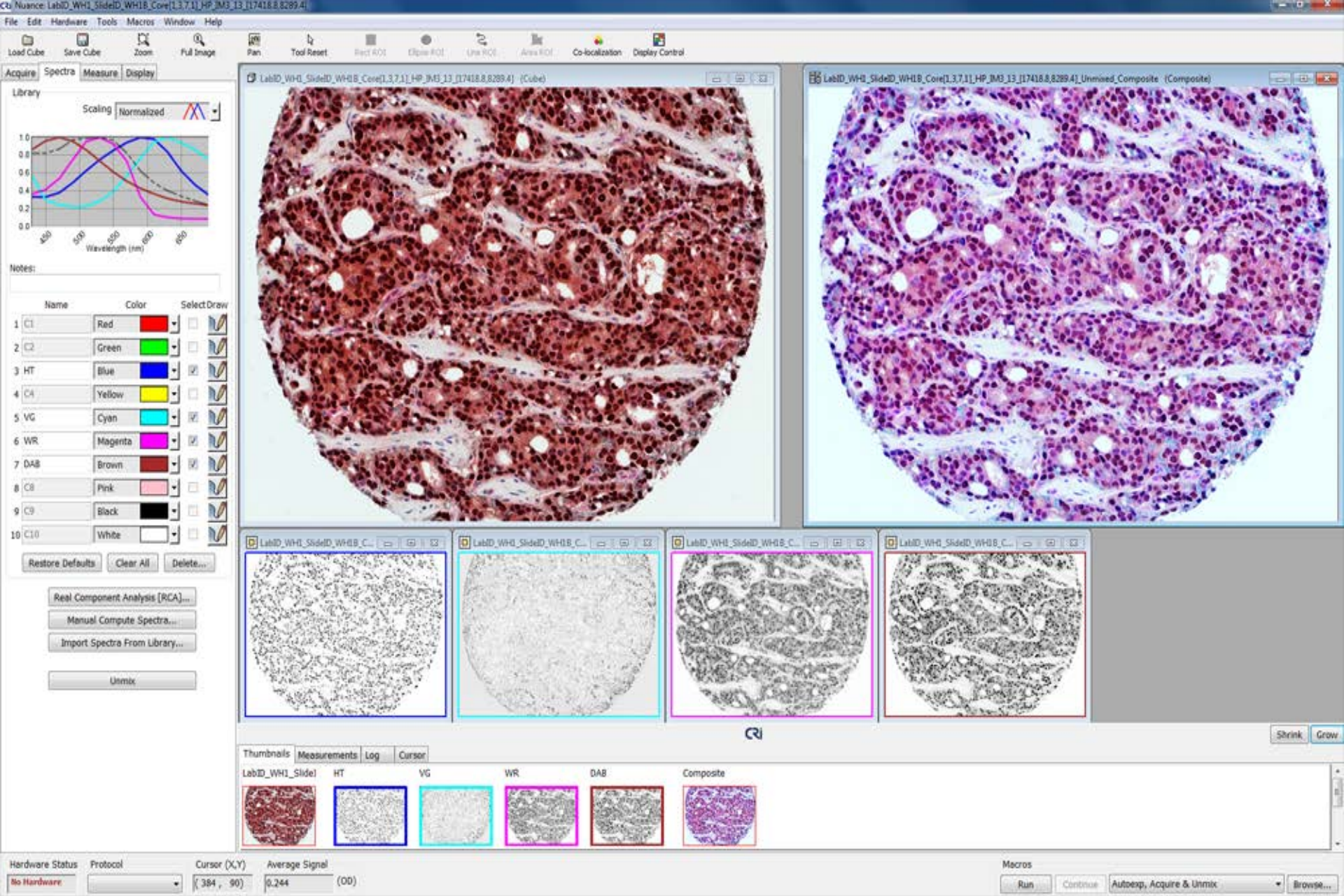
- Nuance multispectral imaging technology enables us to study multiple markers simultaneously with both BF-IHC and IF.
- Each assay has its pros and cons.

A comparison of BF-IHC and IF

	BF-IHC	IF
Maximal markers (tentative)	4-5	6-10
Spectral library	Chromogen-specific	Dye- and tissue-specific
Scanning	easy	Longer time, requires careful determination of optimal exposure times for each channel
Mask antibody	optional	required
Light liableness	minimal	Liable, dye-dependent



A spectral library – 4 fluorephores (Alexa Fluors and DAPI)

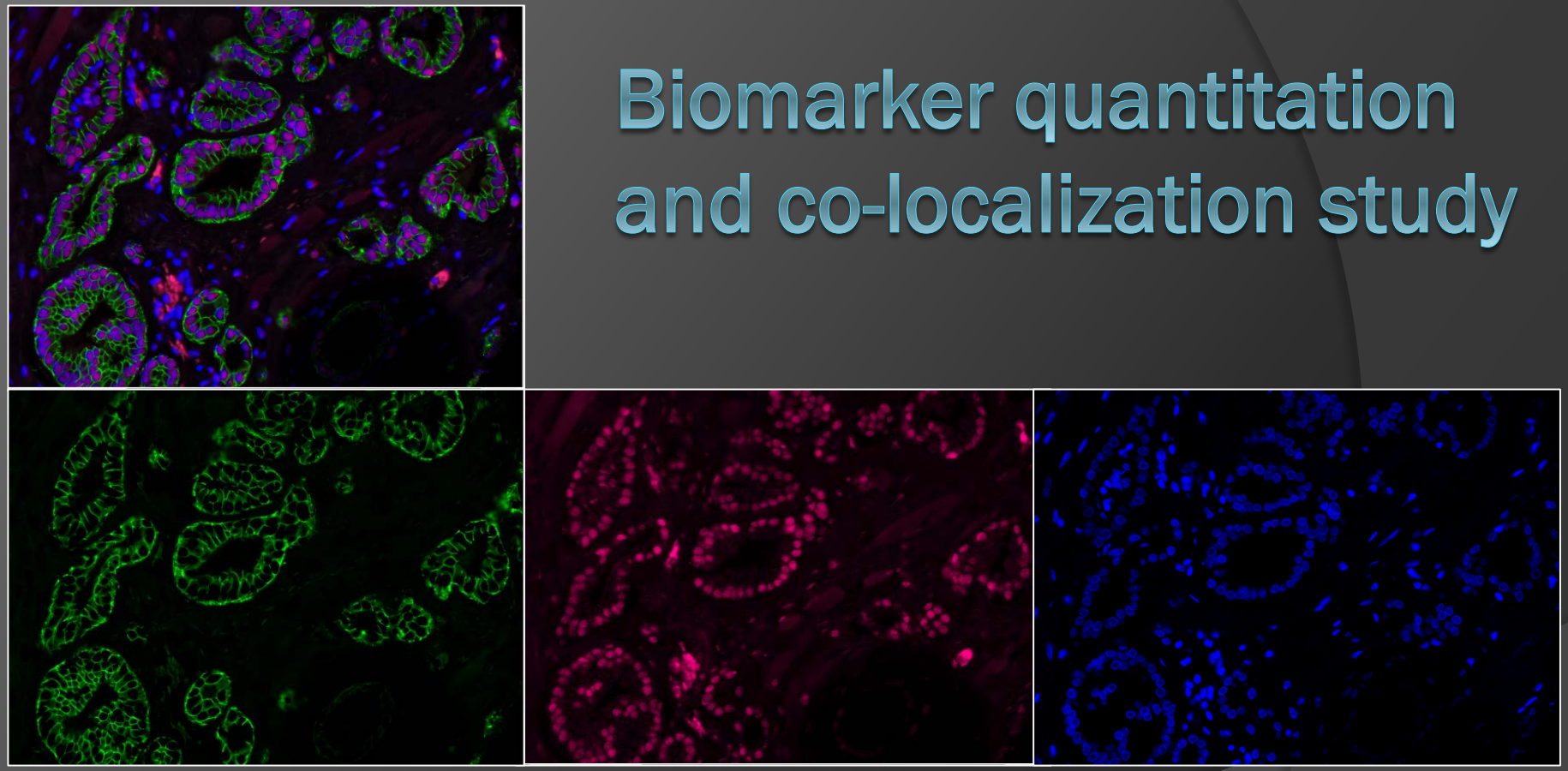


A spectral library - 4 chromogens

Common Applications

- Biomarker quantitation
  - continuous data (mean optical density or mean counts per pixel in the tissue/cell segment of interest in each image)
  - categorical (scoring) data
    - Two-bin: positive, negative
    - Four-bin: 0, 1+, 2+, 3+
    - Ten-bin: 0, 1+, 2+…10+
    - Fifty-bin: 0, 1+, 2+…50+
- Biomarker co-localization analysis
- Tissue segmentation and measurement
- Cell/structure counting (tissue cytometry)

Biomarker quantitation and co-localization study



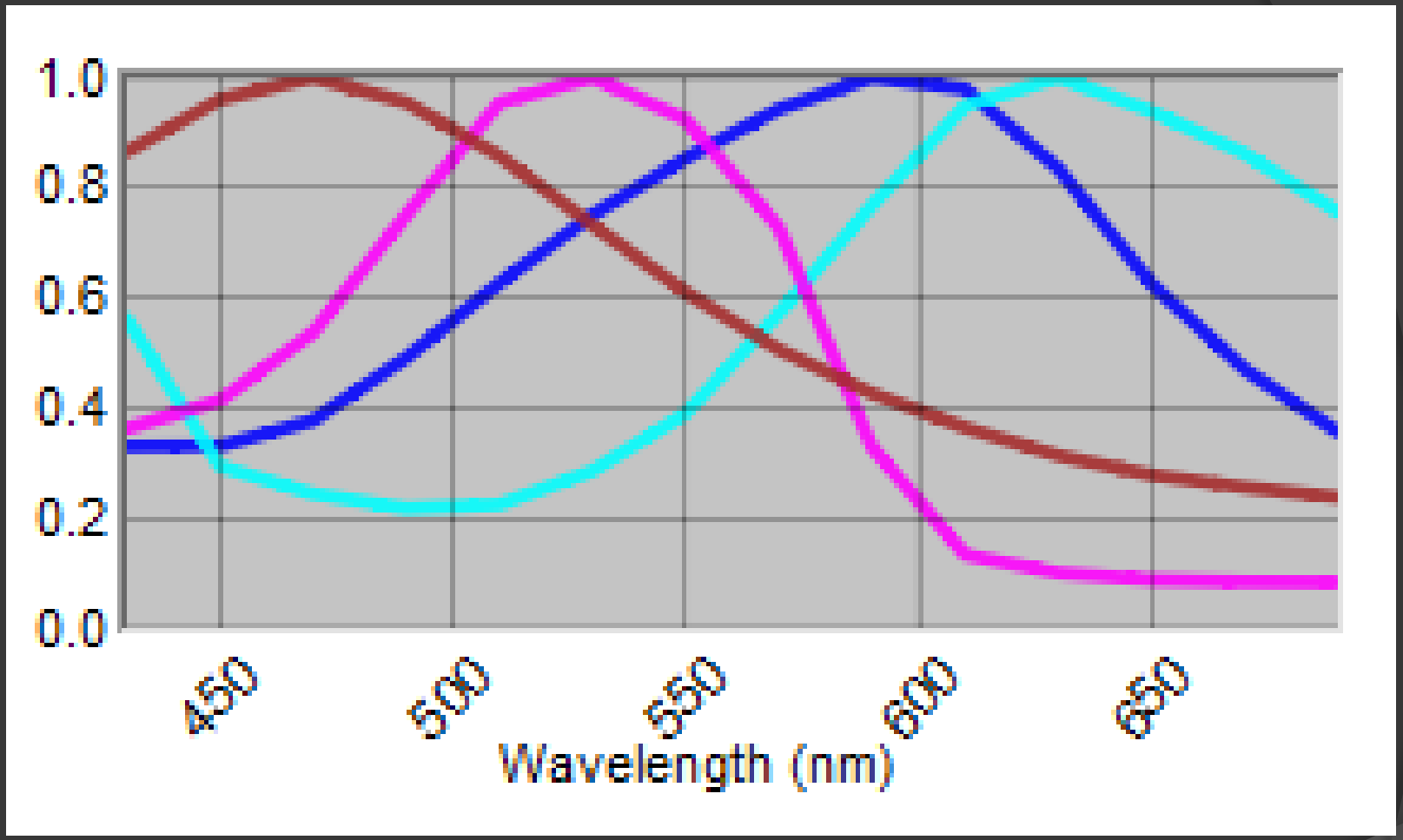
Example 1

- Validate Vectra system with multiplexed IHC assay using E-cadherin and AMACR .
- Study AR and ERG expression levels and co-localization.
- Study AR expression levels in ERG+ and ERG- prostate cancer tissues.

Study Cohorts and TMA Information			
TMA (cohort)	Disease/Recurrence Status (#core)	Gleason Score (mean)	Mean age (y)
pTMA (cohort 1)	BPH (48)		67.8
	BPT (96)		62.8
	HGPIN (50)		
	PCa_local (86)	6-9 (6.9)	
	PCa_aggr (60)	7-9 (7.9)	
	Met (44)		
oTMA (cohort 2)	None (250)	5-9 (6.8)	60
	Biochemical (76)	5-9 (7.4)	
	Cancer (40)	5-9 (7.6)	
	BPT (96)		

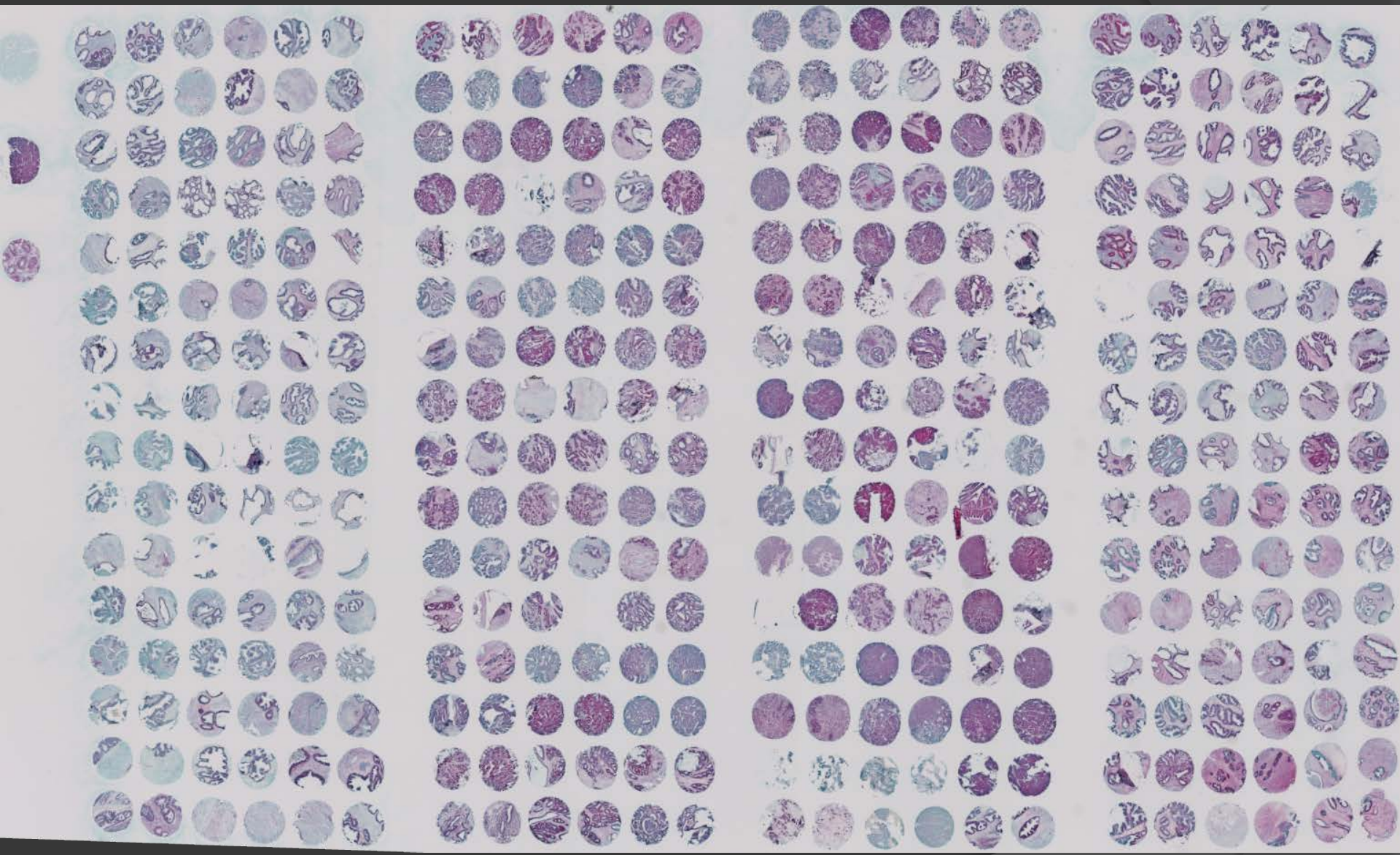
TMA, tissue microarrary; BPH, benign prostatic hyperplasia; BPT, benign prostatic tissue, PCa_local, localized prostate cancer; PCa_aggr, aggressive prostate cancer; Met, metastatic prostate cancer; None, no recurrence; Biochemical, biochemical recurrence; Cancer, cancer recurrence

Three TMA sections were stained with different combination of antibodies					
1st Ab	pTMA1	pTMA2	oTMA1	2nd Ab	Chromogen
AMACR (rabbit mAb, 1:100, Zata Corp)	x			Anti-mouse Ig-HRP	DAB
E-cadherin (mouse mAb, 1:100, Biocare Medical)	x	x	x	Anti-mouse Ig-HRP	Vina Green
AR (mouse mAb, 1:50, Biocare Medical)		x	x	Anti-mouse Ig-HRP	DAB
ERG (rabbit mAb, 1:200, Epitomics)		x	x	Anti-rabbit Ig-AP	Warp Red
Counterstain	x	x	x		Hematoxylin

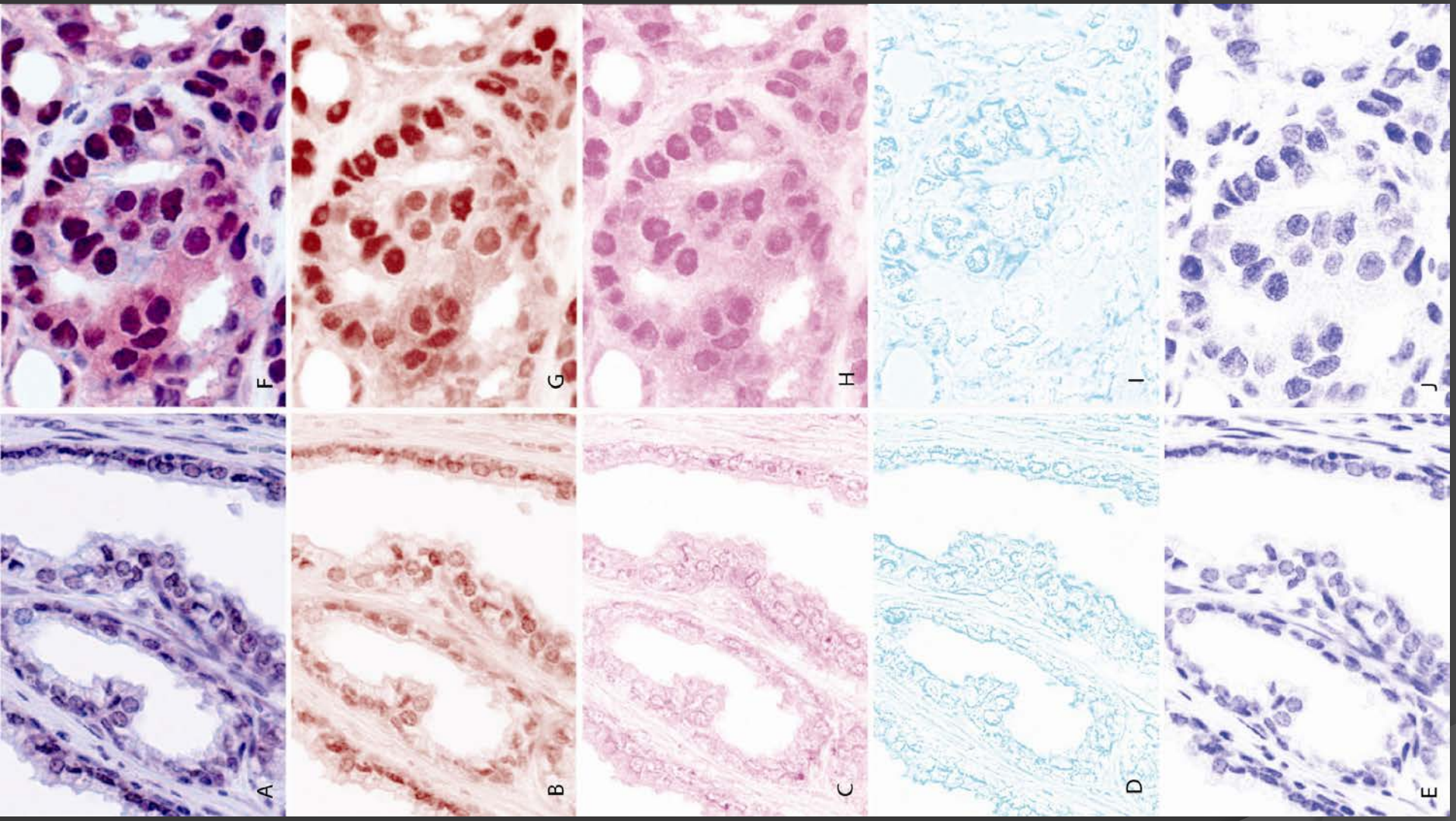


A spectral library of 4 chromogens (DAB, Warp red, Vena green and hematoxylin) built using Nuance software.

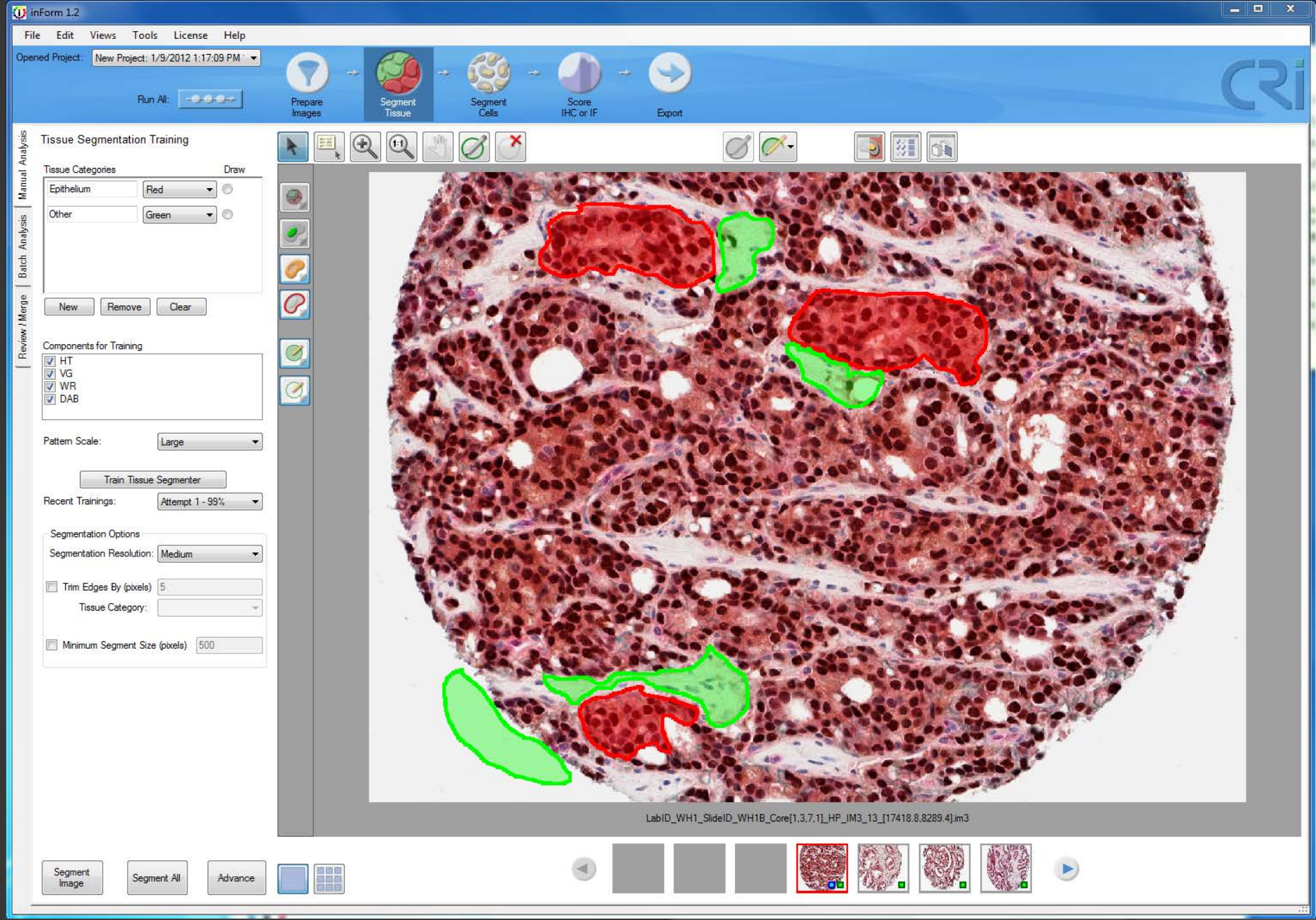
A pTMA section stained with AMACR and E-cadherin



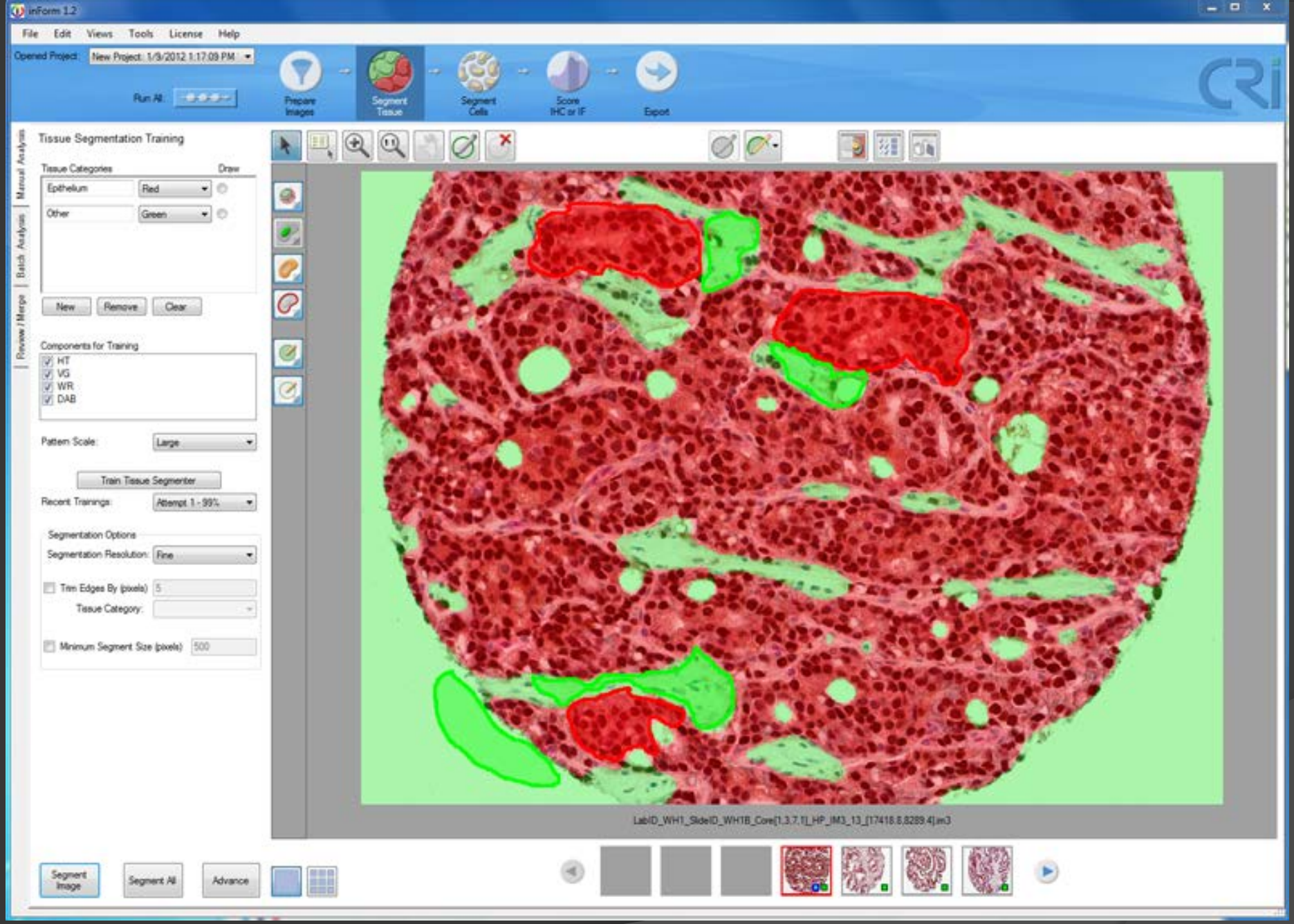
A pTMA section stained with AR, ERG and E-cadherin



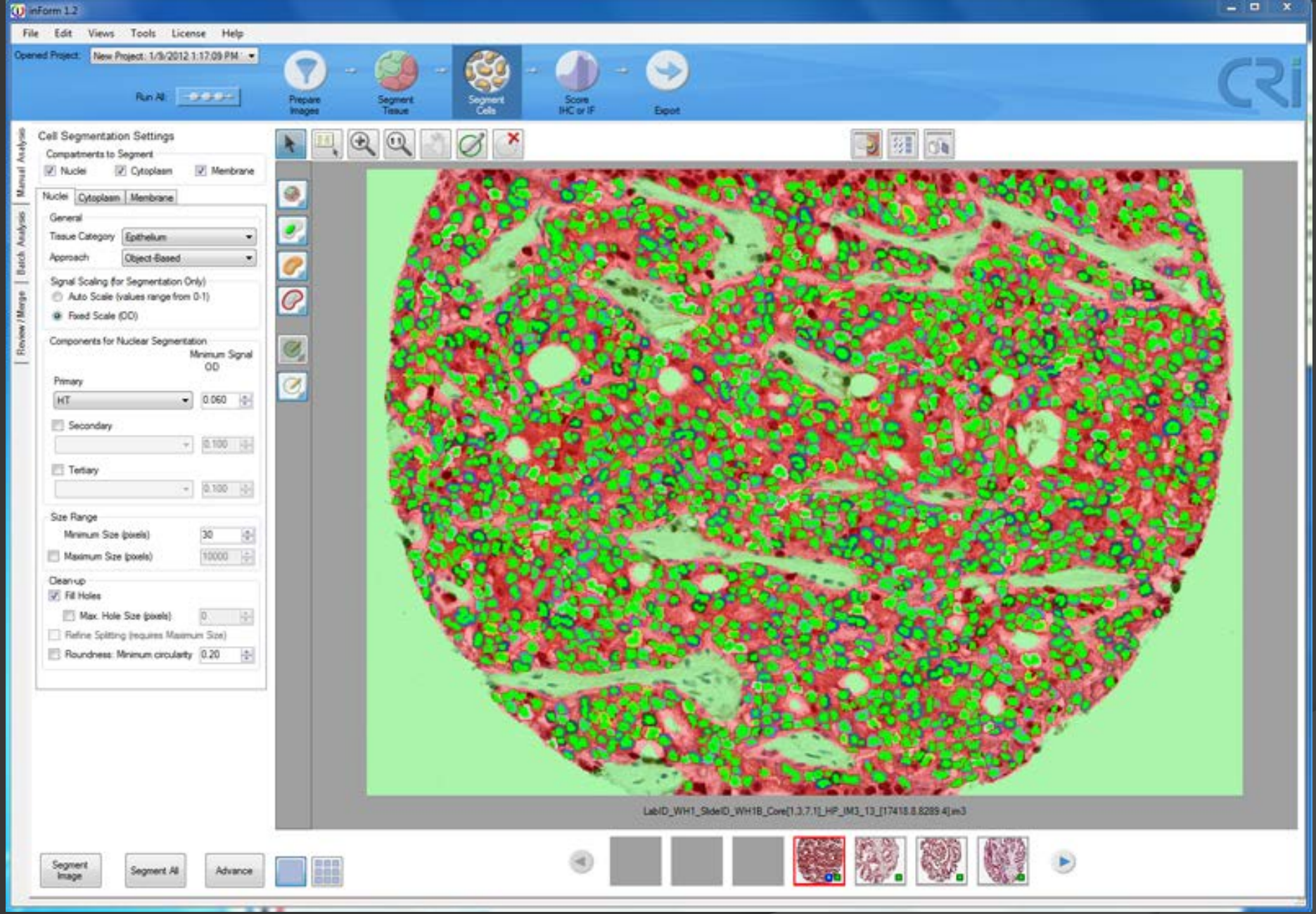
Images of unmixed multispectral signals (DAB, Warp red, Vina green and hematoxylin) of BPT (A-E) and PCa (F-J) using the spectral library. A&F, composite image; B & G, DAB (AR), C & H, Warp red (ERG); D & I, Vina green (E-cad) and E & J, hematoxylin.



A screenshot of inForm software-Tissue segmentation

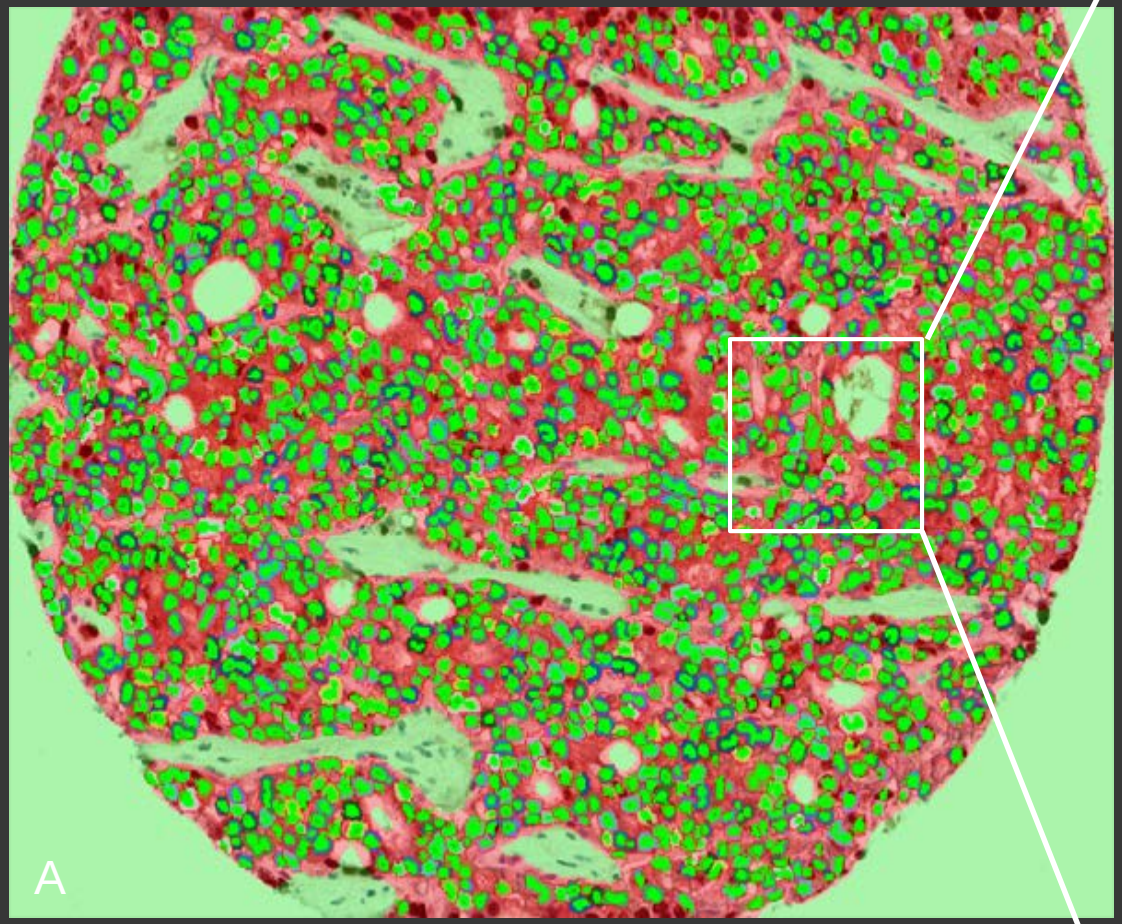


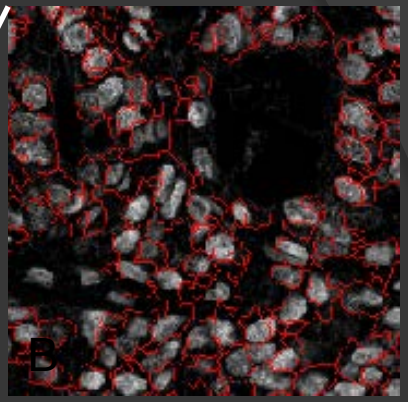
A screenshot of inForm software-Tissue segmentation

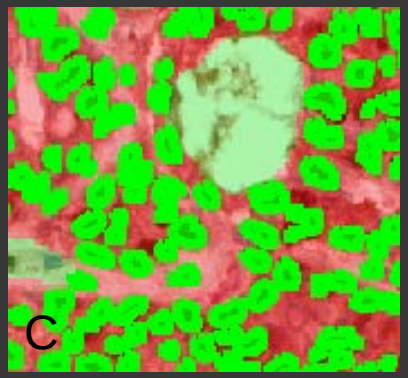


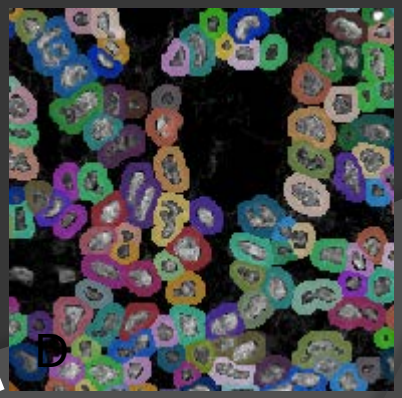
A screenshot of inForm software-cell segmentation

Nuclear-based Cell segmentation



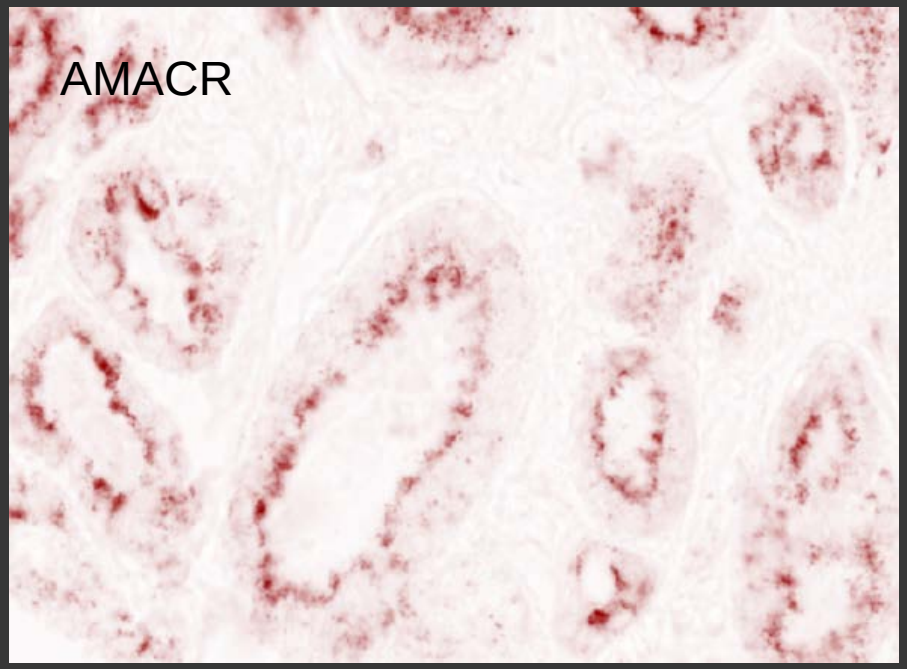


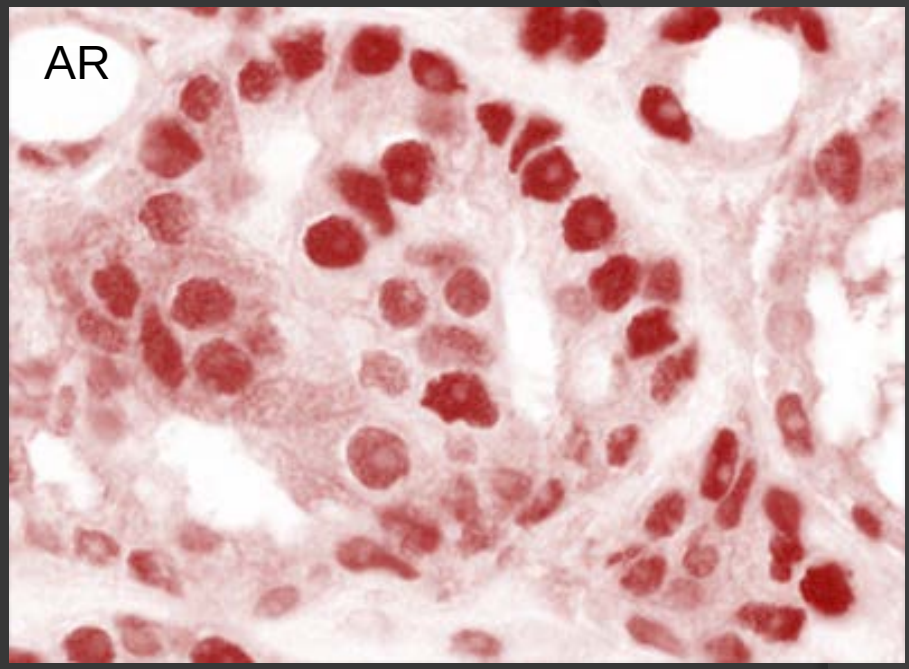


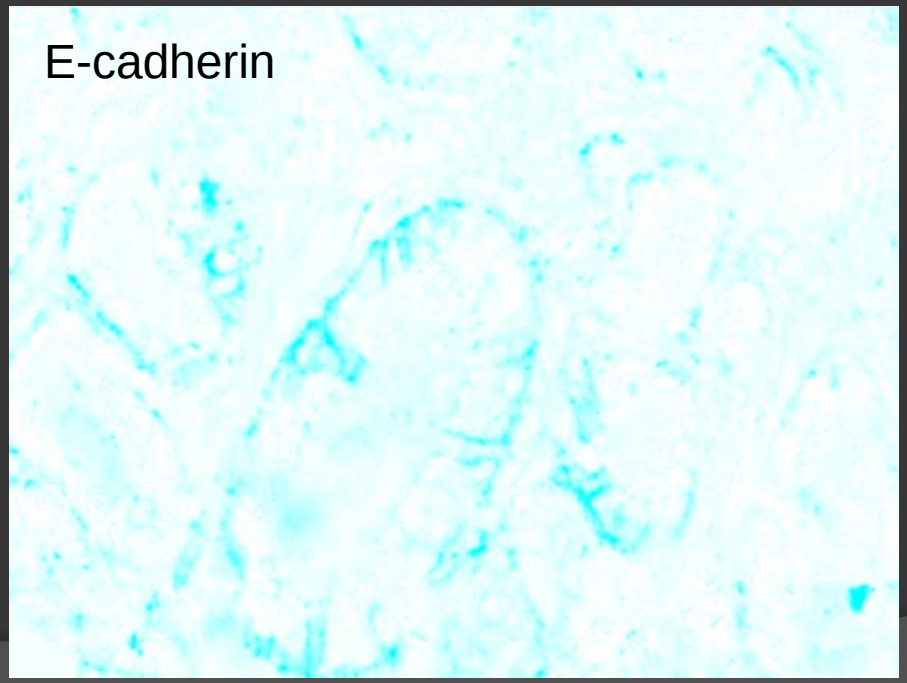


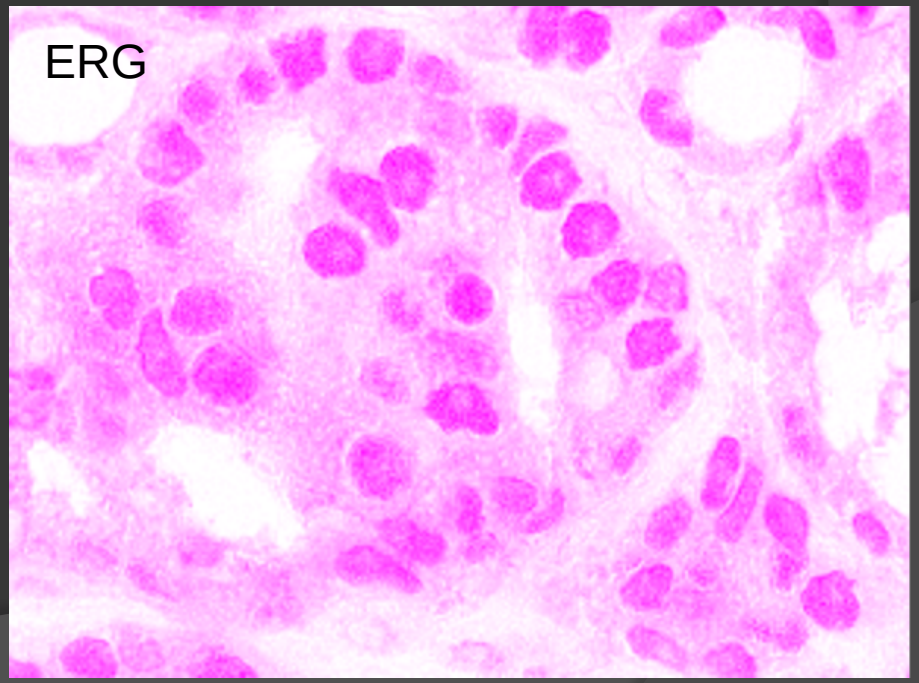
Tissue and cell segmentation using inForm software. A, a composite image of tissue and cell segmentation of prostate epithelium; B, C and D illustrate membranous, nuclear and cytoplasmic compartments, respectively.

- inForm software offers several ways to quantitate biomarker expression levels.
- Understand the tissue and cellular localization of biomarkers that you are studying in order to determine how the analysis should be done and to achieve the best possible results.


AMACR

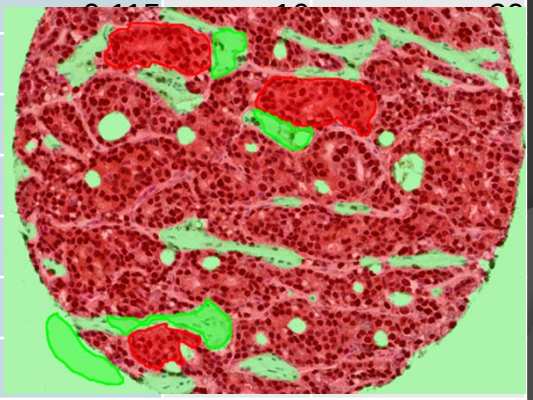

AR

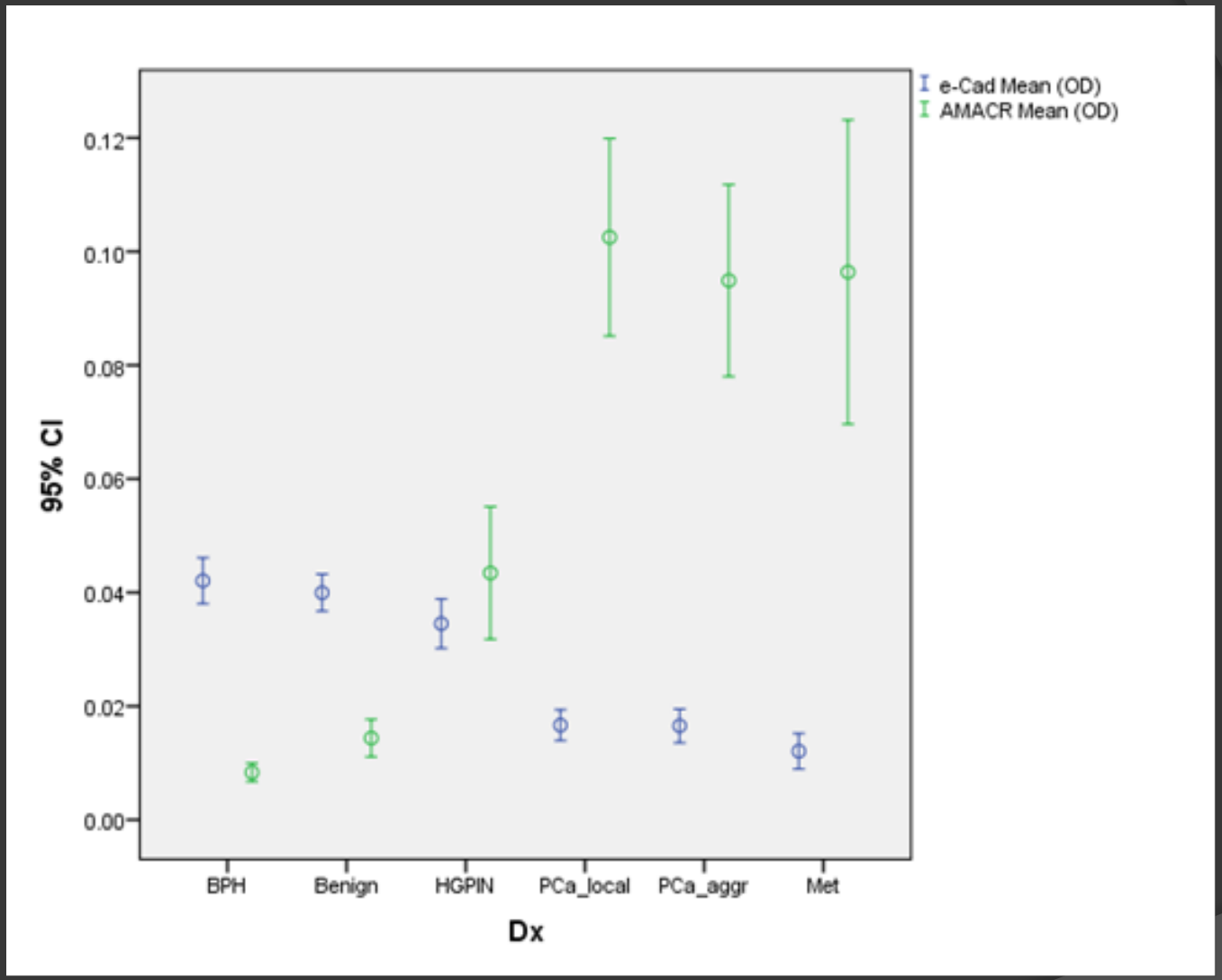

E-cadherin


ERG

Continuous Data per Tissue Segment (AMACR and E-cadherin)

Tissue Category	Total Regions	Pixel Area	Percent Area	VG (E-Cad) Mean (Optical Density)	DAB (AMACR) Mean (Optical Density)	TMA Row	TMA Column
Epithelium	18	463958	32.55%	0.003	0.107	16	26
Epithelium	7	460365	32.30%	0.003	0.137	16	25
Epithelium	7	602214	42.25%	0.004	0.116	16	24
Epithelium	3	1049003	73.59%	0.003	0.118	16	23
Epithelium	12	910034	63.84%	0.005	0.105	16	22
Epithelium	16	583429	40.93%	0.002	0.112	16	21
Epithelium	20	427037	29.96%	0.005			
Epithelium	21	501186	35.16%	0.005			
Epithelium	7	910715	63.89%	0.001			
Epithelium	19	658448	46.19%	0.003			
Epithelium	26	563428	39.53%	0.004			
Epithelium	7	847142	59.43%	0.004			
Epithelium	7	986784	69.23%	0.01			

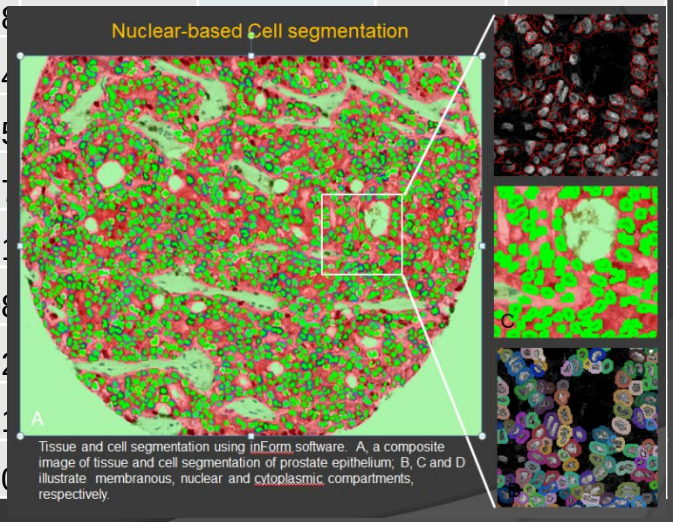


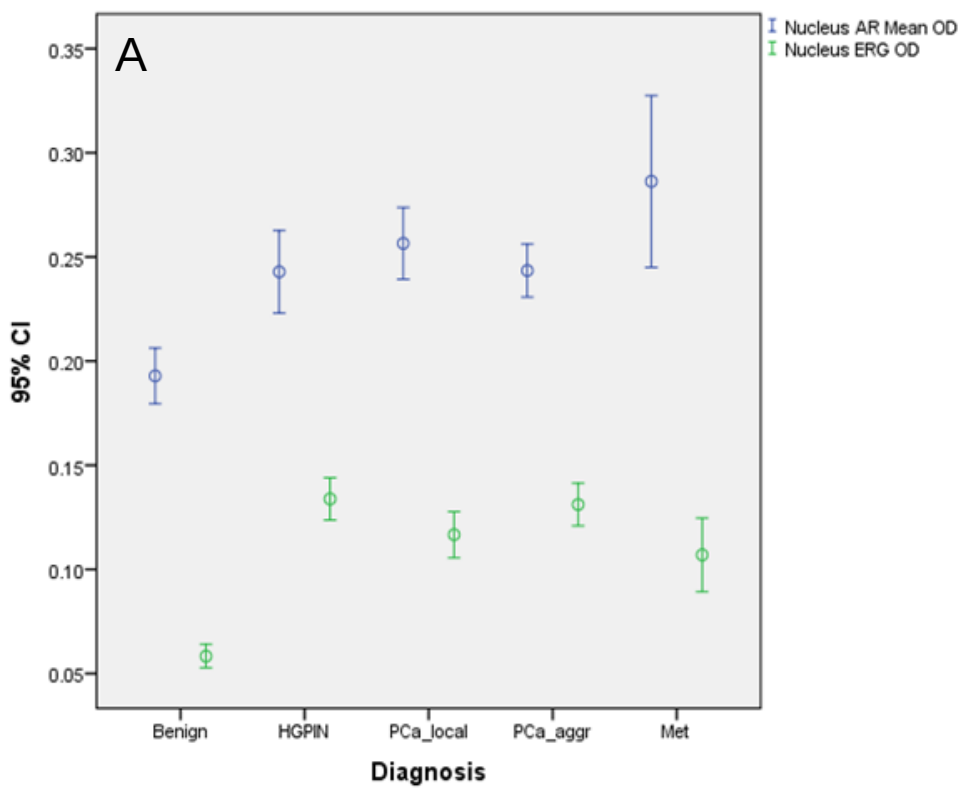


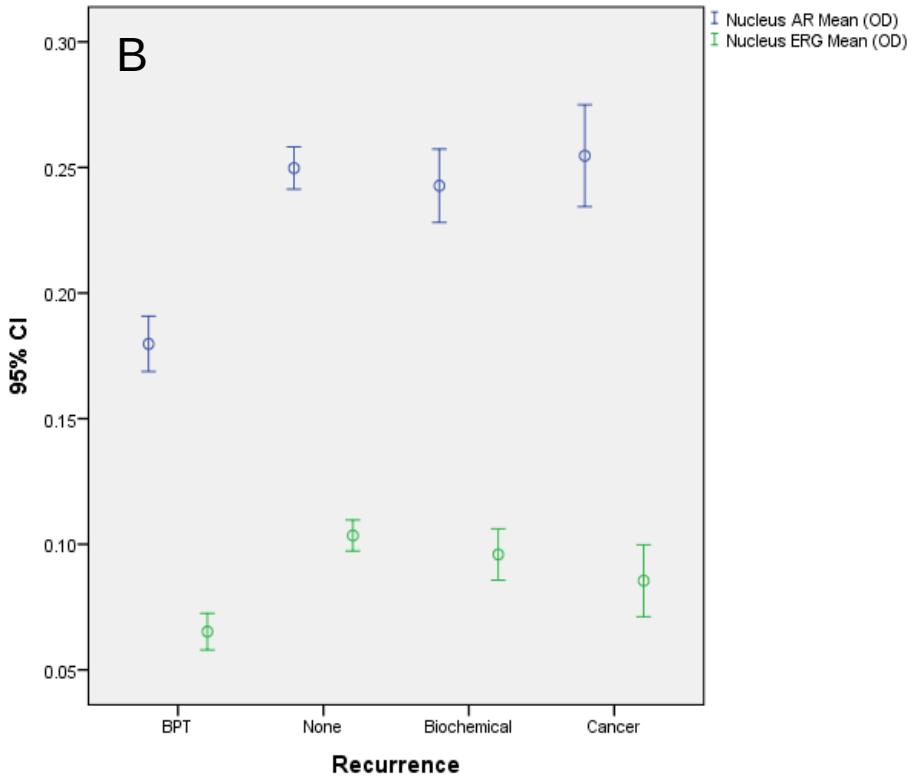
AMACR and E-cadherin expression in prostate tissue and correlation with cancer progression. (IBM SPSS Statistics 20)

Subcellular Continuous Data (ERG and AR)

Tissue Category	Total Cells	Nucleus Pixel Area	Nucleus Percent Area	Nucleus WR (ERG) Mean (Optical Density)	Nucleus DAB (AR) Mean (Optical Density)	Membrane Pixel Area	Membrane Percent Area	Membrane VG (E-Cad) Mean (Optical Density)	TMA Row	TMA Column
Epithelium	1479	138914	15.28%	0.06	0.299	132448	14.57%	0.015	16	30
Epithelium	1384	148770	17.44%	0.056	0.28	125563	14.72%	0.01	16	29
Epithelium	434	44627	11.54%	0.053	0.307	3869				
Epithelium	560	49080	11.58%	0.052	0.266	4629				
Epithelium	296	34208	9.08%	0.034	0.193	2774				
Epithelium	311	31799	9.48%	0.04	0.212	2886				
Epithelium	557	73745	14.62%	0.092	0.27	5150				
Epithelium	1879	213003	21.53%	0.127	0.29	16230				
Epithelium	999	111012	17.31%	0.071	0.277	8420				
Epithelium	718	78139	13.79%	0.062	0.262	6559				
Epithelium	392	42532	12.41%	0.127	0.279	3378				



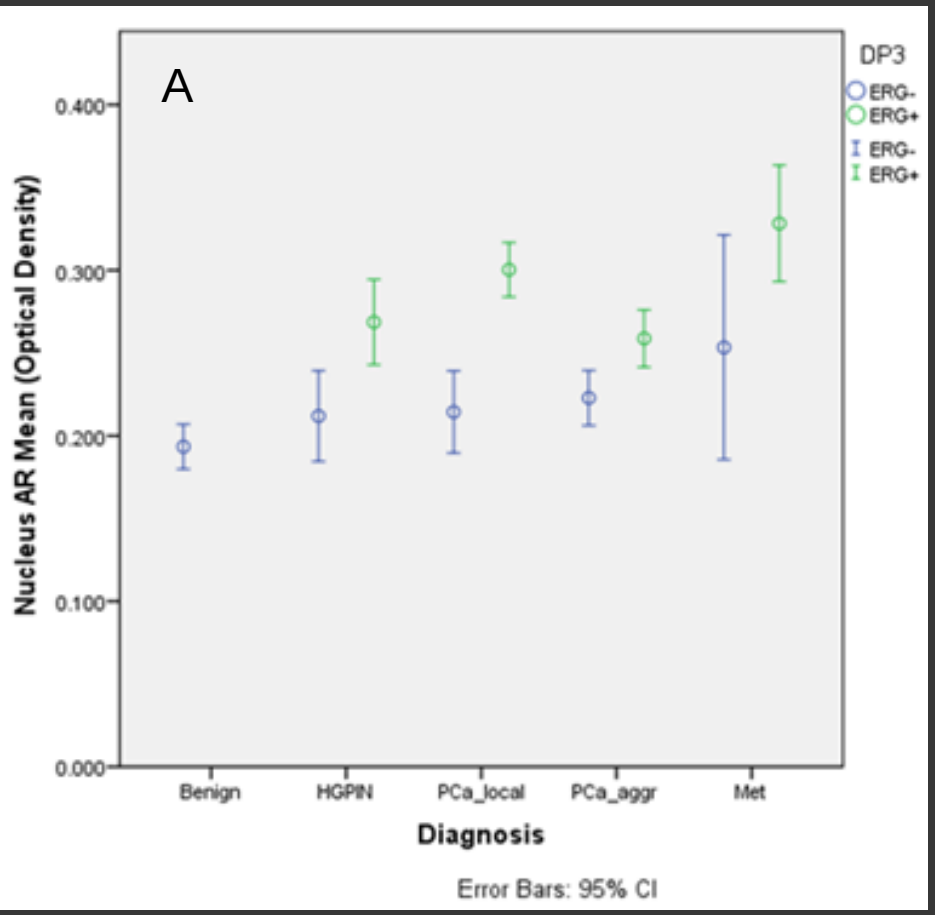


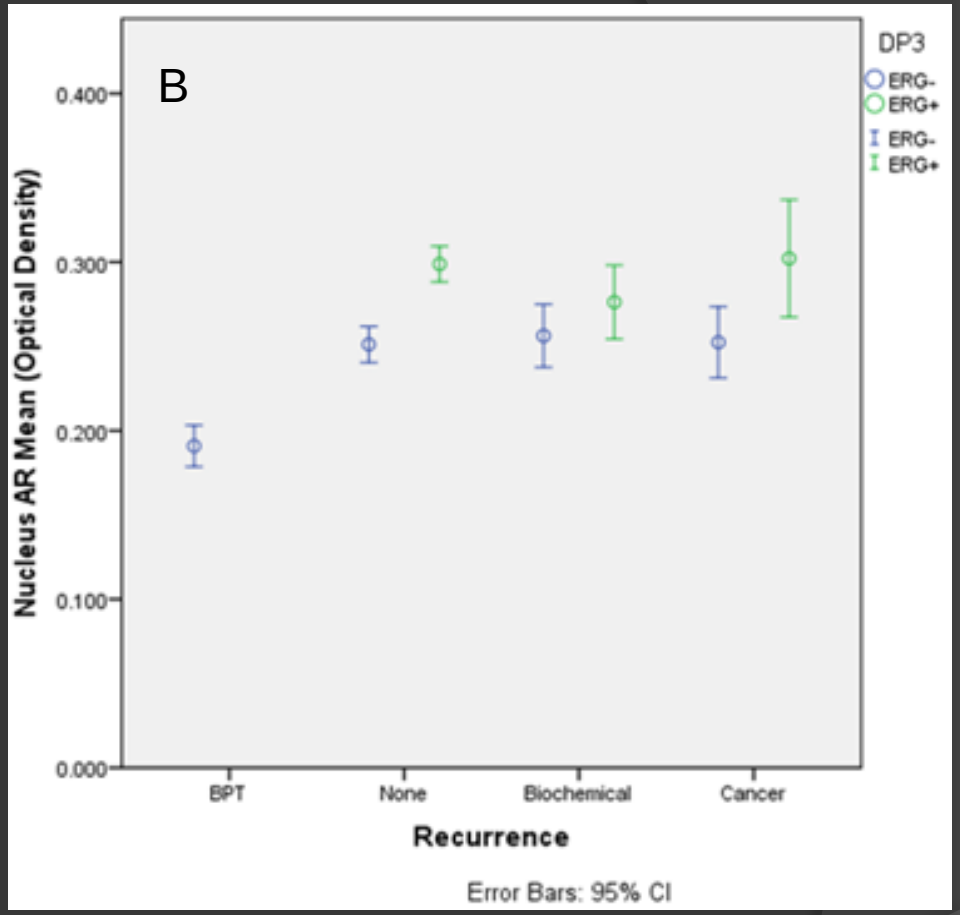


A. Nuclear AR and ERG expression (mean OD/pixel) measured in pTMA.

B. Nuclear AR and ERG expression (mean OD/pixel) measured in oTMA.

(IBM SPSS Statistics 20)





A. Nuclear AR levels in ERG+ and ERG-PCa measured in pTMA.

B. Nuclear AR levels in ERG+ and ERG-PCa measured in oTMA.

(IBM SPSS Statistics 20)

Nuclear AR Expression in Prostate Cancer

Cohort	Dx/Recurrence status	Core# (ERG+/ERG-)	AR Mean OD±SD	
			ERG^+	ERG^-
pTMA	BPT	0/84	0	0.19±0.06
	HGPIN	30/25	0.27±0.07	0.21±0.07
	PCa_local*	41/40	0.30±0.05*	0.21±0.08*
	PCa_aggr	35/26	0.26±0.05	0.22±.04
	Mets	18/23	0.33±0.07	0.25±0.16
oTMA	BPT	0/72		0.19±0.05
	None*	98/128	0.30±0.05*	0.25±0.06*
	Biochemical	31/44	0.28±0.06	0.26±0.06
	Cancer	14/25	0.30±0.06	0.25±0.05

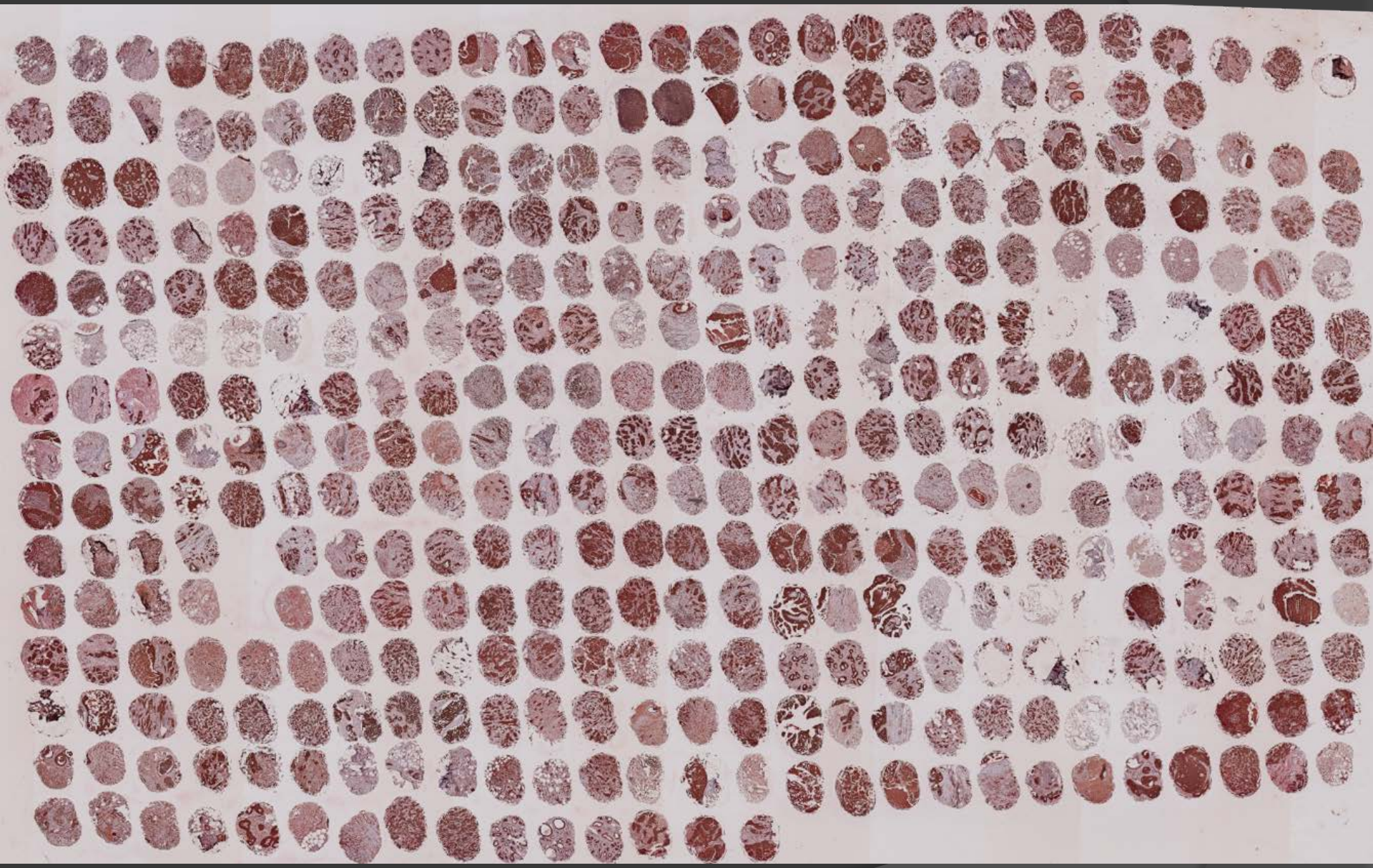
ERG^+ PCa=51% in pTMA cohort, ERG^+ PCa=42% in oTMA cohort; *P<0.001 ERG^+ vs. ERG^-

Biomarker co-localization data (AR and ERG)

Tissue Category	First Cell Compartment	First Stain Component	Second Cell Compartment	Second Stain Component	Double Negative	Single WR (ERG)	Single DAB (AR)	Double Positive	Tissue Category Area (percent)	Number of Cells	WR Threshold	DAB Threshold
Epithelium	Nucleus	WR	Nucleus	DAB	7.91%	0.00%	92.09%	0.00%	63.76%	1482	0.18	0.12
Epithelium	Nucleus	WR	Nucleus	DAB	9.10%	0.00%	90.90%	0.00%	59.86%	1385	0.18	0.12
Epithelium	Nucleus	WR	Nucleus	DAB	3.92%	0.00%	96.08%	0.00%	27.12%	434	0.18	0.12
Epithelium	Nucleus	WR	Nucleus	DAB	5.36%	0.00%	94.64%	0.00%	29.73%	560	0.18	0.12
Epithelium	Nucleus	WR	Nucleus	DAB	2.03%	0.00%	97.97%	0.00%	26.44%	296	0.18	0.12
Epithelium	Nucleus	WR	Nucleus	DAB	4.18%	0.00%	95.82%	0.00%	23.54%	312	0.18	0.12
Epithelium	Nucleus	WR	Nucleus	DAB	3.05%	0.00%	96.77%	0.18%	35.39%	557	0.18	0.12
Epithelium	Nucleus	WR	Nucleus	DAB	7.18%	0.05%	87.87%	4.90%	69.41%	1879	0.18	0.12
Epithelium	Nucleus	WR	Nucleus	DAB	9.21%	0.00%	90.69%	0.10%	44.99%	999	0.18	0.12
Epithelium	Nucleus	WR	Nucleus	DAB	5.43%	0.00%	94.57%	0.00%	39.76%	719	0.18	0.12

Example 2

- Explore ERβ as a predictive marker for breast cancer progression
  - Breast TMA slides are stained with antibody against ERβ and visualized with DAB.
  - Hematoxylin is used as counter stain to define the nuclei.
  - A four-tiered positivity of ERβ in each core is quantitated.

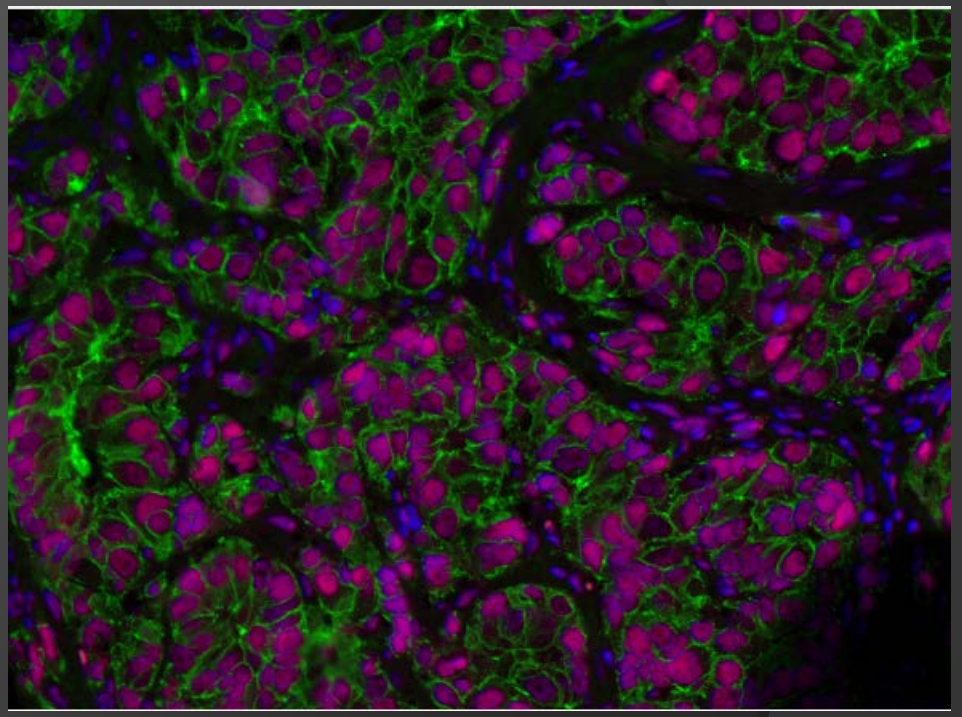


A breast cancer TMA stained with ERβ

Subcellular Categorical (scoring) Data (ERbeta)

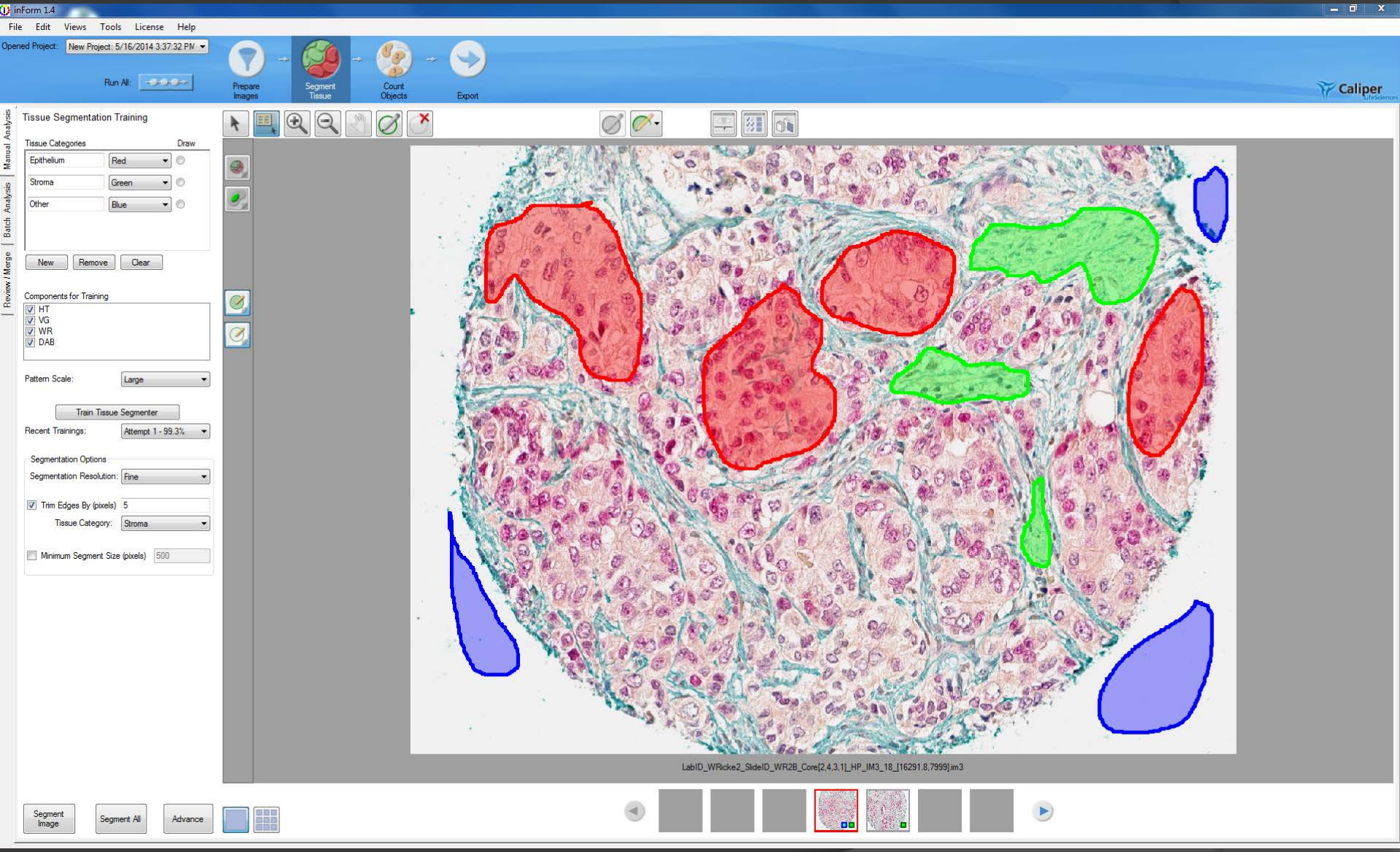
Tissue Category	Cell Compartment	Stain Component (ER beta)	0+	1+	2+	3+	Tissue Category Area (percent)	Number of Cells	Threshold 0/1+	Threshold 1+/2+	Threshold 2+/3+
Cancer	Cytoplasm	DAB	72.25%	23.20%	4.26%	0.29%	36.30%	681	0.1	0.2	0.3
Cancer	Cytoplasm	DAB	77.15%	21.02%	1.74%	0.10%	50.68%	1037	0.1	0.2	0.3
Cancer	Cytoplasm	DAB	49.49%	44.23%	5.69%	0.58%	31.64%	685	0.1	0.2	0.3
Cancer	Cytoplasm	DAB	69.60%	25.76%	3.95%	0.68%	36.83%	885	0.1	0.2	0.3
Cancer	Cytoplasm	DAB	54.35%	29.69%	11.50%	4.46%	47.91%	896	0.1	0.2	0.3
Cancer	Cytoplasm	DAB	51.04%	37.21%	11.75%	0.00%	46.66%	962	0.1	0.2	0.3
Cancer	Cytoplasm	DAB	56.99%	28.42%	13.98%	0.60%	60.12%	1323	0.1	0.2	0.3
Cancer	Cytoplasm	DAB	31.54%	51.64%	16.69%	0.13%	32.92%	761	0.1	0.2	0.3
Cancer	Cytoplasm	DAB	26.28%	40.00%	32.73%	0.99%	26.45%	605	0.1	0.2	0.3
Cancer	Cytoplasm	DAB	71.89%	27.88%	0.23%	0.00%	63.02%	1309	0.1	0.2	0.3
Cancer	Cytoplasm	DAB	72.82%	27.18%	0.00%	0.00%	43.31%	927	0.1	0.2	0.3
Cancer	Cytoplasm	DAB	98.42%	1.58%	0.00%	0.00%	49.79%	952	0.1	0.2	0.3
Cancer	Cytoplasm	DAB	98.11%	1.89%	0.00%	0.00%	62.03%	1167	0.1	0.2	0.3

Cell counting



Example 3

- A prostate TMA slide is stained with antibodies against AR (Warp Red), ER (DAB), Smooth muscle actin (Vina Green).
- Hematoxylin is used as counter stain to define nuclei.
- ER positive cells in epithelial compartment are counted.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Count Objects
Export
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Manual Analysis
Batch Analysis
Review / Merge
Tissue Segmentation Training
Tissue Categories
Draw
Epithelium
Red
Stroma
Green
Other
Blue
New
Remove
Clear
Components for Training
HT
VG
WR
DAB
Pattern Scale:
Large
Train Tissue Segmenter
Recent Trainings:
Attempt 1 - 99.3%
Segmentation Options
Segmentation Resolution:
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Tissue Category:
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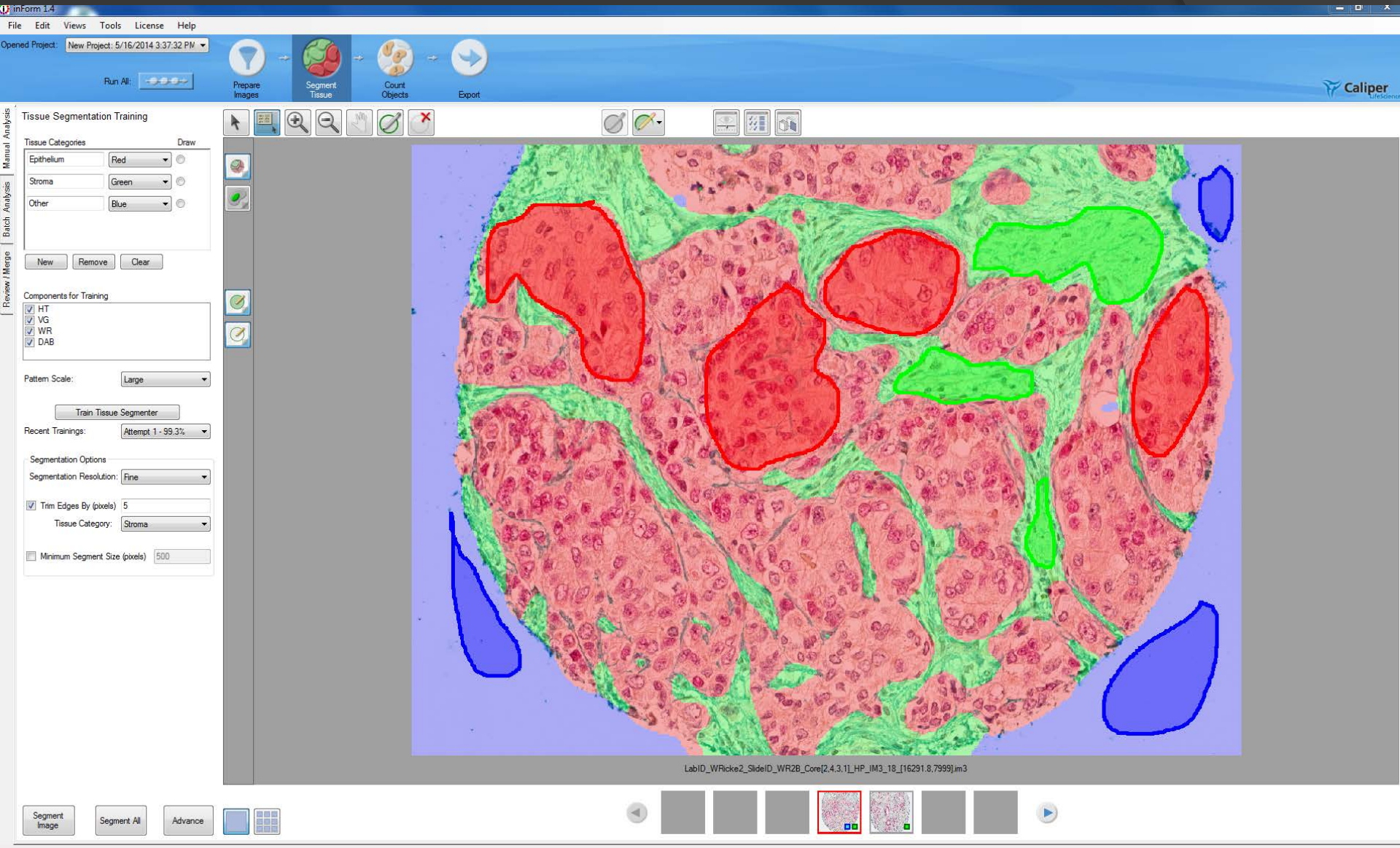
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Tissue Categories
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Epithelium Red
Stroma Green
Other Blue
New
Remove
Clear
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VG
WR
DAB
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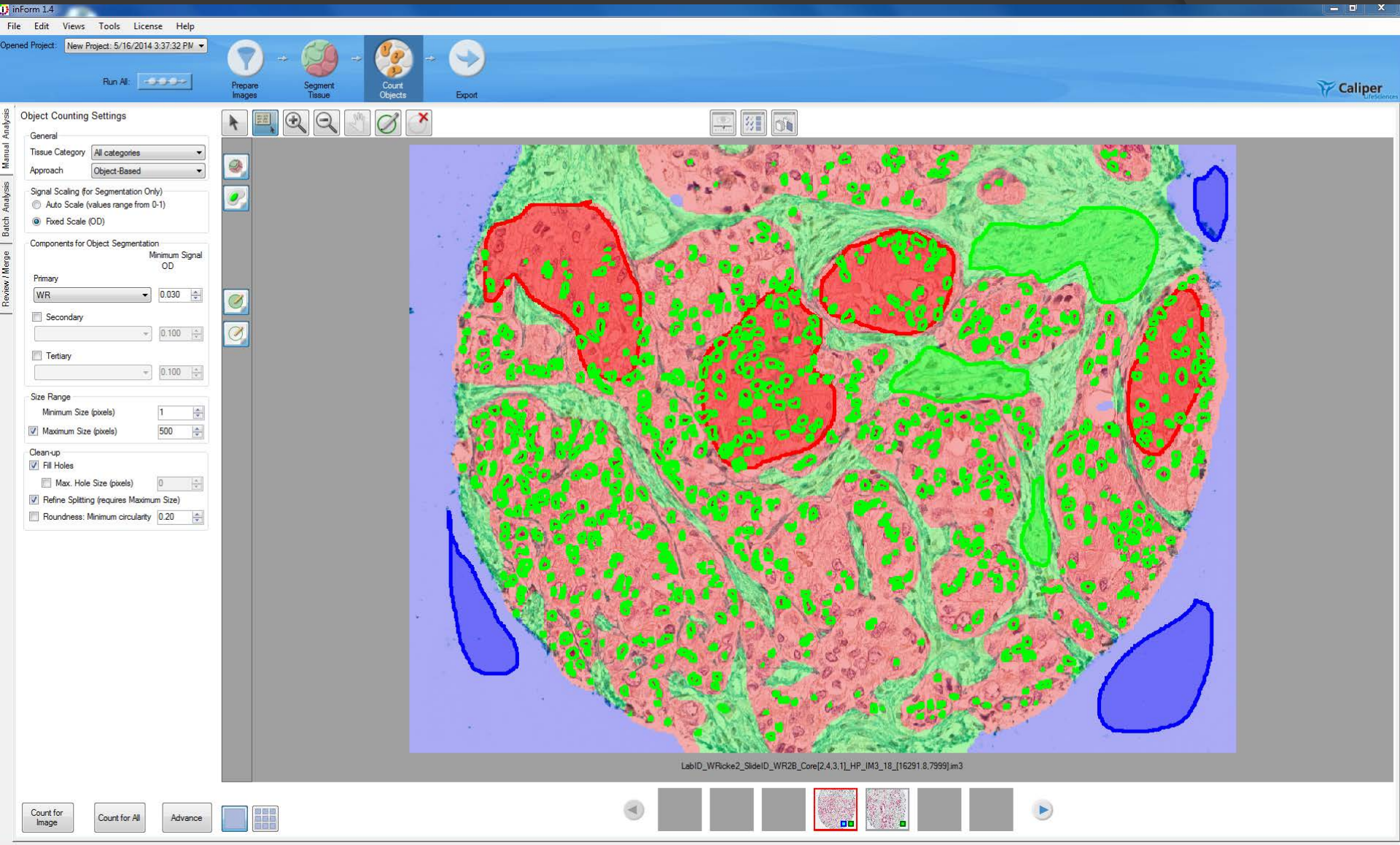
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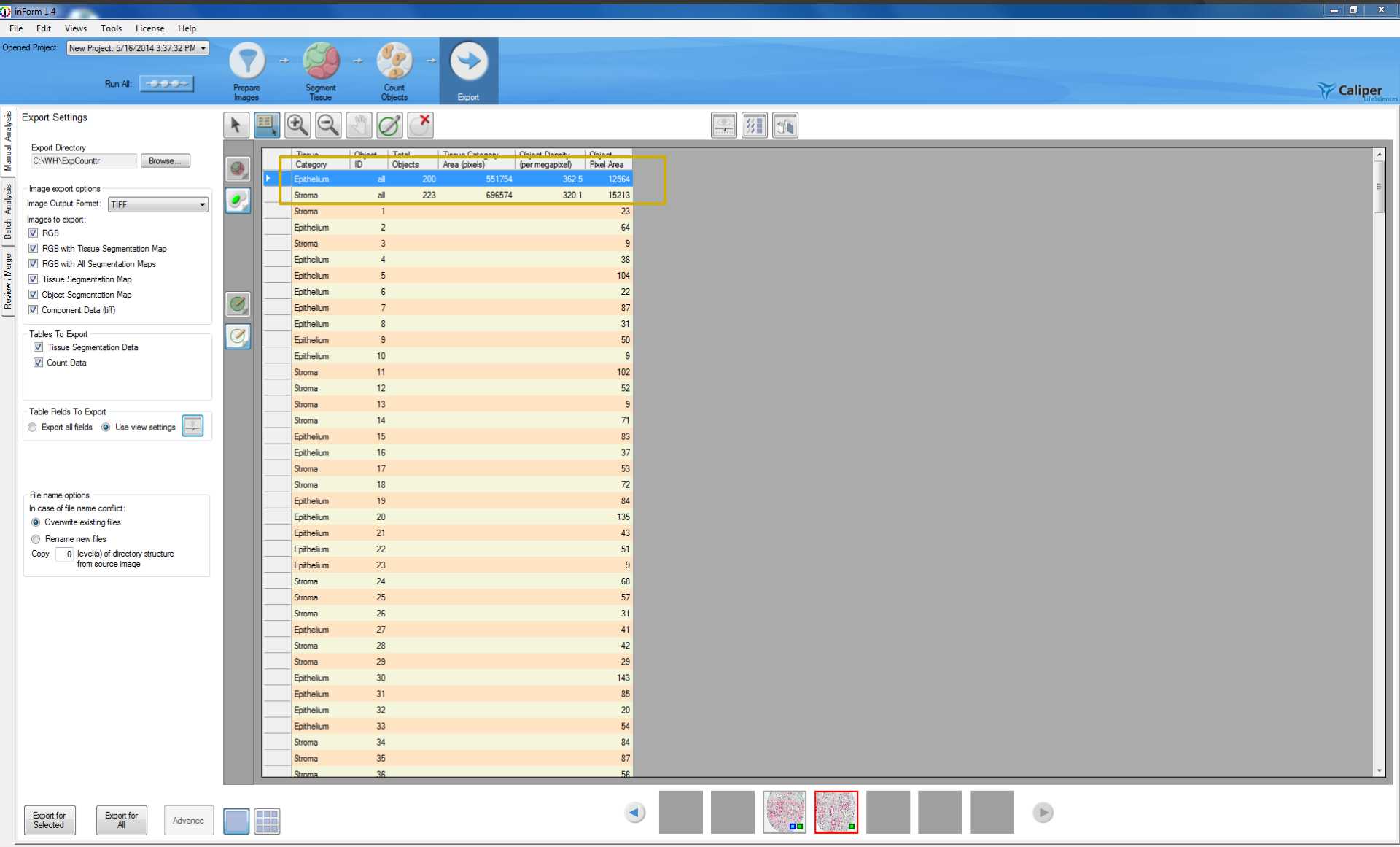
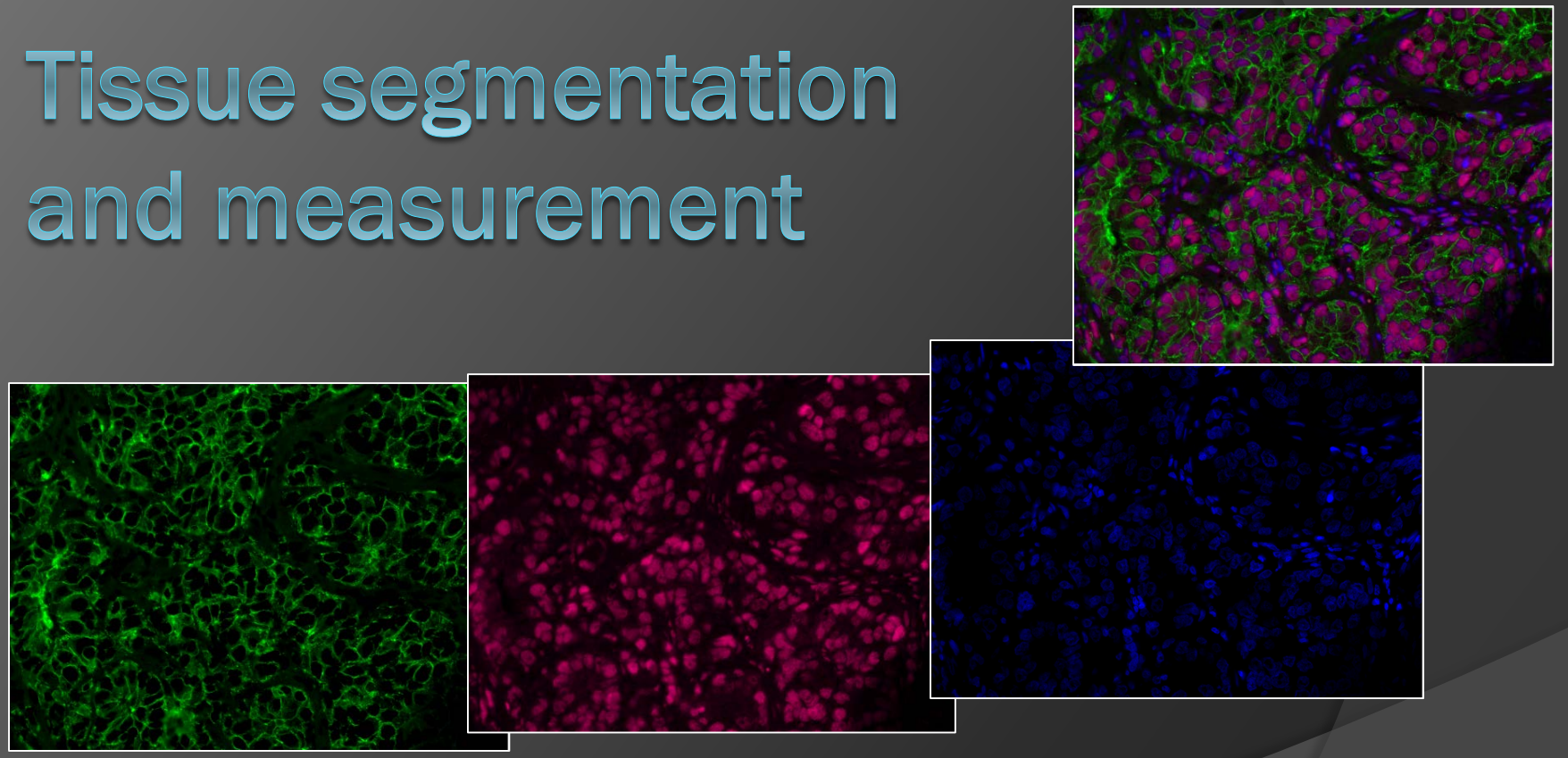
Run All:
Prepare Images
Segment Tissue
Count Objects
Export
Caliper
Object Counting Settings
General
Tissue Category
All categories
Approach
Object-Based
Signal Scaling (for Segmentation Only)
Auto Scale (values range from 0-1)
Fixed Scale (OD)
Components for Object Segmentation
Minimum Signal OD
Primary
WR
0.030
Secondary
0.100
Tertiary
0.100
Size Range
Minimum Size (pixels)
1
Maximum Size (pixels)
500
Clean-up
Fill Holes
Max. Hole Size (pixels)
Refine Splitting (requires Maximum Size)
Roundness: Minimum circularity
0.20
Manual Analysis
Batch Analysis
Review / Merge
LabID_WRicke2_SlideID_WR2B_Core[2,4,3,1]_HP_IM3_18_[16291.8,7999].im3
Count for Image
Count for All
Advance

Cell Counting Data from a Single Image



Tissue segmentation and measurement



Example 4

- Assessment of prostate atrophy associated with finasteride treatment
  - Ratio of epithelium (pixel area) to stroma (pixel area).
  - Ratio of luminal epithelium (pixel area) to basal cells (pixel area).

- Whole section slides of prostate chips are stained with antibodies against CK5, PSA and AR.
  - CK5 is used to define prostate basal cells.
  - PSA is used to define prostate luminal epithelium.
  - Hematoxylin counter stain is used to define nuclei.

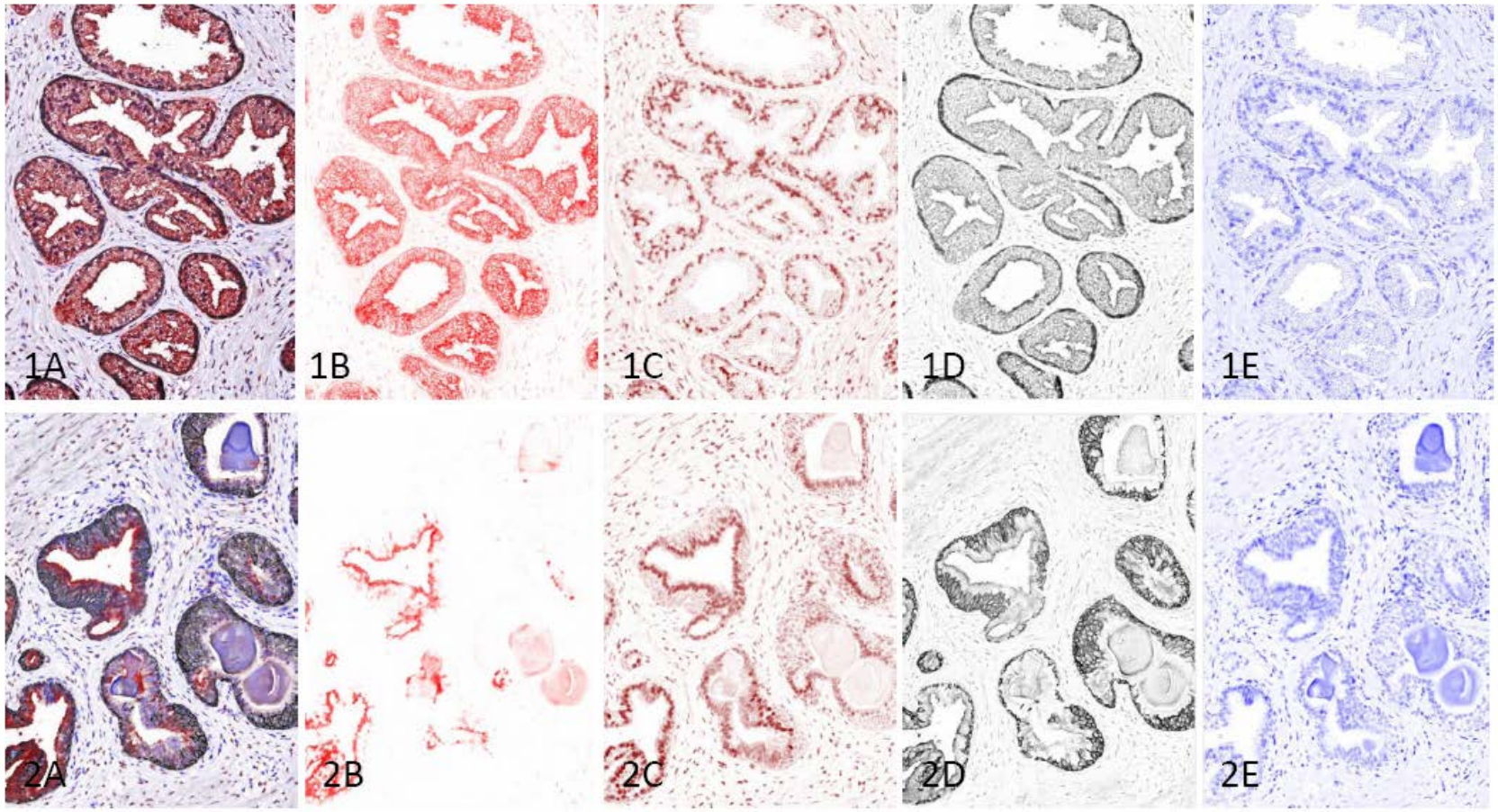
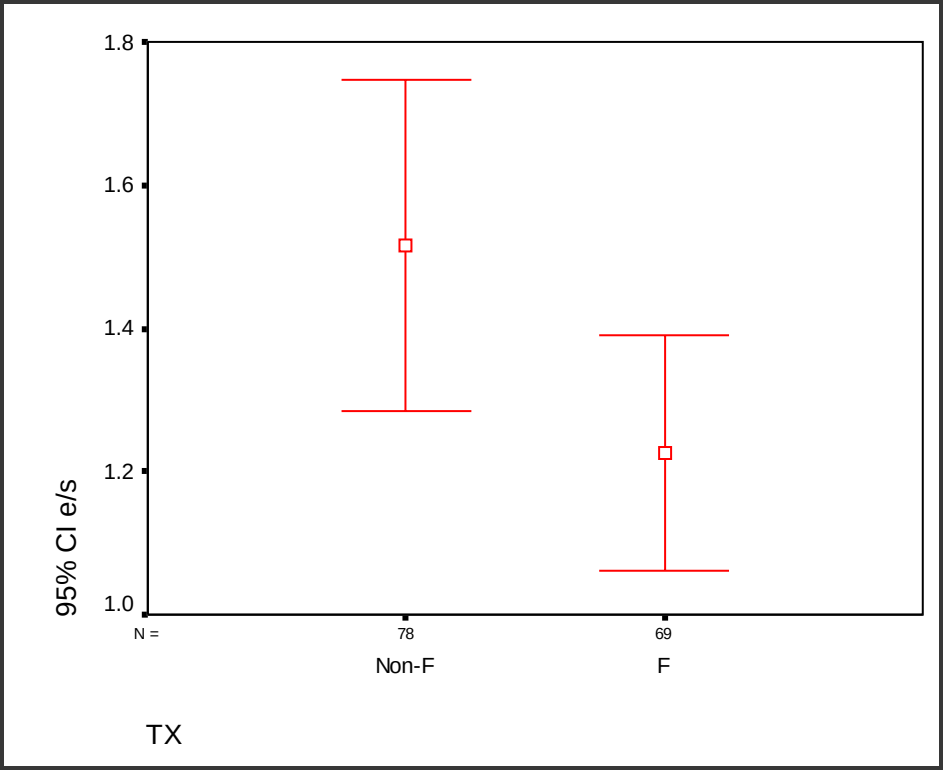


Figure 3. Prostatic glands from patient without finateride treatment (1A -1E), with finasteride treatment (2A-2E).

Tissue Segmentation and Measurement

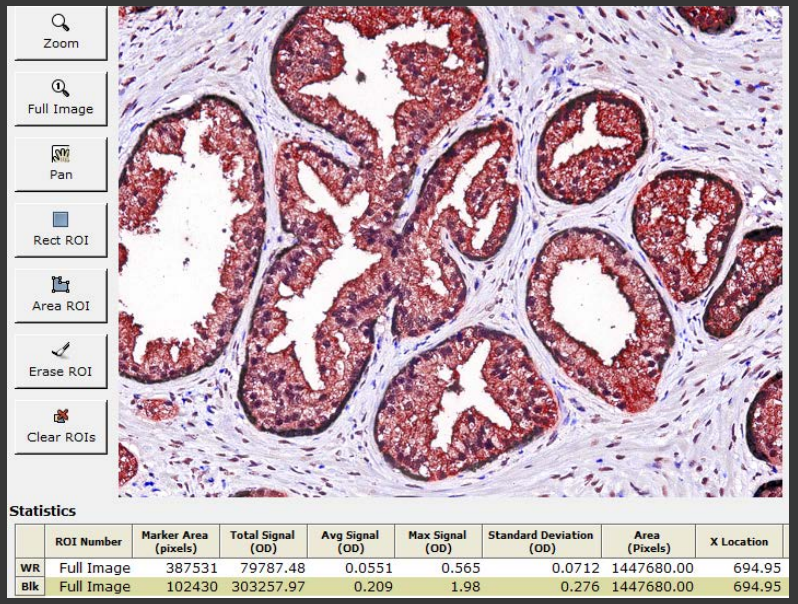
Tissue Category	Pixel Area	Percent Area	WR Mean (Optical Density)	DAB Mean (Optical Density)	Blk Mean (Optical Density)
epithelium	704064	48.63%	0.039	0.213	0.219
epithelium	837808	57.87%	0.031	0.191	0.225
epithelium	862576	59.58%	0.011	0.281	0.132
epithelium	656576	45.35%	0.049	0.212	0.269
epithelium	901920	62.30%	0.046	0.253	0.328
epithelium	772192	53.34%	0.06	0.239	0.278
epithelium	832384	57.50%	0.02	0.255	0.403
epithelium	765488	52.88%	0.008	0.216	0.376

Tissue Category	Pixel Area	Percent Area	WR Mean (Optical Density)	DAB Mean (Optical Density)	Blk Mean (Optical Density)
stroma	505664	34.93%	0.005	0.042	0.064
stroma	298416	20.61%	0.006	0.054	0.059
stroma	371328	25.65%	0.003	0.048	0.053
stroma	623488	43.07%	0.008	0.044	0.063
stroma	336000	23.21%	0.005	0.074	0.073
stroma	405872	28.04%	0.014	0.046	0.089
stroma	497040	34.33%	0.002	0.053	0.065
stroma	422080	29.16%	0.002	0.05	0.057



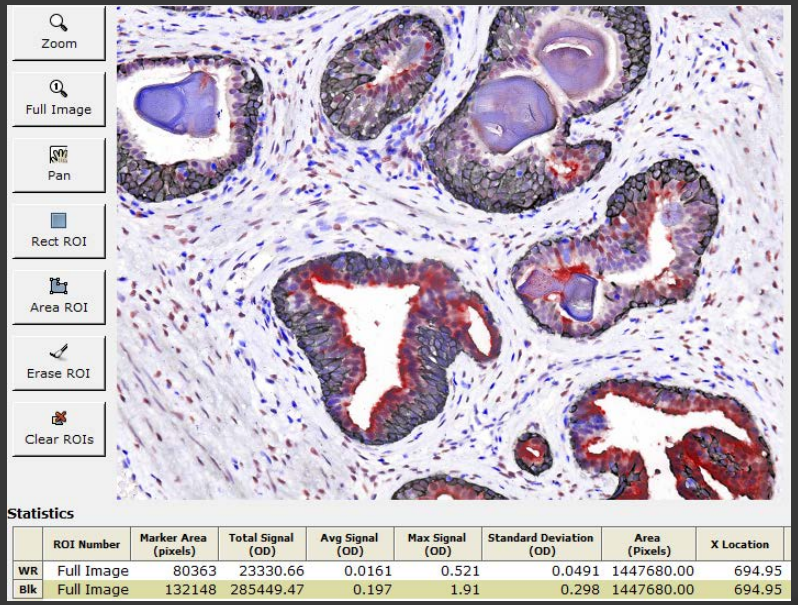
Ratio of prostate epithelium to stroma with(F) and without finasteride treatment (non-F). (IBM SPSS Statistics 20)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.102	1	3.102	3.965	.048
Within Groups	113.434	145	.782		
Total	116.537	146			



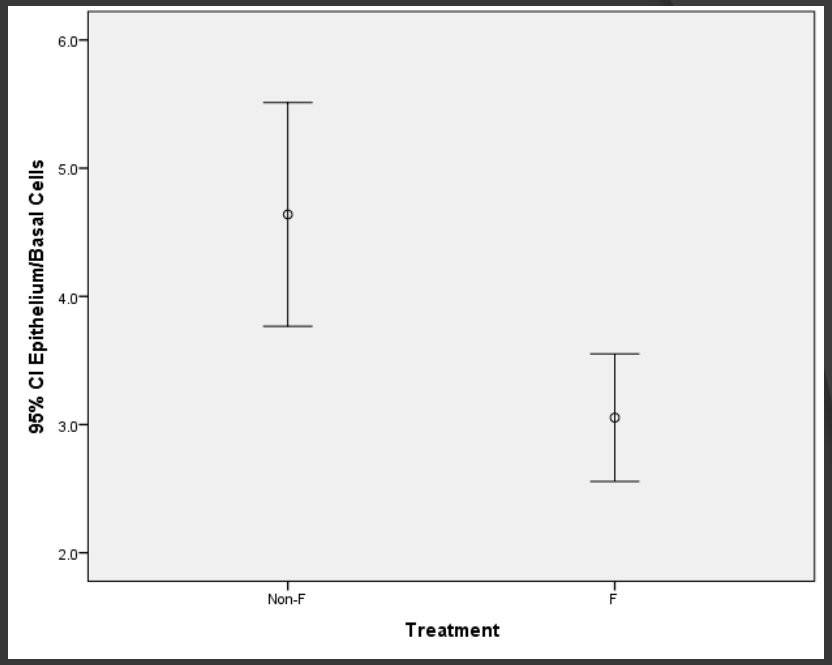
Statistics

	ROI Number	Marker Area (pixels)	Total Signal (OD)	Avg Signal (OD)	Max Signal (OD)	Standard Deviation (OD)	Area (Pixels)	X Location
WR	Full Image	387531	79787.48	0.0551	0.565	0.0712	1447680.00	694.95
Blk	Full Image	102430	303257.97	0.209	1.98	0.276	1447680.00	694.95



Statistics

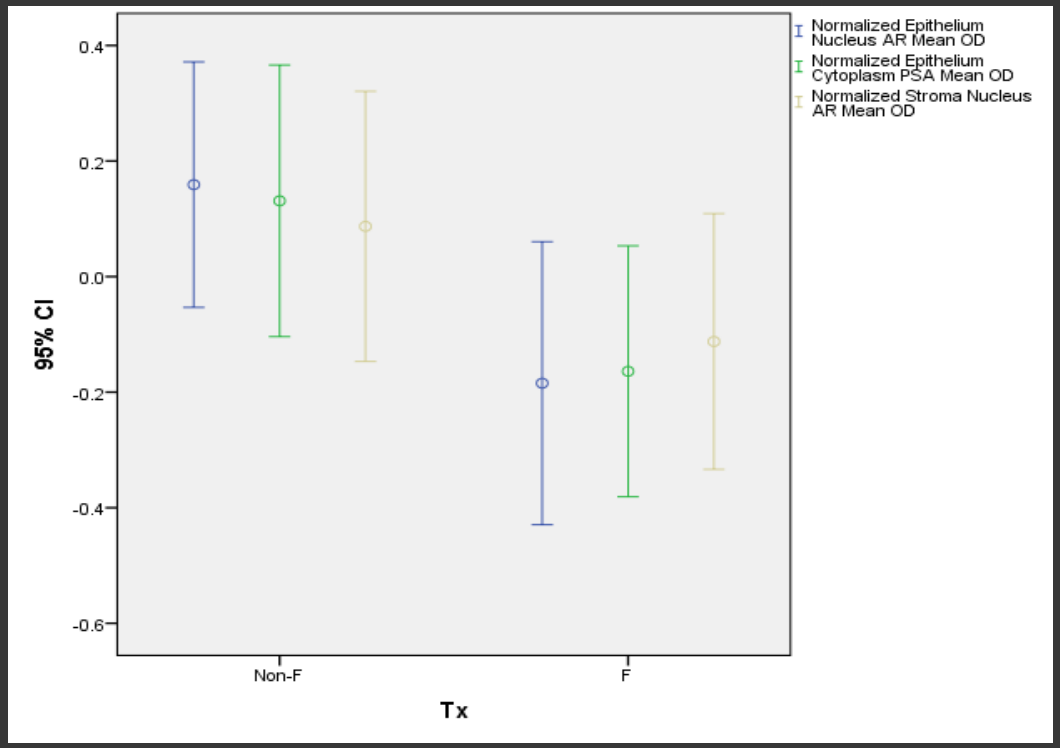
	ROI Number	Marker Area (pixels)	Total Signal (OD)	Avg Signal (OD)	Max Signal (OD)	Standard Deviation (OD)	Area (Pixels)	X Location
WR	Full Image	80363	23330.66	0.0161	0.521	0.0491	1447680.00	694.95
Blk	Full Image	132148	285449.47	0.197	1.91	0.298	1447680.00	694.95



Pronounced glandular atrophic change and basal cell hyperplasia were observed in TURP chips with finasteride treatment (IBM SPSS Statistics 20)

Ratio of Epithelium to Basal Cells

TX	N	Mean	Std. Deviation
F	68	3.05	2.05
Non-F	78	4.64	3.87



Finasteride effect on androgen regulated protein (PSA and AR) expression in prostate epithelium and/or stroma. (IBM SPSS Statistics 20)

Table 3. Mean OD Z-Score

Tx	Epithelial Nucleus AR	Epithelial Cytoplasm PSA	Stromal Nuclear AR
Non-F (78)	.16±0.94[a]	.13±1.04[b]	.09±1.04[c]
F (69)	-.18±1.02[a]	-.16±0.90[b]	-.11±0.92[c]

[a]P=0.035, [b]P=0.071, [c]P=0.223

Summary

- Vectra is a powerful system for automated morphometric analysis of tissue/cell samples.
  - High throughput and precise
  - Generating continuous and categorical data at cellular and subcellular levels
  - Biomarker co-localization study
  - Cell and structure counting

Caveats

- Know the distribution and localization of biomarkers of interest.
- Understand the limitation of Vectra system.
- Design your experiment well and select appropriate form(s) of analysis to generate and maximize reliable data.

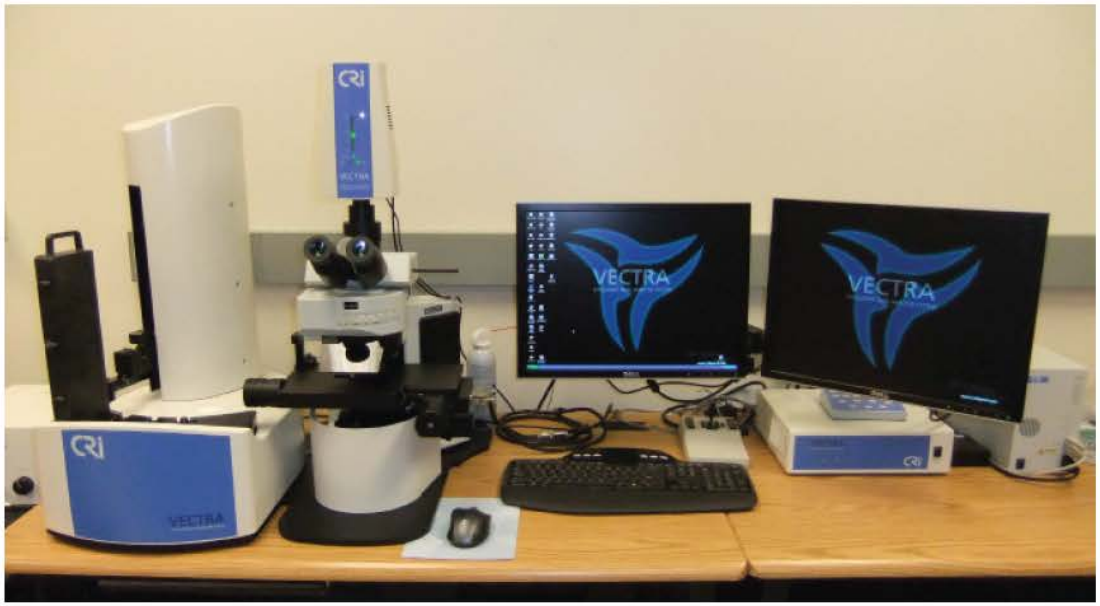
If you decide to use the imaging systems

- You may choose to do the analysis yourself or find a collaborating pathologist to do it for you.
- You may contact Pathology TRIP lab by e-mail or phone to get detailed instructions on how to proceed and to get an estimated budget.
- TRIP lab will provide
  - Project consultation
  - Training for using Nuance and inForm software (if you choose to do it yourself)
  - Affordable fee-for-service

For more information about Vectra application

Huang, W., K. Hennrick, et al. "A colorful future of quantitative pathology: validation of Vectra technology using chromogenic multiplexed immunohistochemistry and prostate tissue microarrays." Hum Pathol 44(1): 29-38.

Thank you!



CONTACT US

Email: trip@pathology.wisc.edu

http://www.pathology.wisc.edu/research/trip