



Prognostic and Diagnostic Evaluation of Histopathologic Images by Multispectral Imaging

“Cytomics on Slide”

Or

Slow Cytometry...

Michael Feldman, MD, PhD

feldmanm@mail.med.upenn.edu

Assistant Professor Pathology
University Pennsylvania



The Need

Current method for assessing tumor cell signaling or other proteins by IHC

- Immunostain for proteins
- Pathologist-dependent, subjective (0-3+)
 - “quantified” by stain area, intensity and estimated percent
 - Very subjective and not reproducible
- Proteins assessed one at a time

Assessment of inhibition of tumor cell signaling

- Similar problems
- Need objective quantification of changes in cell signaling



Platform for assessing activity of TC signaling pathways

Immunohistologic staining of pathway components

Pathways analyzed on a cellular basis ("cytometry")

Results quantitated computationally ("automated")

Multiple pathways analyzed together ("multiparameter")

Components

1. *Multiparameter immunostaining*

2. *Multispectral image capture*

3. *Image processing to resolve individual stains based on spectra*

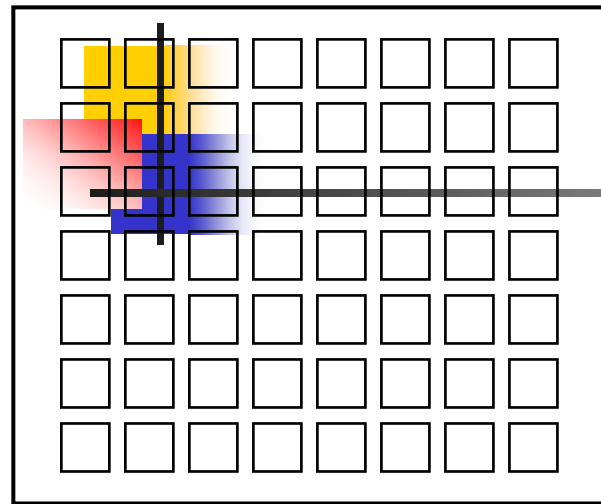
4. *Tumor segmentation*

5. *Computational identification of nuclei and cells in image*

6. *Computational assignment of immunostains to each nucleus or cell*

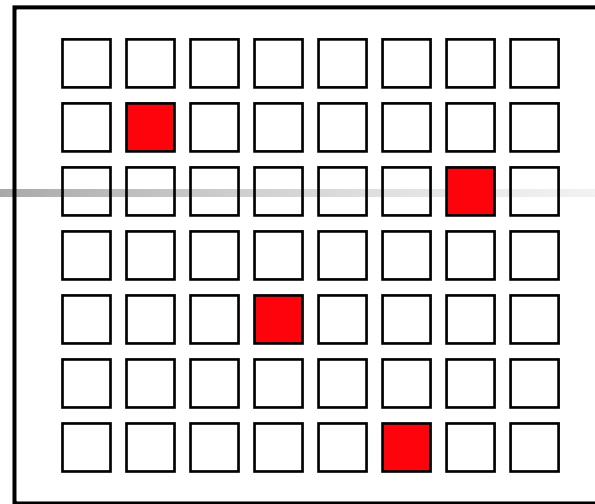
7. *Data display and Analysis*

1. Multiparameter immunostaining

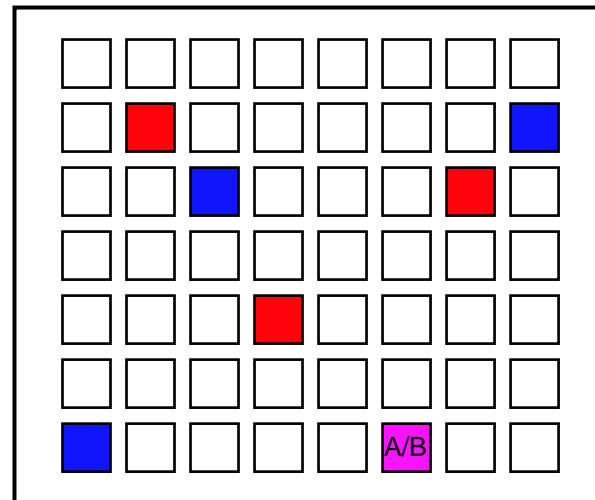


Paraffin section

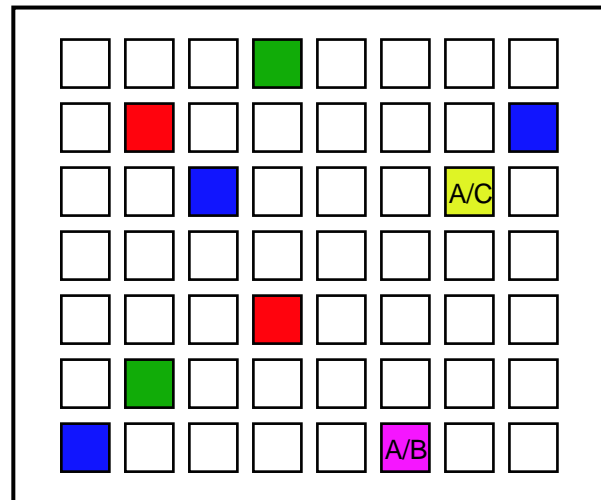
Stain A
→
■



Stain B
↓
■



Stain C
←
■



Current State

3 Colors, no more than 2 colors in same spatial compartment

In Development

3 Colors in same spatial compartment

Future

High order multiplexing with nanotechnology

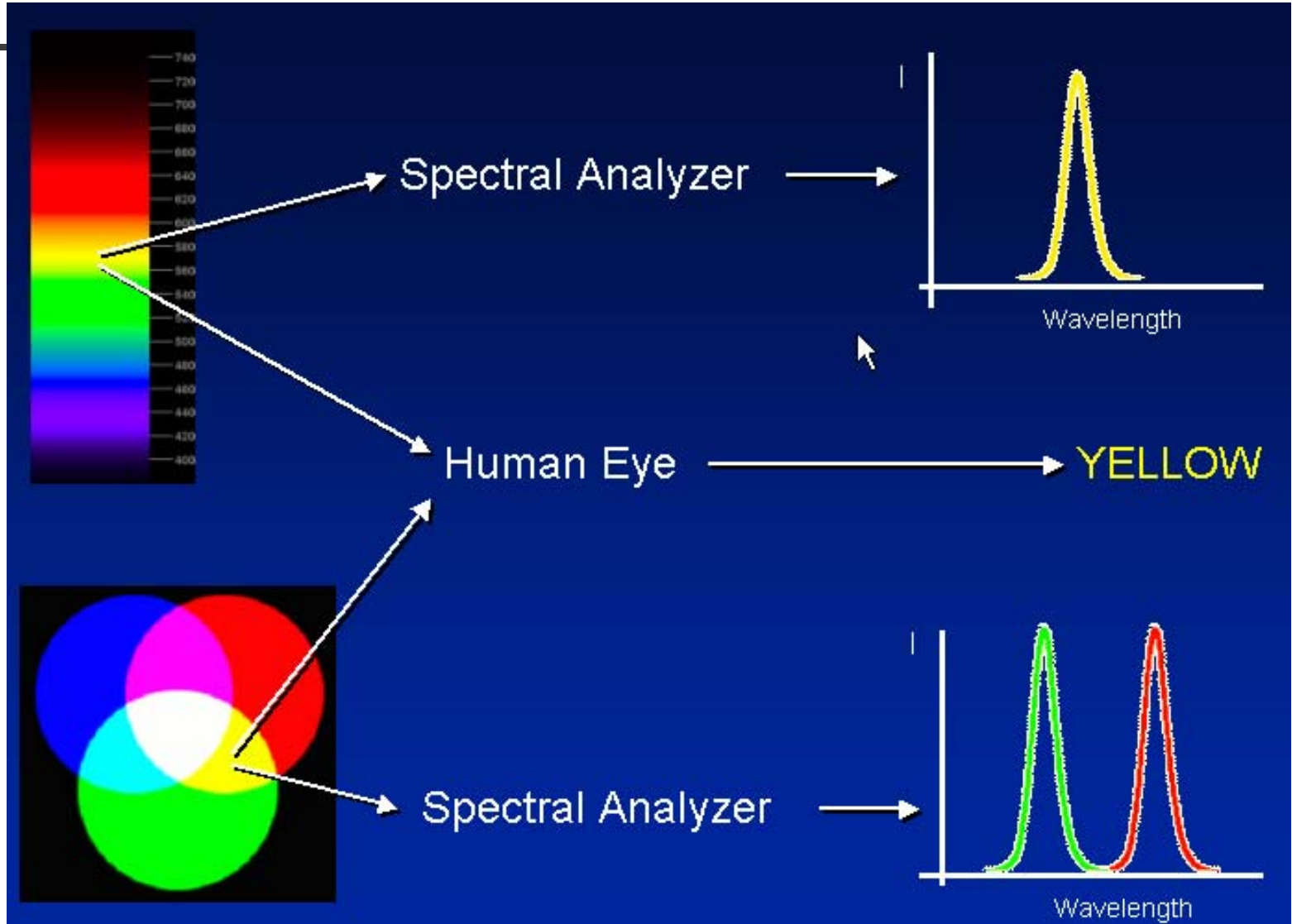
2. Multispectral Hardware

- Dispersive Elements
 - Prism – PARISS
 - <http://www.lightforminc.com/>
 - Dispersive
- Tunable Liquid Filters
 - CRI -
 - www.cri-inc.com



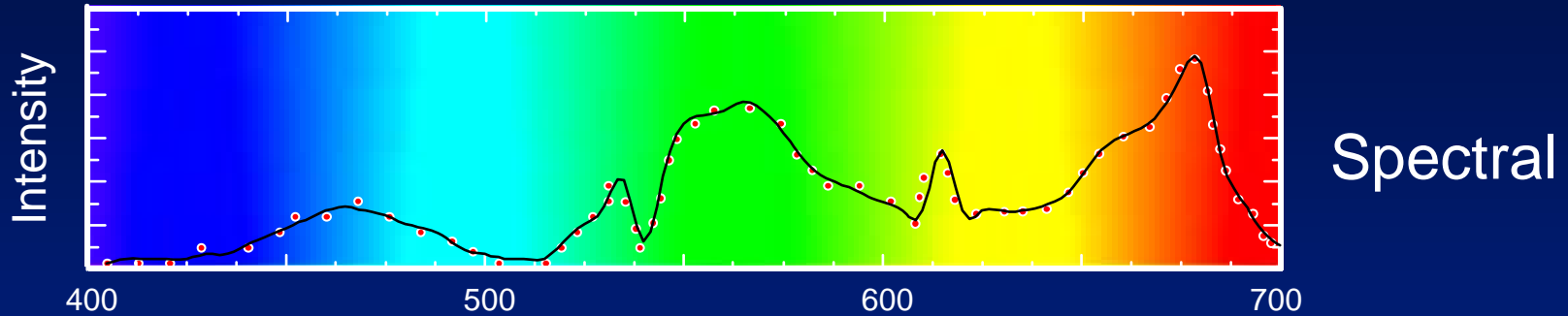
Color and Spectra

Light has no color. "Color" is an observer interpretation.
These two yellows appear identical to the human eye, yet they have very different spectral components.

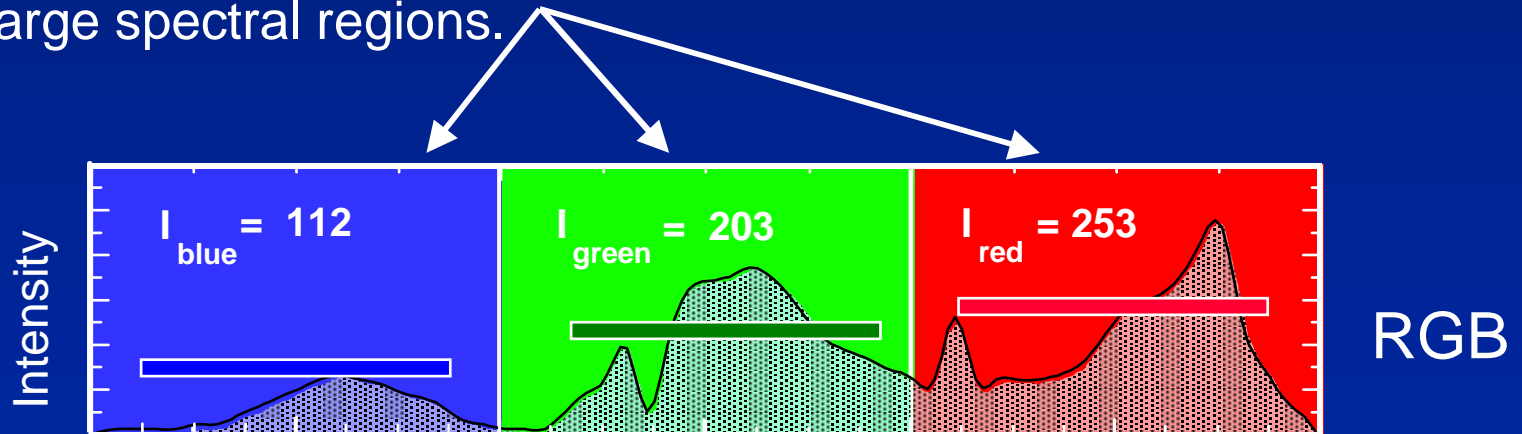


MSI vs. Traditional Color Imaging

Spectroscopy captures the entire spectrum (light intensity as a function of wavelength).

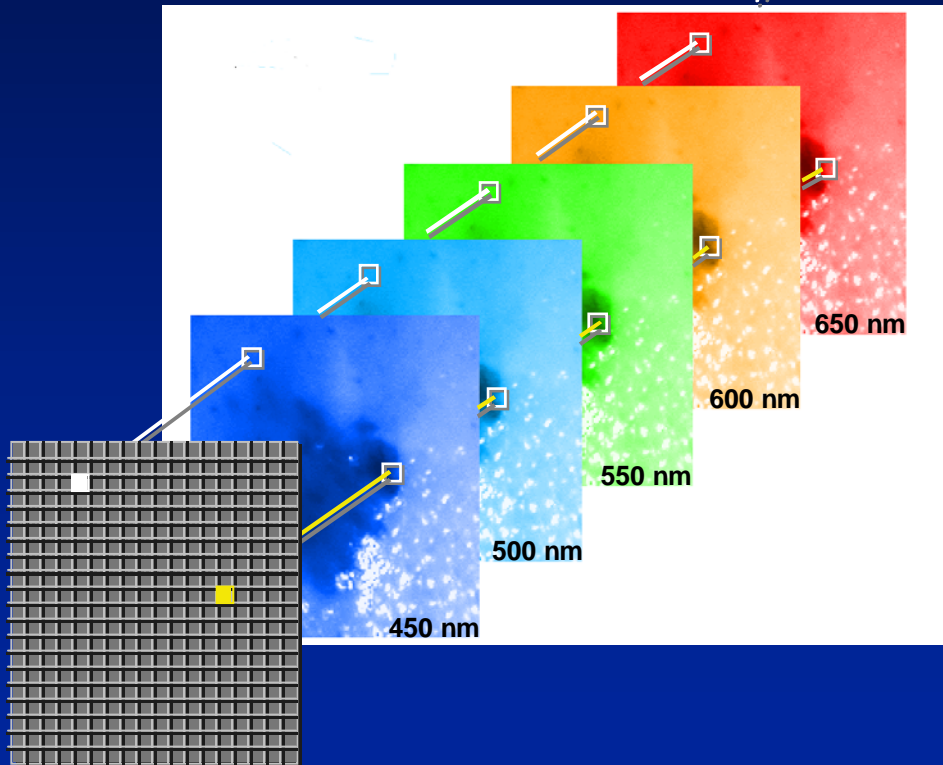


But with RGB-based instrumentation, this complex spectrum will be described using only 3 values (bins) averaged over large spectral regions.

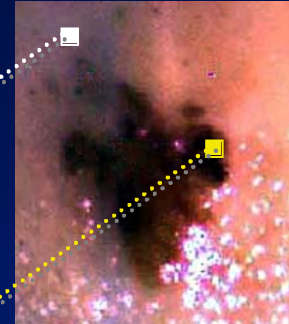


Multispectral Imaging

Take images at different wavelengths using a CCD



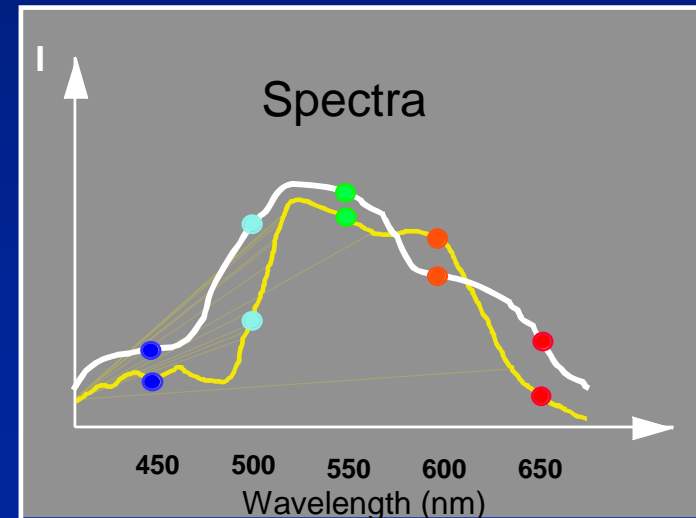
Melanoma



Assemble the data into a "cube" in computer memory

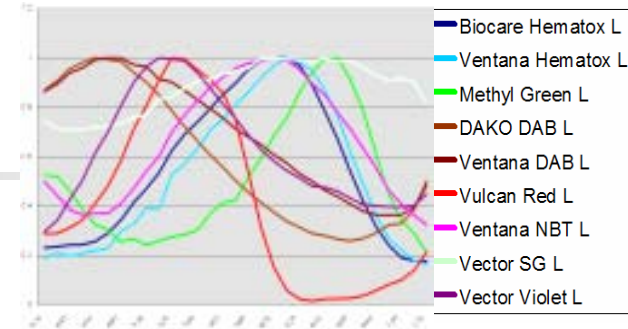
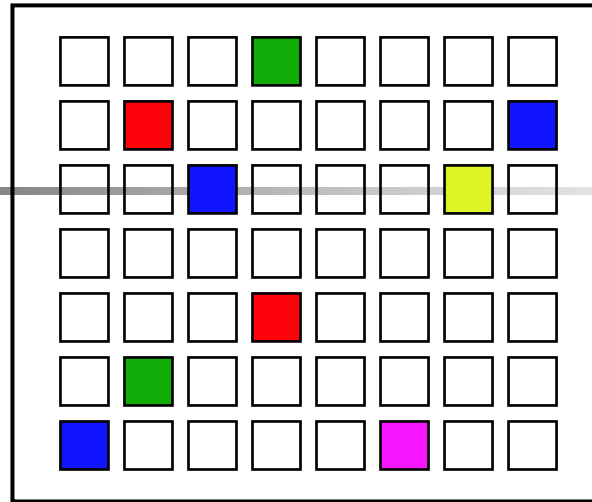
CCD

This creates an optical spectrum at every pixel of the image



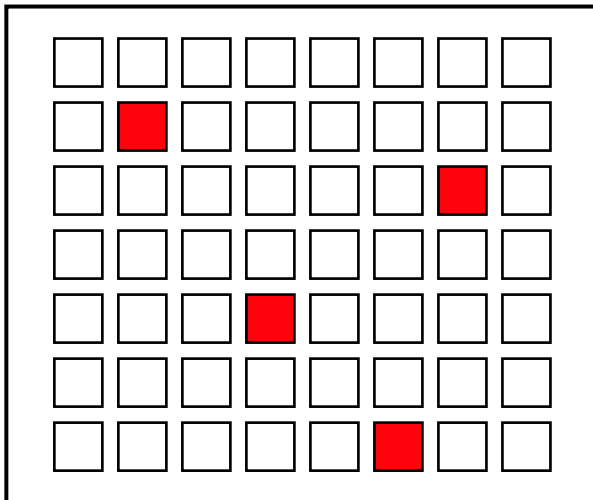
3. Image processing to resolve individual stains based on spectra

teach computer the spectral profile of chromogen/fluorophore A, B, C

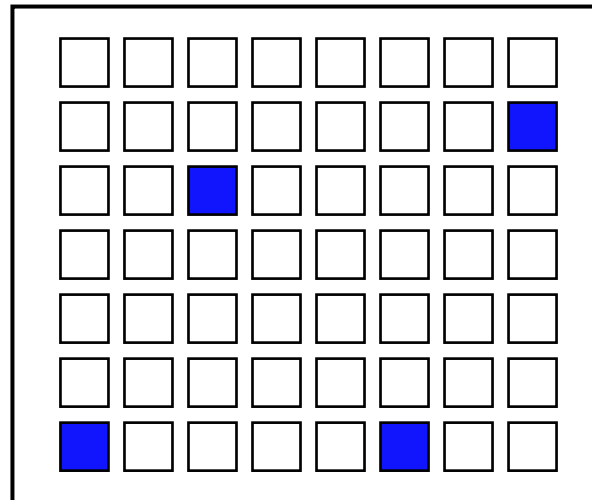


“extract” spectra of individual stains

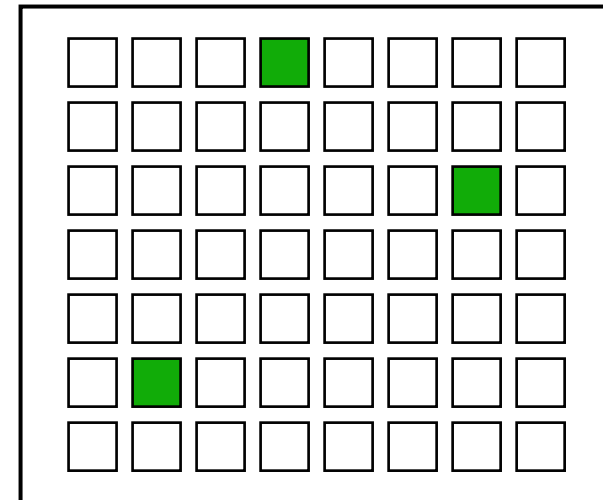
A



B

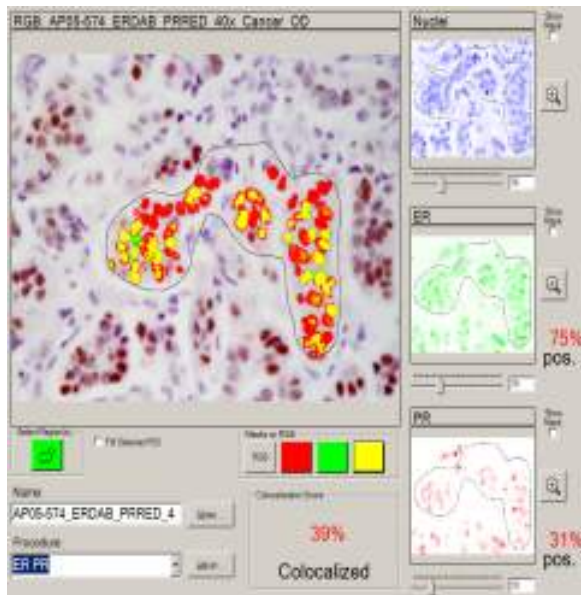


C

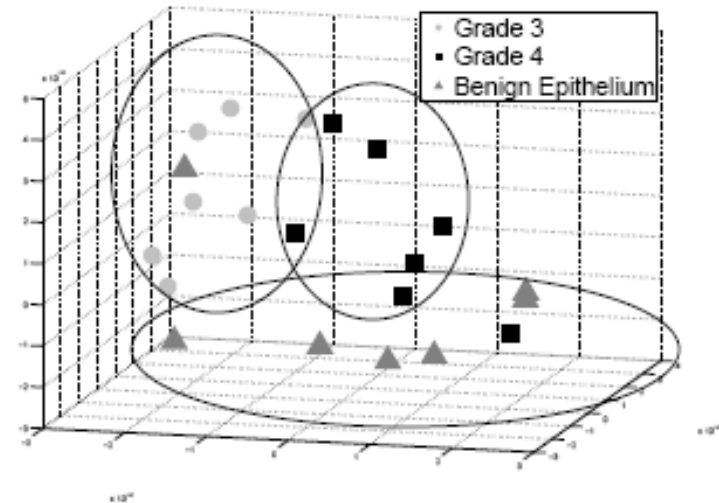


4. Tumor Segmentation

Manual Segmentation

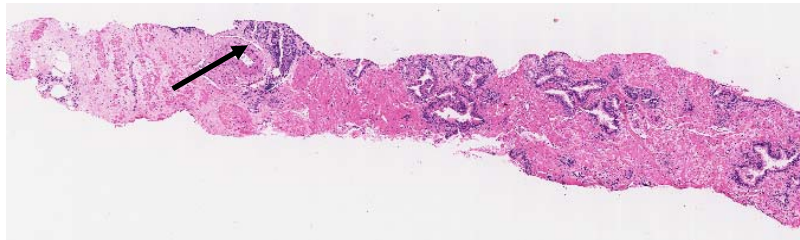


Machine Segmentation



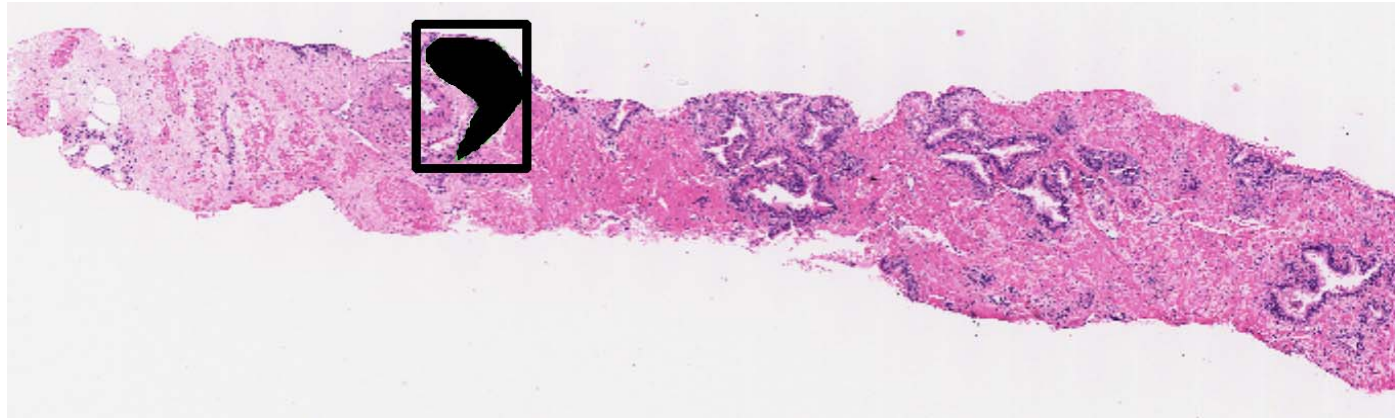
(b)

CAD – Computer Assisted Dx



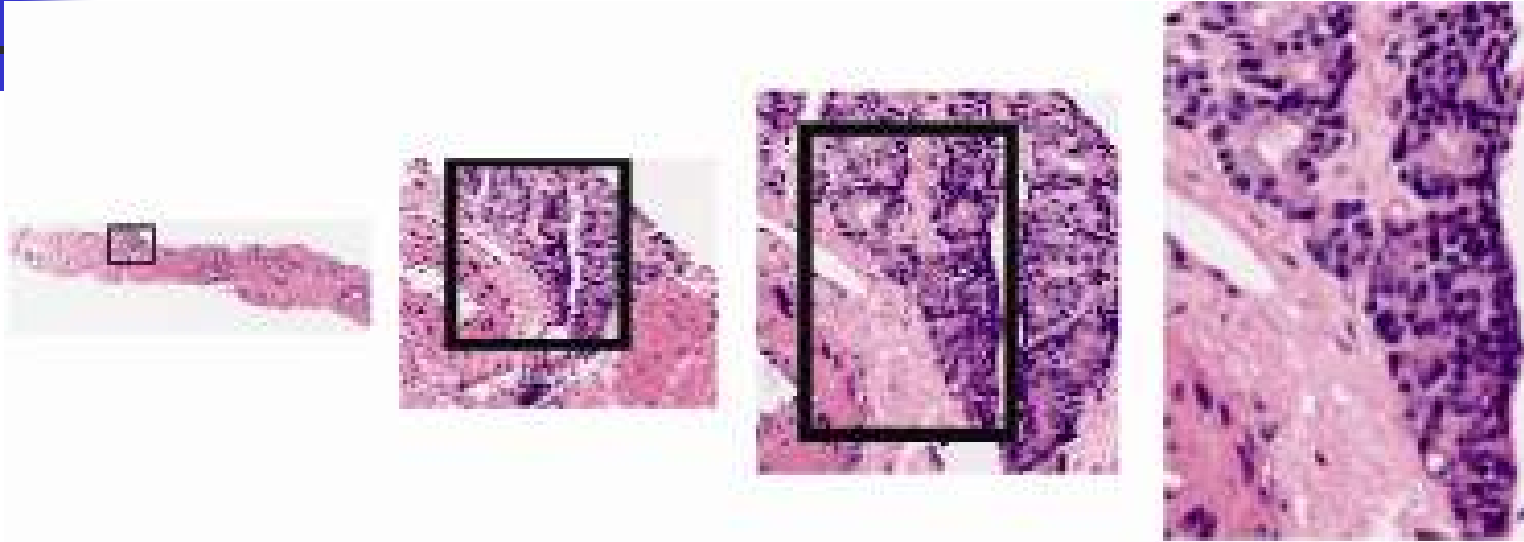
- Can we develop software to identify Prostate Cancer in whole digitized slides?
- Utility
 - Disease level Segmentation for IHC/IF,
 - CAD for Rescreening or Primary Screening

Introduction



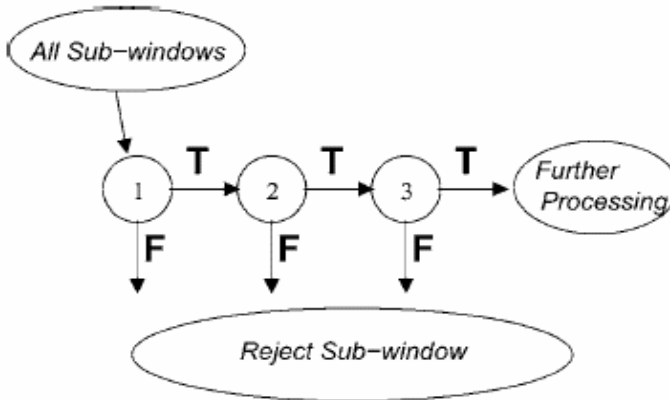
- Most of the image is benign
- Can we reduce image size by excluding benign areas
- Combine this with scale information to greatly increase the efficiency of the detection procedure

Introduction



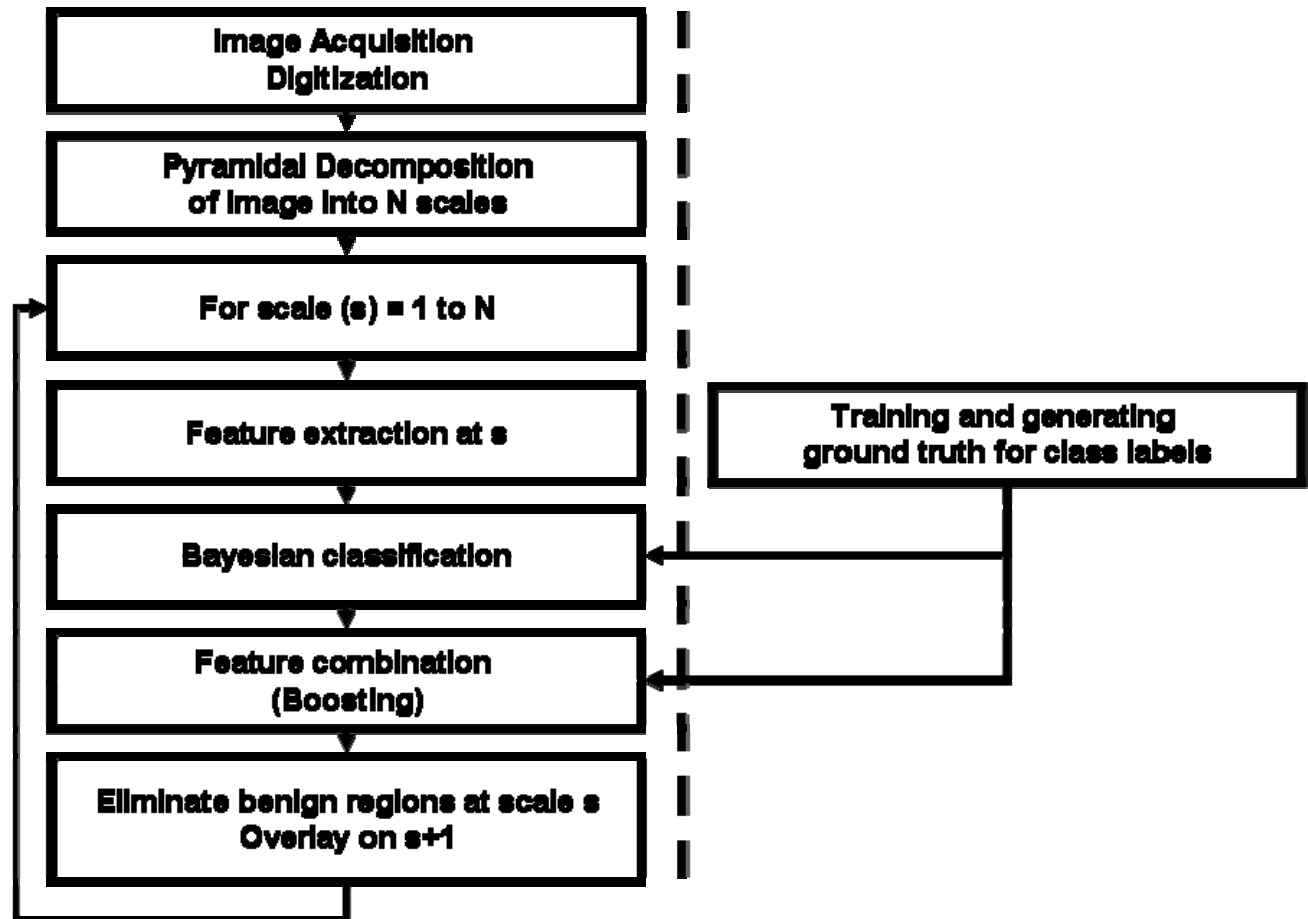
- Each “pass” of the image rejects pixels, and then only the positive pixels are analyzed at higher scales
- This allows us to efficiently analyze the image at higher scales by only looking at “interesting” pixels
- Increasing accuracy does NOT increase execution time

Introduction



- Viola and Jones [1] introduced the "attention cascade"
- By quickly rejecting image regions that are definitely negative using a minimum of features, we can concentrate computation on regions that might be positive

Methods



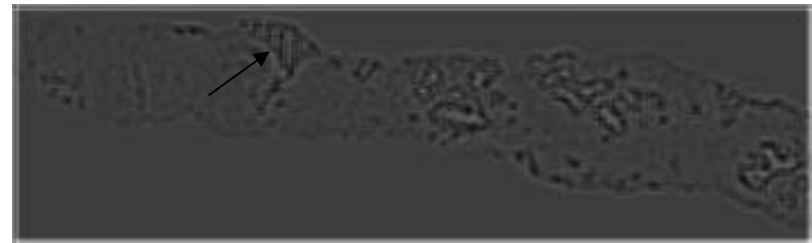
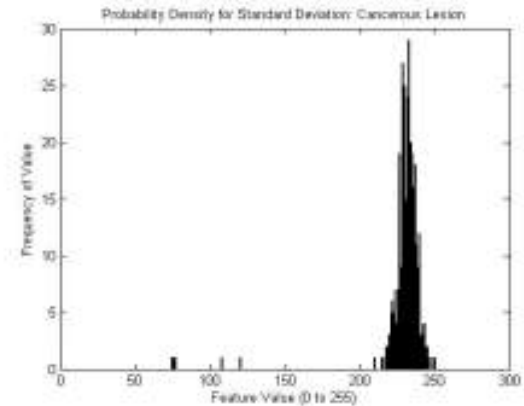


Methods

- To describe each pixel, we extract ~ 600 image features from the image
- There are three categories of features:
 - Statistical Features
 - Co-occurrence (texture) Features
 - Gabor Filter Features
- Each of these is extracted from the three channels of the image, at three different window sizes

Methods

- Probability density functions are constructed using the ground truth pixels provided by a pathologist
- These PDFs are used to assign a likelihood value to each pixel based on the feature value



Standard Deviation



Methods

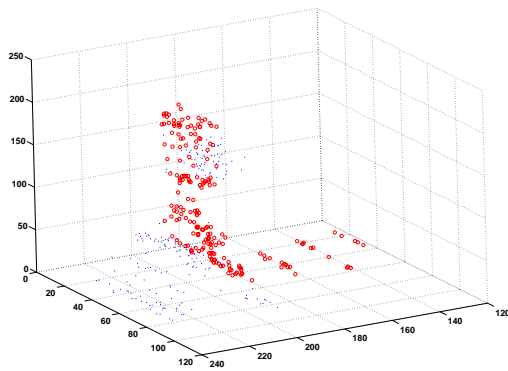
- The individual feature likelihoods are combined by AdaBoost [3] to get a likelihood ensemble
- A small number of features are used at scale 0 to obtain the ensemble, which is then thresholded
- The process begins over again at the next scale; only pixels labeled as positive at the previous scale are analyzed



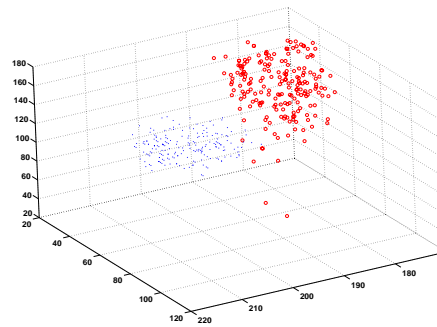
Results

- Graph of the computation time used to visualize efficiency in using the cascade
- Visual comparison of the likelihood ensembles with the tumor mask
- Receiver operating characteristic (ROC) curves were used to measure accuracy
- Scatter plots visualize the separation of classes

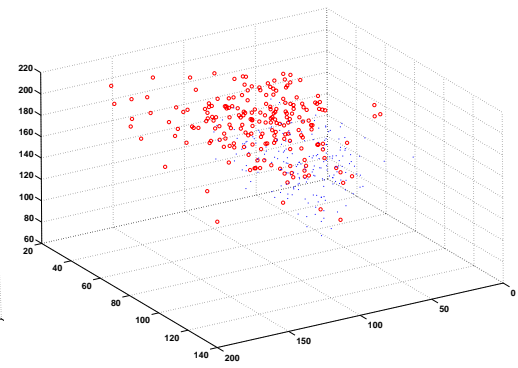
Results



Separation at Scale 0

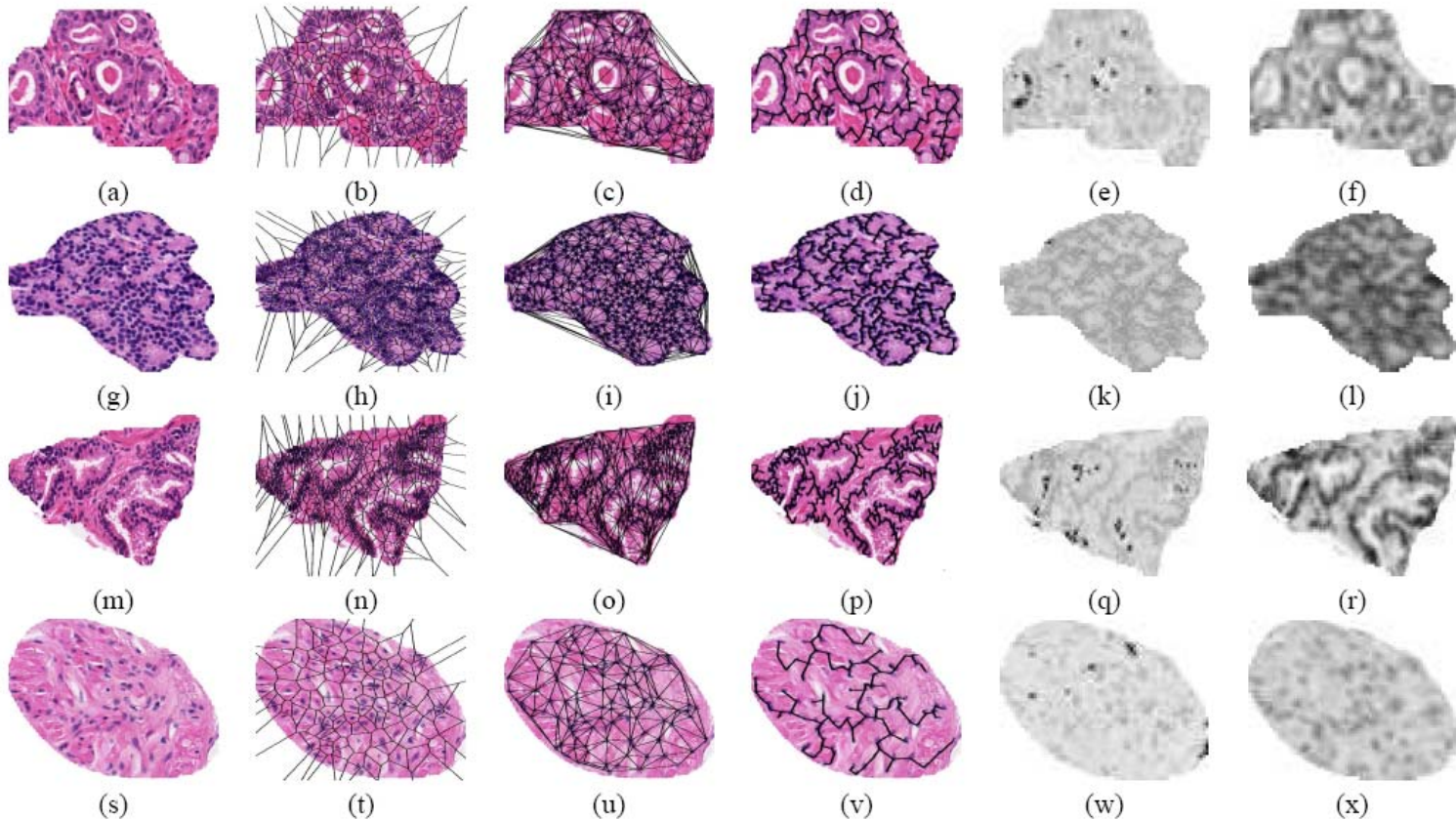


Separation at Scale 1

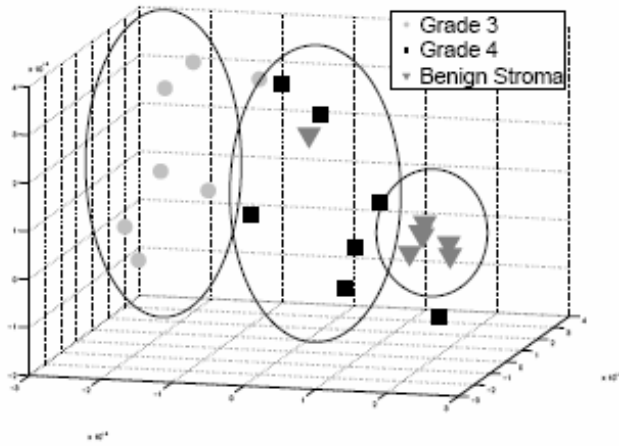


Separation at Scale 2

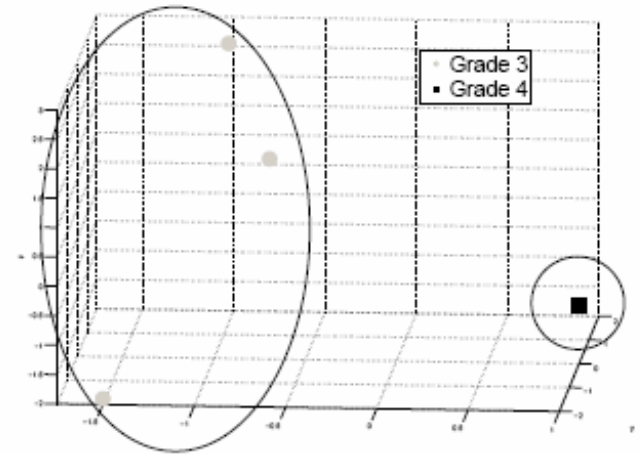
Higher scale analyses



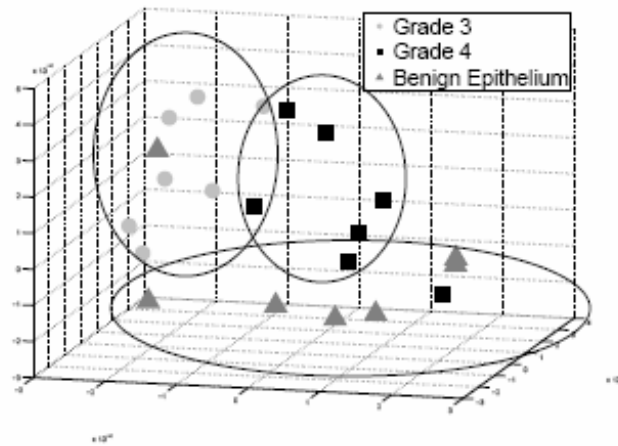
Higher scale



(a)



(c)



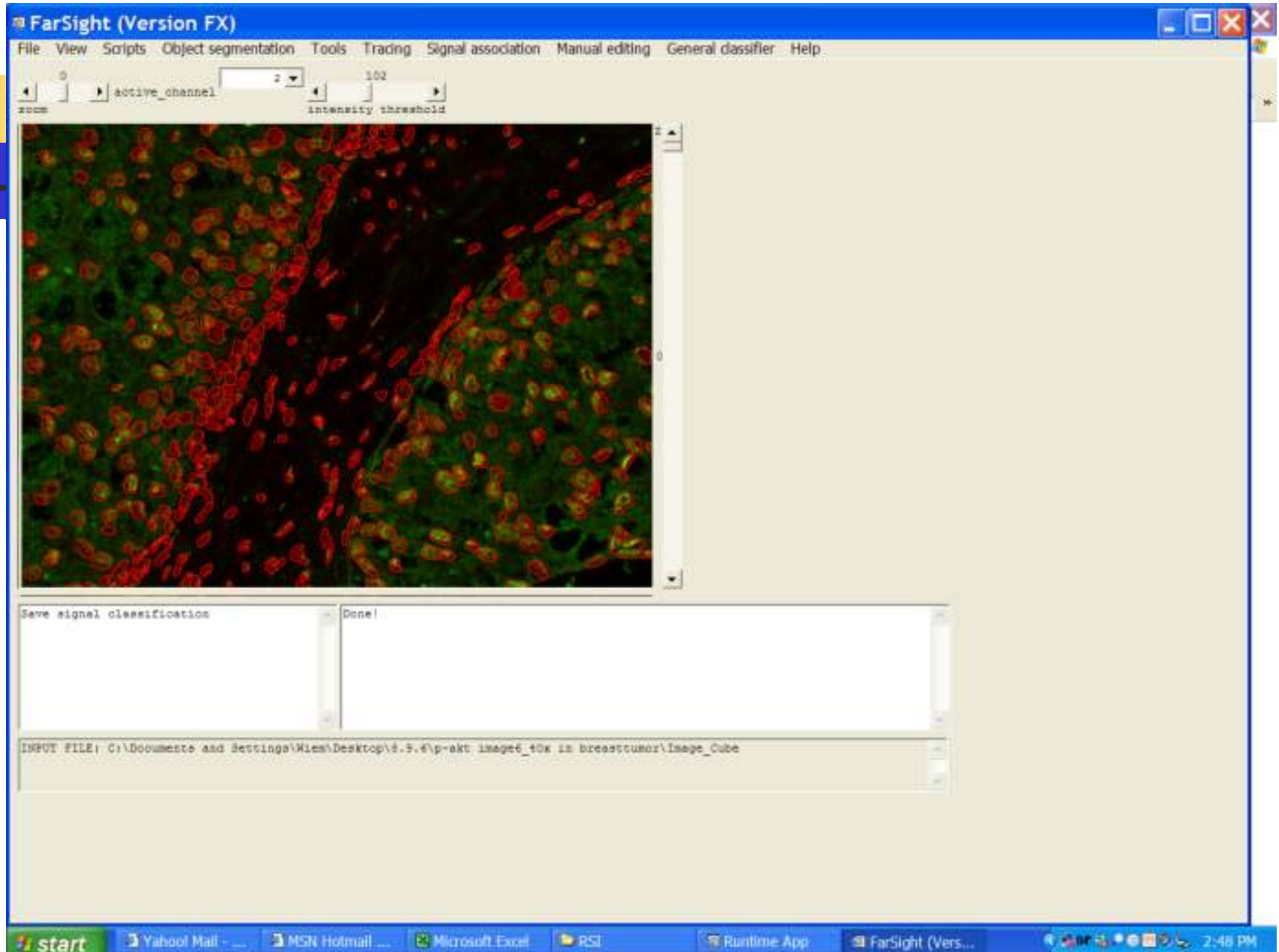
(b)



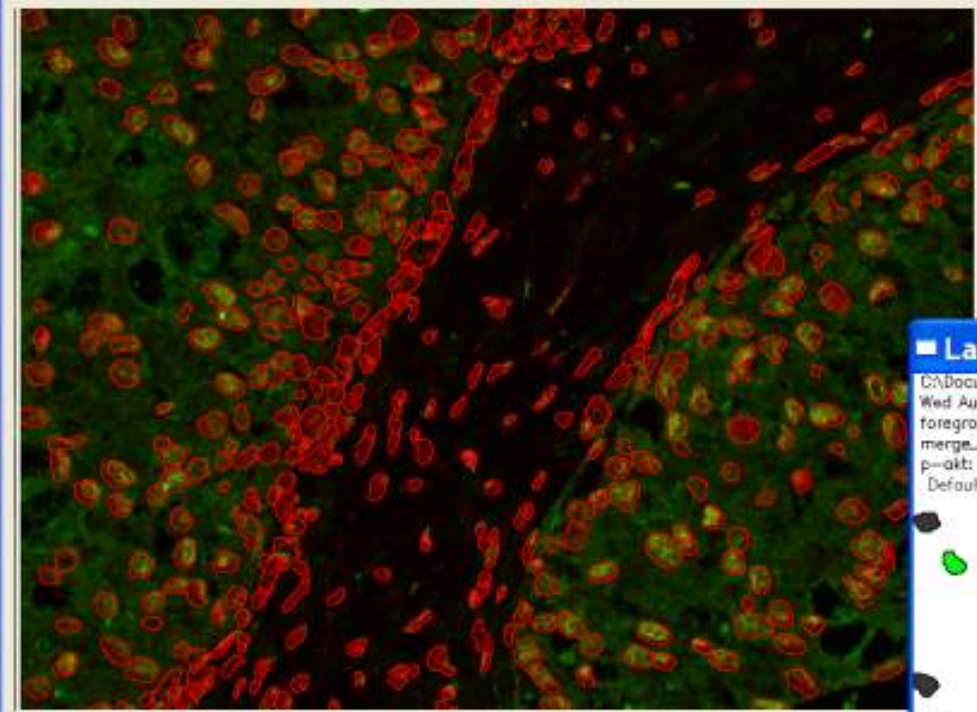
CAD Conclusions

- Using the Hierarchical cascade allows for fast, accurate analysis of large biomedical images
- Such a methodology could be employed in a number of scale-sensitive image analysis systems
- Both the cascade and the analysis itself can be applied to various pathologies and imaging modalities
- The separation of classes as the scales increase indicates that more discriminatory information is available at higher scales
- Higher scales studies demonstrate the capacity to distinguish stroma vs benign glands from Gleason grade 3 and grade 4 carcinoma

5. Computational identification of nuclei in image using Farsight software



0 2 102
active_channel intensity threshold



Save signal classification Done!

INPUT FILE: C:\Documents and Settings\Wiem\Desktop\8.9.6\p-akt image6_40x in bree

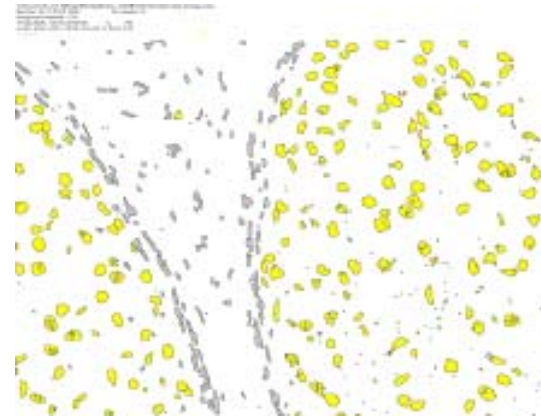
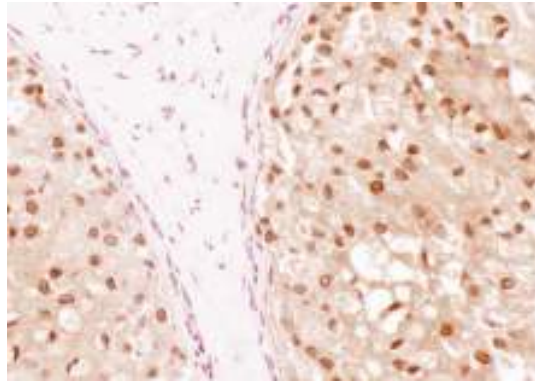
Label image...

C:\Documents and Settings\Wiem\Desktop\8.9.6\p-akt image6_40x in breastumor\image_Cube
 Wed Aug 09 14:50:03 2006 GUI version: FX
 foreground threshold: 0.20
 merge_depth, volume_threshold: 3, 33
 p-akt: zone: 0.00<->5.00, max_int: 0, thresh: 2.00
 Default: p-akt+

6. Computational assignment of immunostains to each nucleus

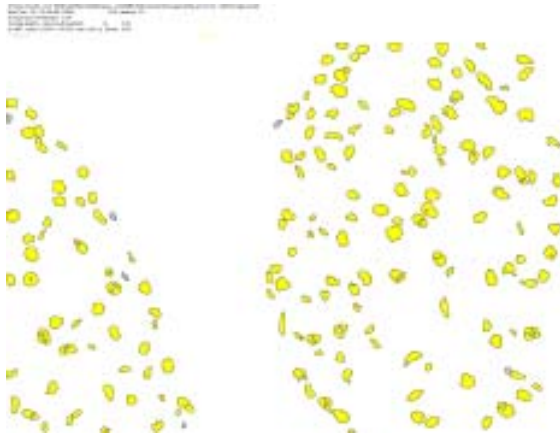
breast tumor stained
for p-ERK (DAB) & hematoxylin

segmentation of nuclei
(based on hematoxylin)



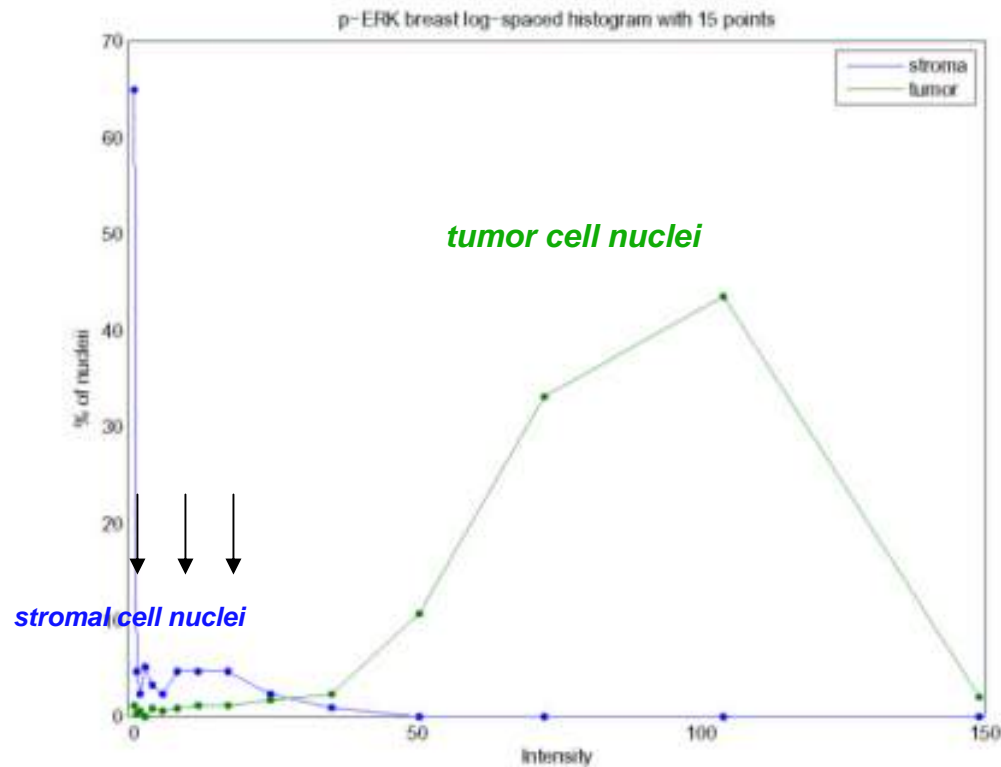
p-ERK+ tumor cells (yellow)

p-ERK- stromal cells (grey)



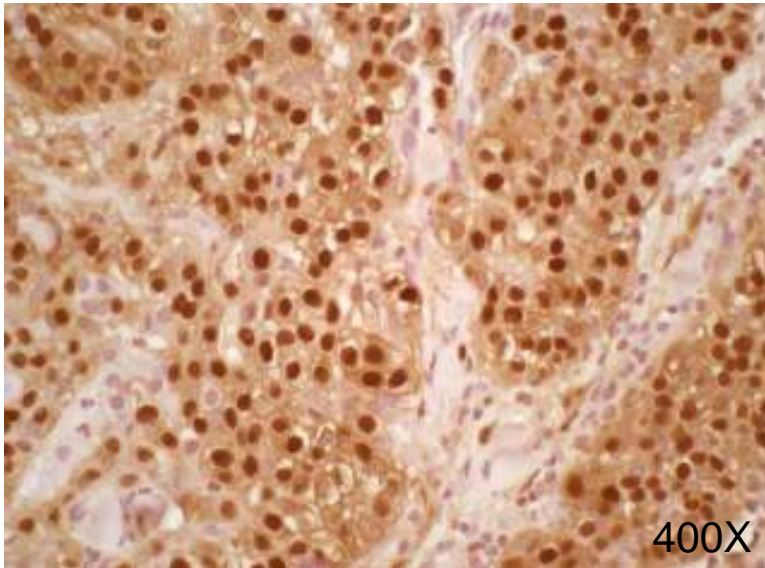
7. Data display & Analysis:

Frequency histogram of intensity of p-ERK staining of stromal and tumor cell nuclei in a breast tumor

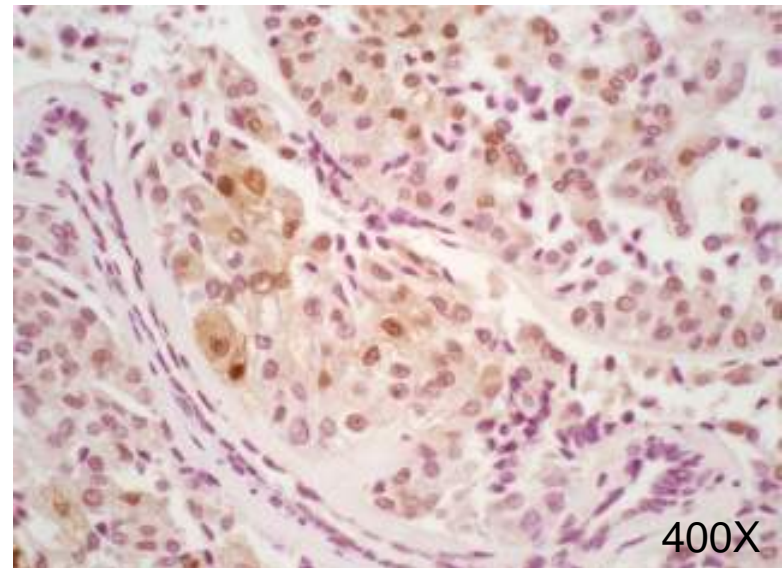


Pharmacodynamics:
p-ERK immunostaining in thyroid carcinoma treated with sorafenib

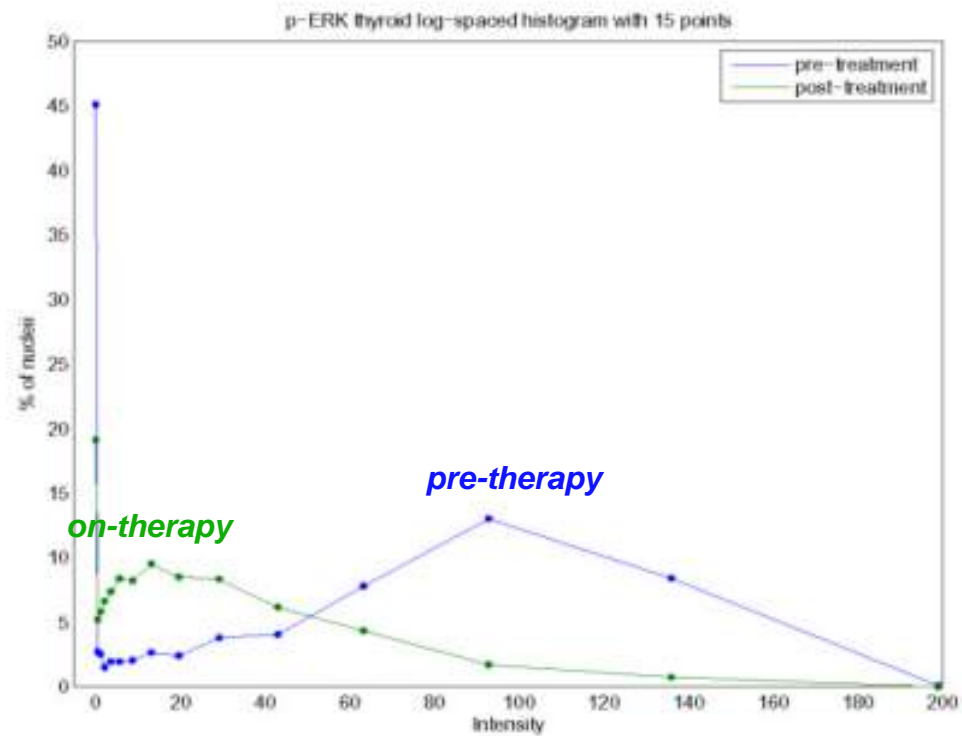
Pre-therapy



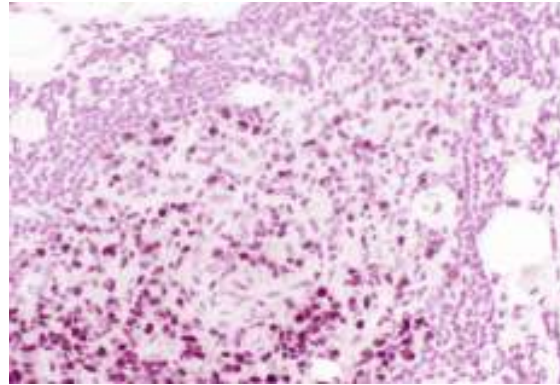
On-therapy (1 week)



Frequency histogram of intensity of p-ERK staining of thyroid carcinoma nuclei



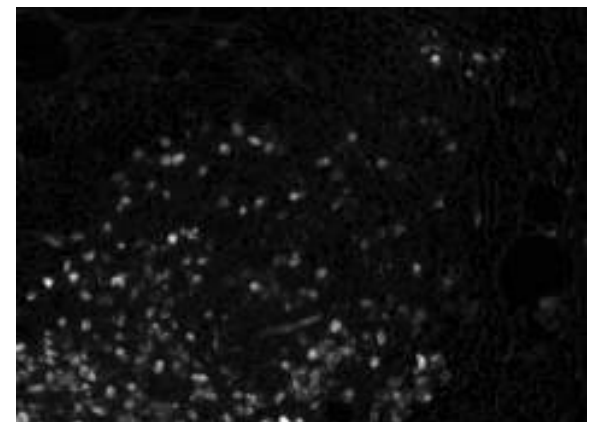
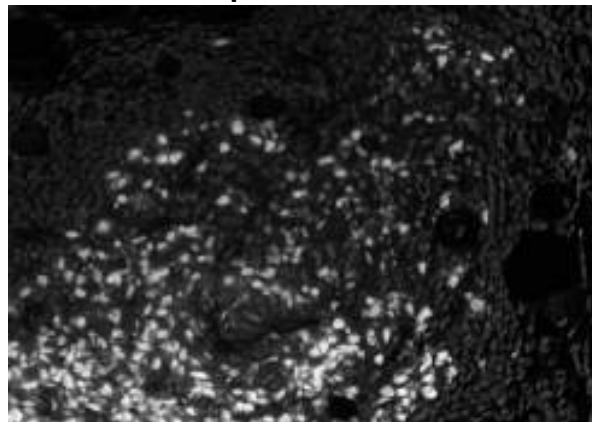
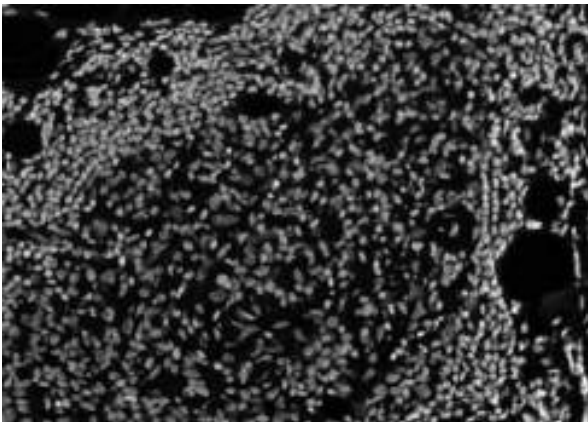
Two-parameter analysis: Germinal center stained
p-ERK (SG blue), Ki-67 (VIP) and hematoxylin



Hematoxylin

p-ERK

Ki-67



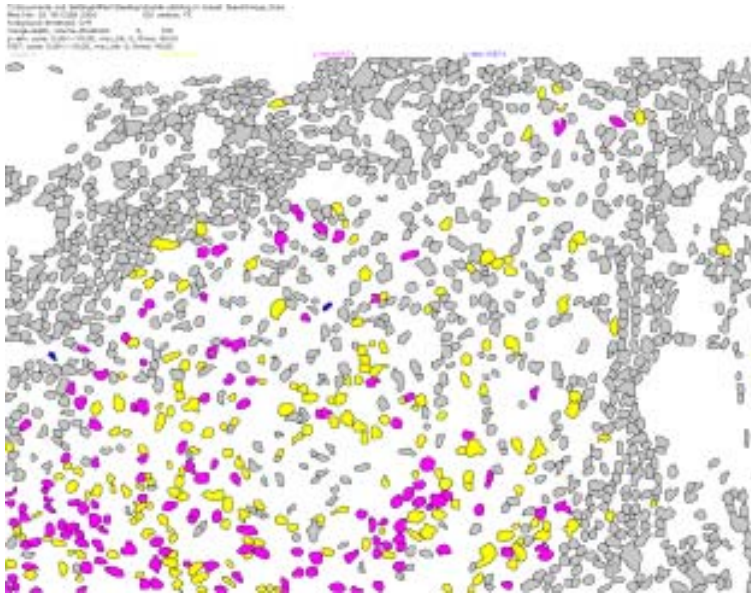
A pathologist would find it very hard to classify nuclei as positive or negative from RGB image

Nuclear segmentation

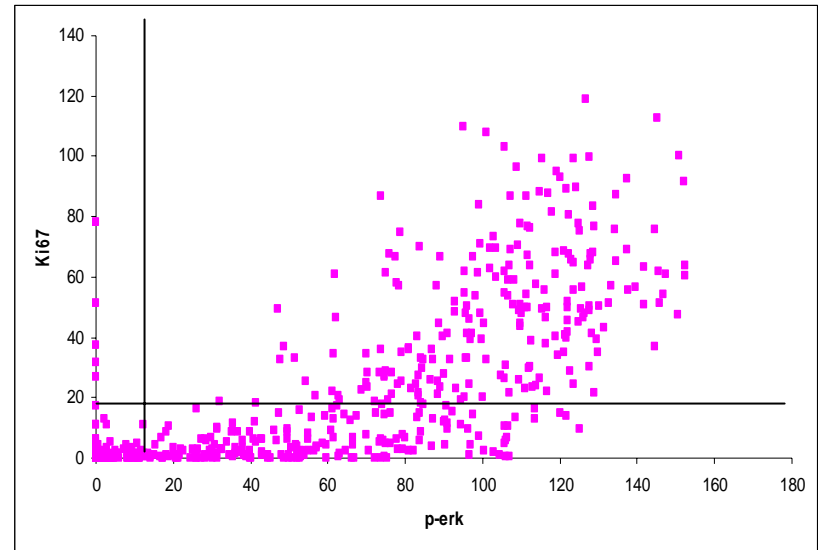
p-ERK+/Ki-67- (yellow)

p-ERK-/Ki-67+ (blue)

p-ERK+/Ki-67+ (magenta)

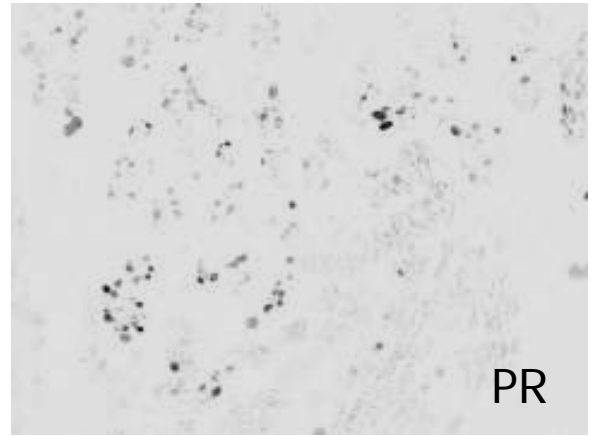
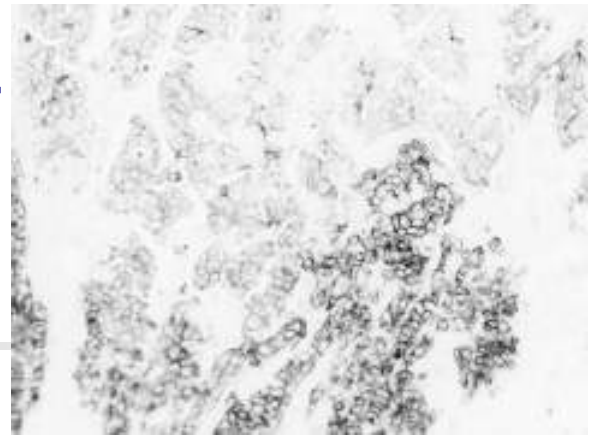
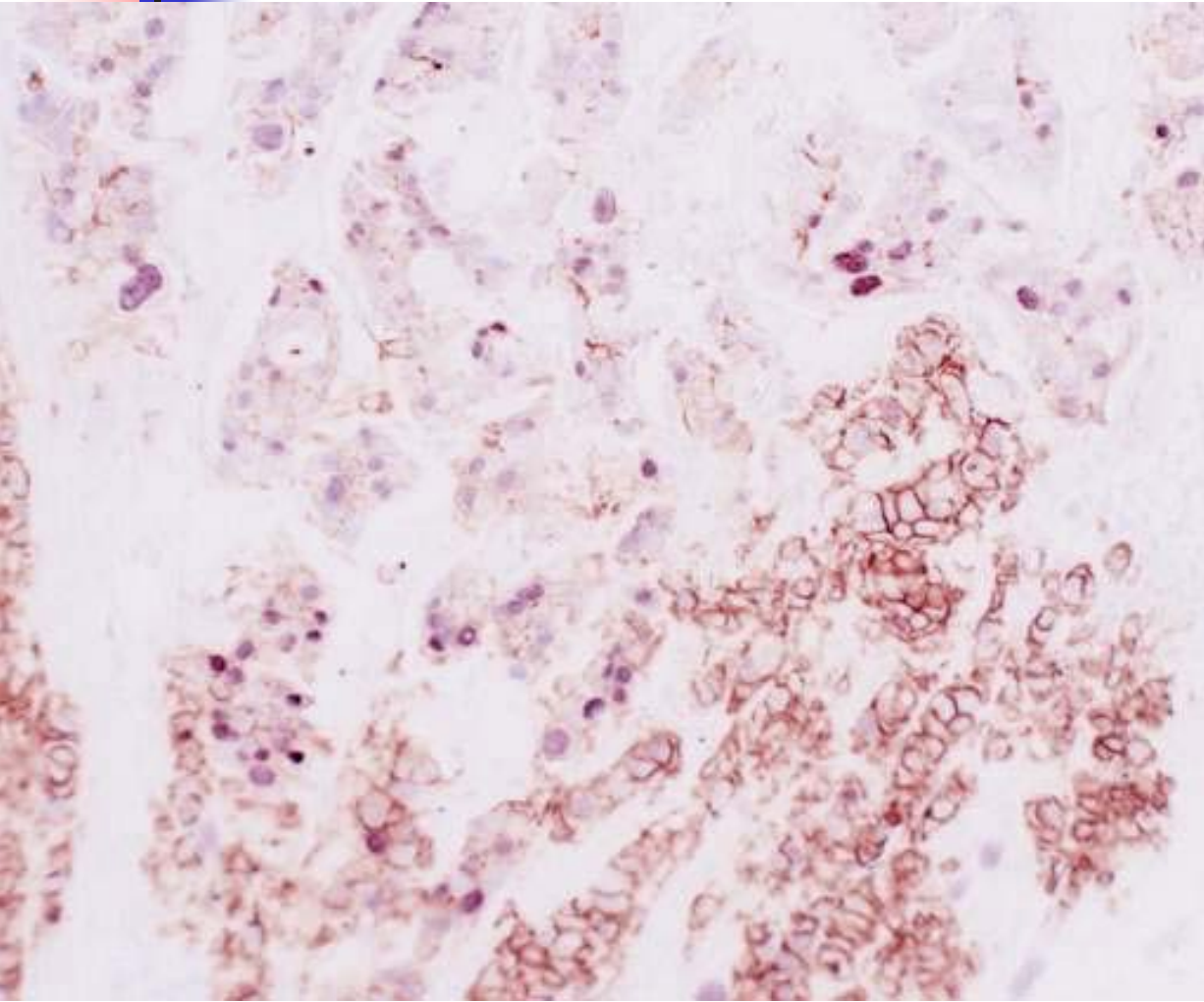


2-antigen scatter plot (from 1 image)

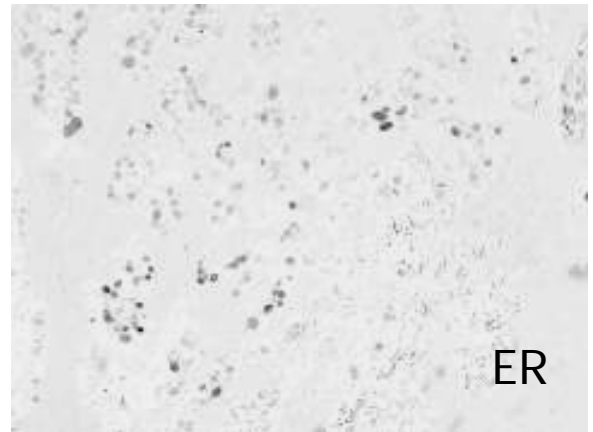


Analysis of antigen association (apparently present) in cells in tissues

Triple stain for breast (ER/PR/Her2)



PR



ER



Breast Carcinoma

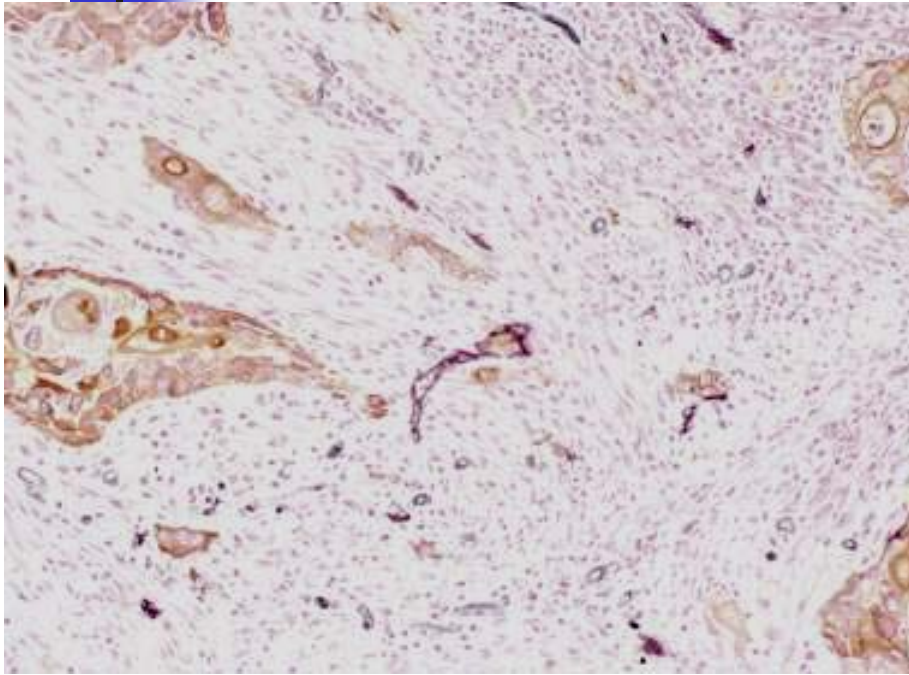
- Lapatinib – Dual inhibitor EGFR and Her2
 - Who responds?
 - How to select?
 - Co-expression of ErbB2 and IGFR
 - Poor response to Trastuzumab (Herceptin)
 - Favorable response to Lapatinib
 - Low PTEN and ErbB2 poor response to Trastuzumab (Herceptin)
 - Key is going to be use of multiple biomarkers
 - Extend segmentation beyond nuclei to include membrane



MSI Applications

- Immunohistochemistry
 - Routine tests
 - New tests
- Immunofluorescence – Clinical Trials
- Background Fluorescence

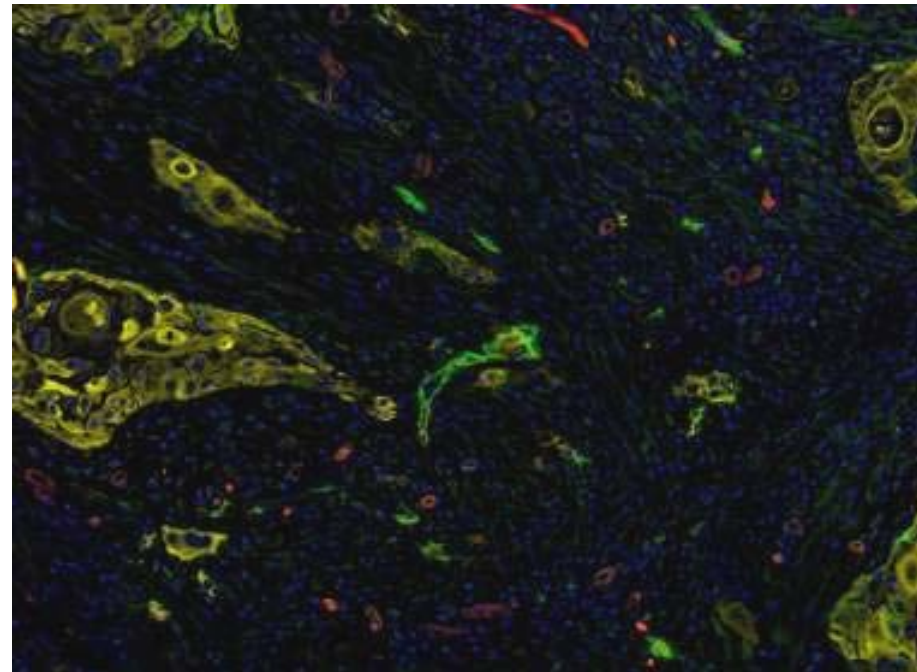
Lymphovascular Invasion Oral SCC



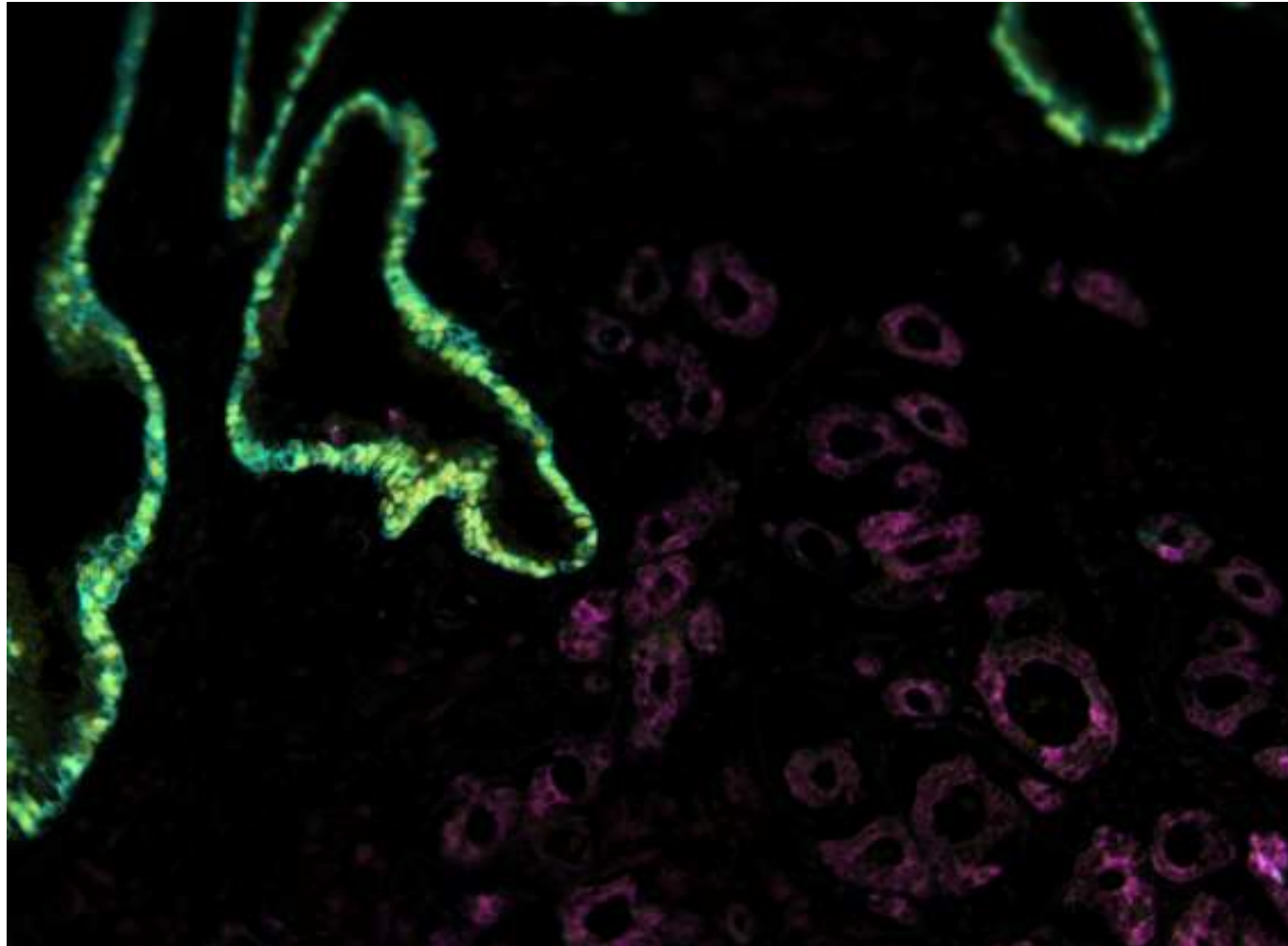
Cytokeratin – DAB (brown)

CD34 – Vector SG (blue)

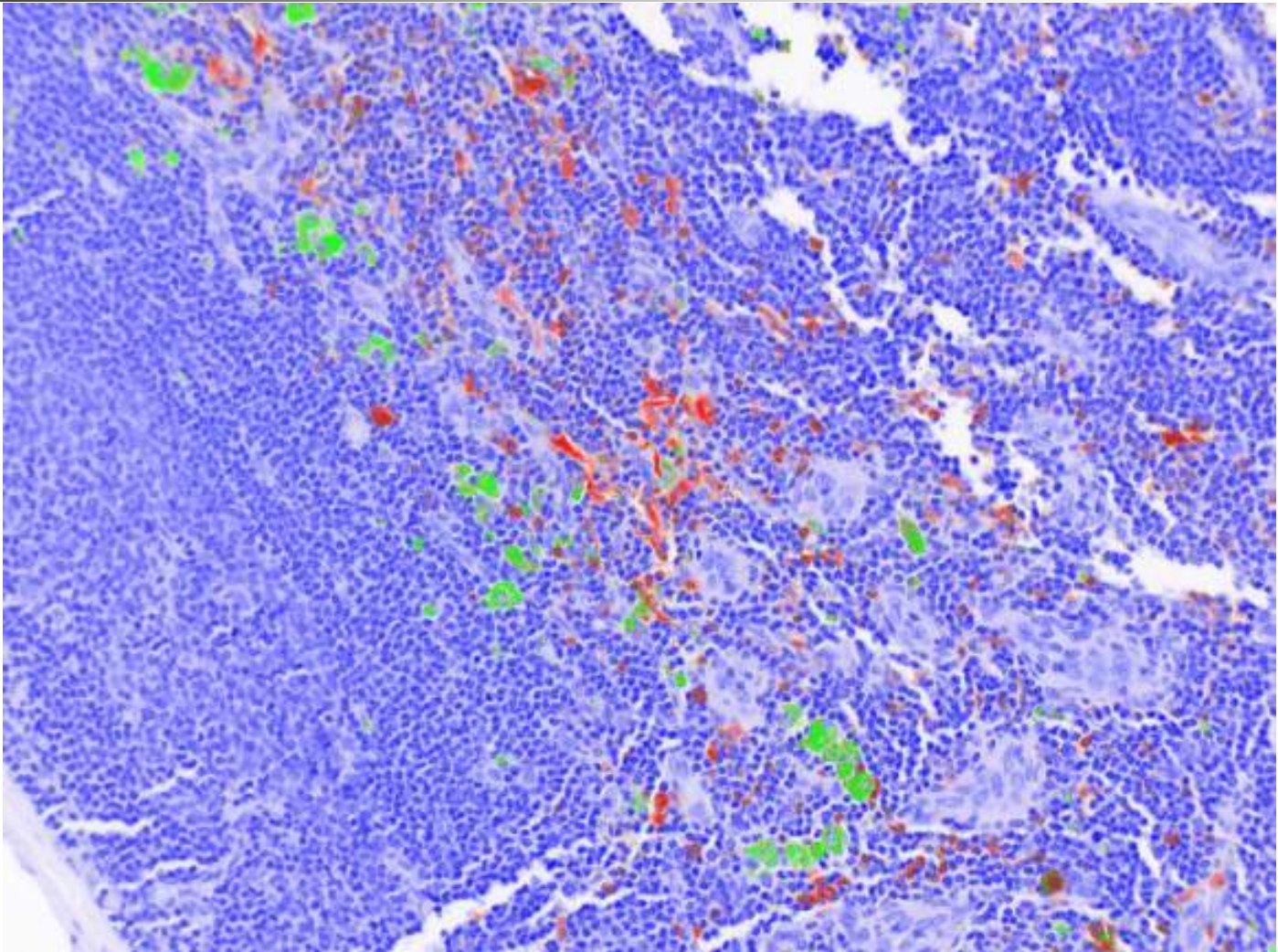
Podoplanin – Vector VIP (purple)



Prostate Cancer – Triple stain



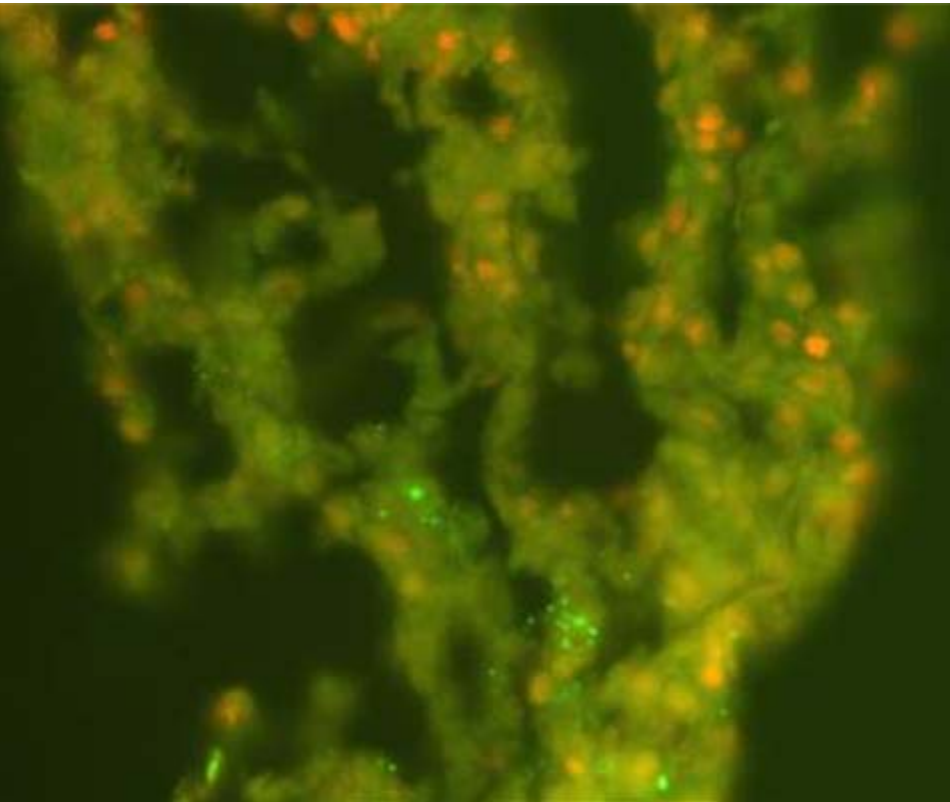
SLN Melanoma



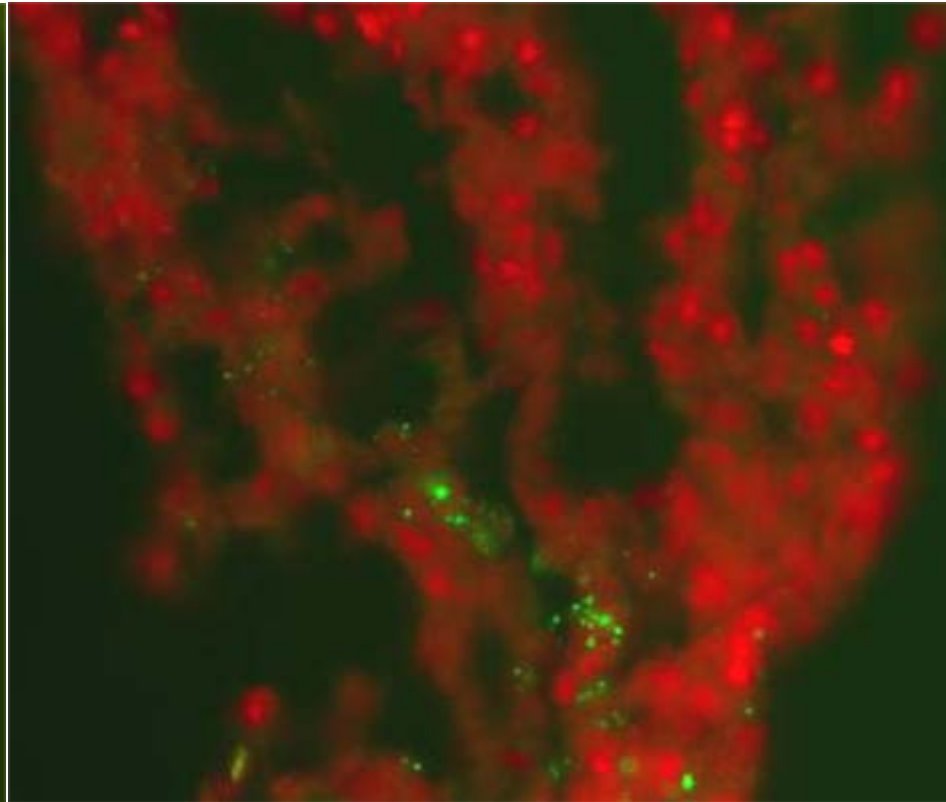


Autofluorescence

Original



Spectrally separated





Future directions

- Develop automated analysis package
 - Pathway analysis (5-6 colors) on FFPE tissue
 - Endothelial cells
 - Tumor cells
 - More routine analysis
 - ER, PR, Her2, EGFR, cKIT...
 - C4d in transplant rejection
 - Interact with Clinical Trial Workspace



Collaborators

- MSI work
- Penn - Bill Lee, Wiem Lassoud
- RPI - Badri Roysam, Gang Lin
- CRI - Richard Levenson, Cliff Hoyt

- CAD
- Anant Madhabushi
 - Scott Doyle