

Microvessel Analysis of Angiogenesis

Aperio European Science Seminar Series

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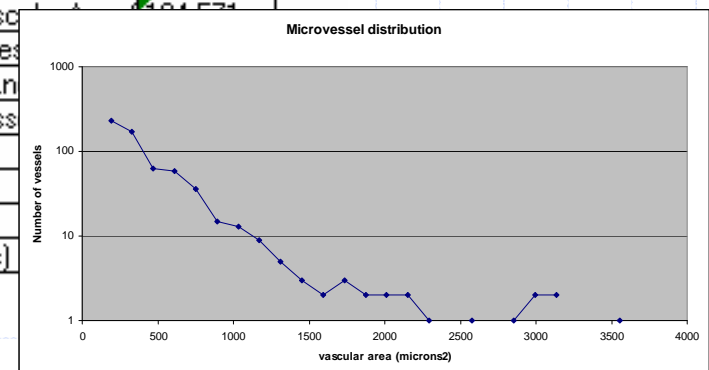
Objectives:

- Definition of terms
- The need for a multipurpose algorithm
- How it works: from finding endothelial cells to vessels to lumens and output statistics
- Examples
 1. Cardiovascular: matrigel plug assay in mouse skin for modeling of angiogenesis with Factor VIII (courtesy Reg Valdez, Novartis NIBR)
 2. Oncology: CD31 stained xenografts
 3. Ophthalmology: Factor VIII stained rodent eyes (courtesy Kim Paes, Lexicon Genetics)
 4. Hematology: Clinical AML bone marrows & Factor VIII (courtesy Maher Albitar, Quest Diagnostics)
 5. Kidney with endothelial stain (courtesy Children's Hospital, Boston)

Definitions

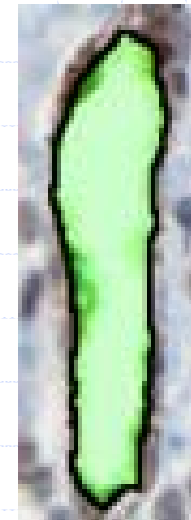
Output Histogram		1 - Vessel Area
Number of Histogram bins		0 - None
Endothelial Stain - Red Component		1 - Vessel Area
Endothelial Stain - Green Component		2 - Vessel Perimeter
Endothelial Stain - Blue Component		3 - Lumen Area
Background Stain 1 - Red Component		4 - Vascular Area
Background Stain 1 - Green Component		5 - Average Vessel Wall Thickness

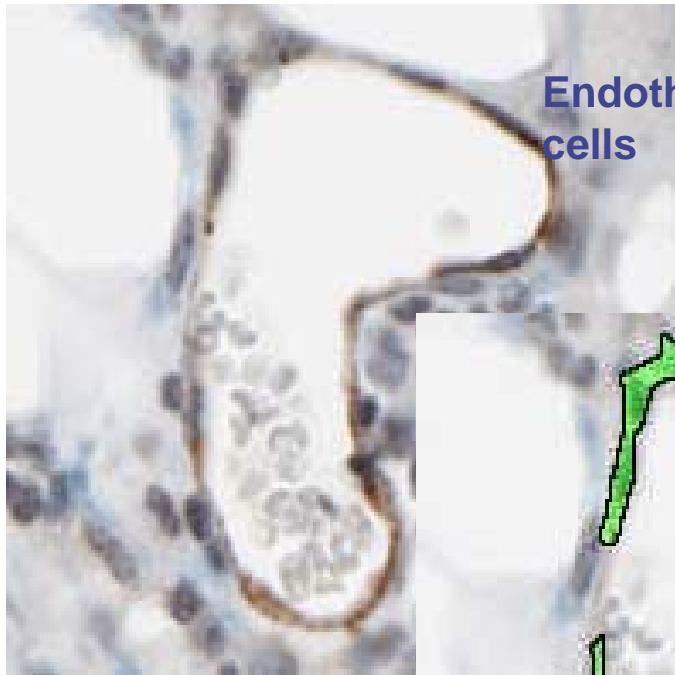
Tumor		Normal	
Number of Vessels	704	Number of Vessels	352
Total Analysis Area (um ²)	12811306.5361	Total Analysis Area (um ²)	4110861.42736
Total Stain Area (um ²)	95351.0895476	Total Stain Area (um ²)	62105.4188384
Average Stain Intensity	158.667	Average Stain Intensity	191.048
Mean Vessel Area (um ²)	143.066	Mean Vessel Area (um ²)	213.132
Median Vessel Area (um ²)	99.6743	Median Vessel Area (um ²)	120.566
Standard Deviation of Vessel Area (um ²)	135.16	Standard Deviation of Vessel Area (um ²)	301.661
Mean Vessel Perimeter (um)	65.1583	Mean Vessel Perimeter (um)	77.859
Median Vessel Perimeter (um)	50.17	Median Vessel Perimeter (um)	58.1972
Standard Deviation of Vessel Perimeter (um)	44.7857	Standard Deviation of Vessel Perimeter (um)	66.3825
Mean Lumen Area (um ²)	16.9771	Mean Lumen Area (um ²)	46.2962
Median Lumen Area (um ²)	0.	Median Lumen Area (um ²)	0.
Standard Deviation of Lumen Area (um ²)	65.3677	Standard Deviation of Lumen Area (um ²)	196.278
Mean Vascular Area (um ²)	114.144	Mean Vascular Area (um ²)	157.154
Median Vascular Area (um ²)	82.3068	Median Vascular Area (um ²)	98.4158
Standard Deviation of Vascular Area (um ²)	105.778	Standard Deviation of Vascular Area (um ²)	284.574
Mean Vessel Wall Thickness (um)	1.5273	Mean Vessel Wall Thickness (um)	1.5273
Median Vessel Wall Thickness (um)	1.47374	Median Vessel Wall Thickness (um)	1.47374
Standard Deviation of Vessel Wall Thickness (um)	0.402804	Standard Deviation of Vessel Wall Thickness (um)	0.402804
Average Red OD	0.510168	Average Red OD	0.510168
Average Green OD	0.782813	Average Green OD	0.782813
Average Blue OD	0.356275	Average Blue OD	0.356275
Total Analysis Area (Pixels)	50898531	Total Analysis Area (Pixels)	50898531



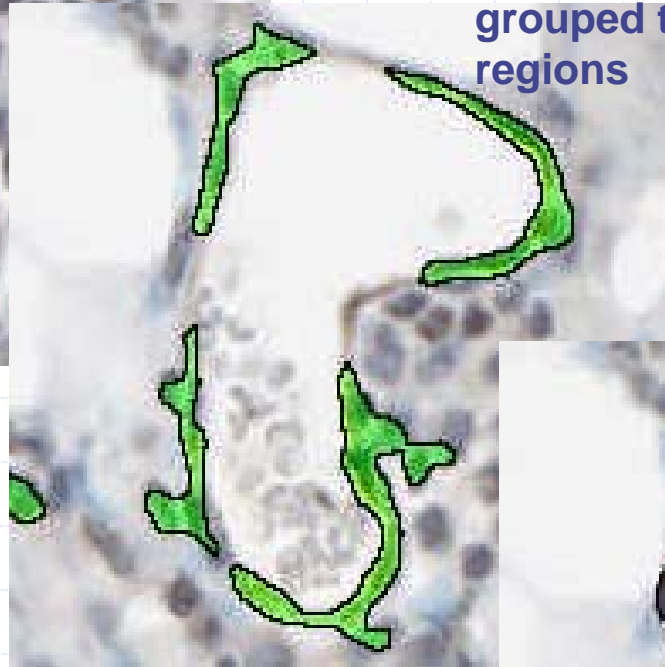
Definitions:

- Endothelial cell: epithelial cell that lines circulatory vessel
- Region (computer putting endothelial cells together into one intermediate object)
- Vessel: connection of regions into one object, typically with a lumen
- Incomplete vessel – does not have a lumen
- Vessel wall: endothelial cells that form this wall
- Vascular area: sum of all endothelial cells
- Vessel area: vascular area + lumen area (approx)
 - We use vessel area = vascular area/2 + lumen area



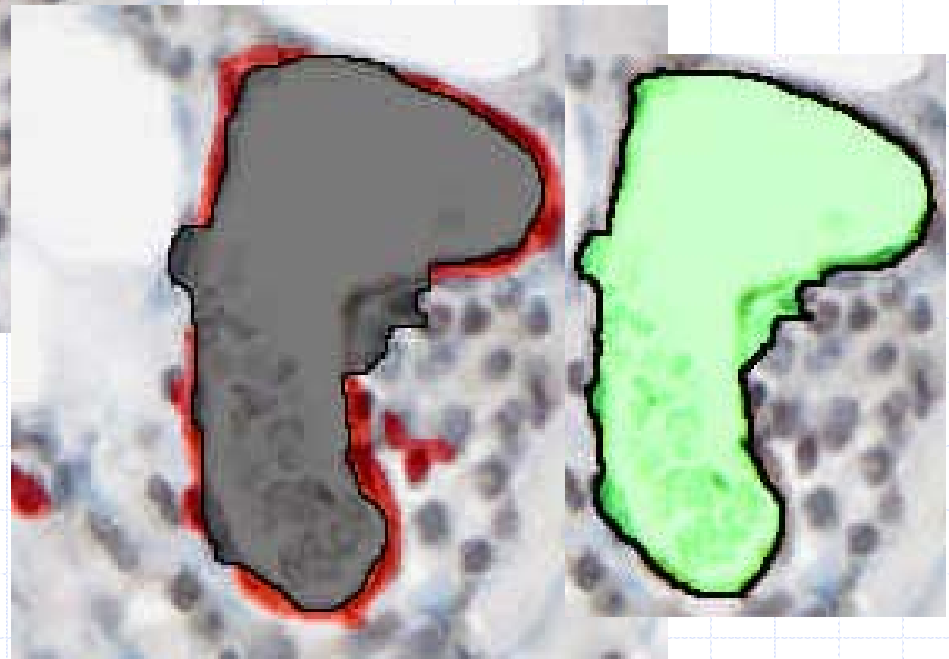


Endothelial cells



Endothelial cells grouped to make regions

Vessel perimeter drawn in middle of vessel walls



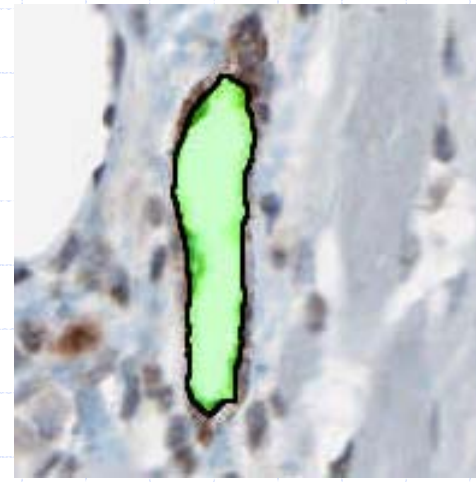
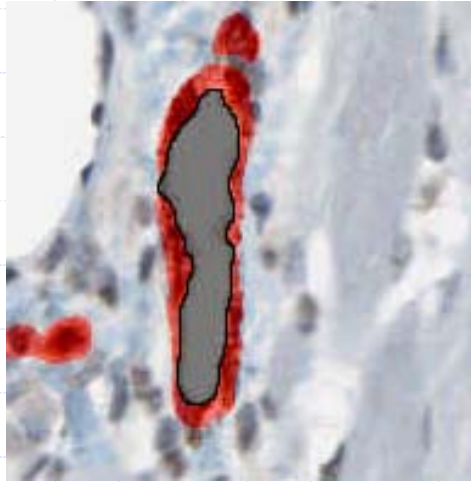
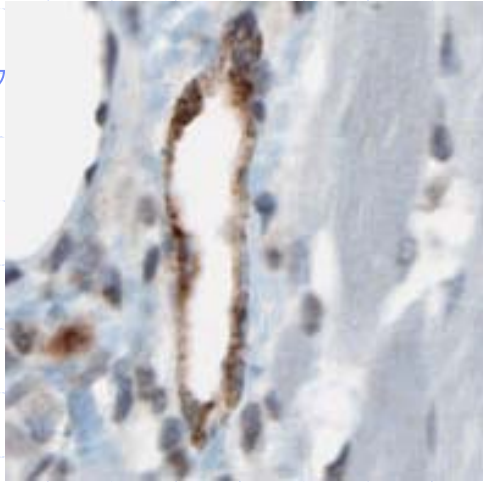
Processing



Red = vessel wall, vascular area
Gray = lumen

Green = vessel area
Black = vessel perimeter

Definitions:



Red = vessel wall, vascular area
Gray = lumen

Green = vessel area
Black = vessel perimeter

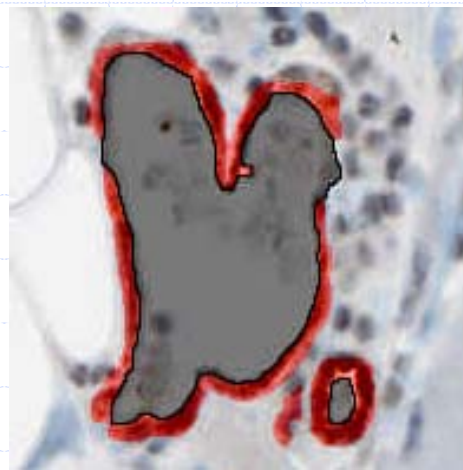
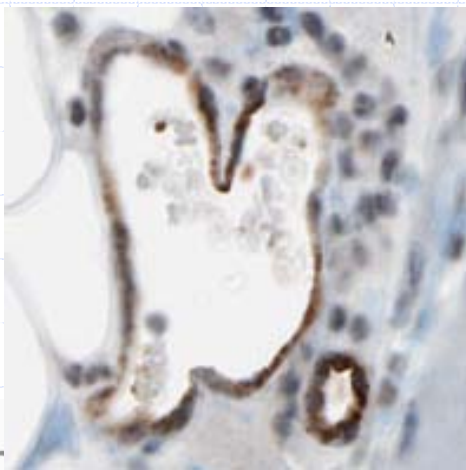
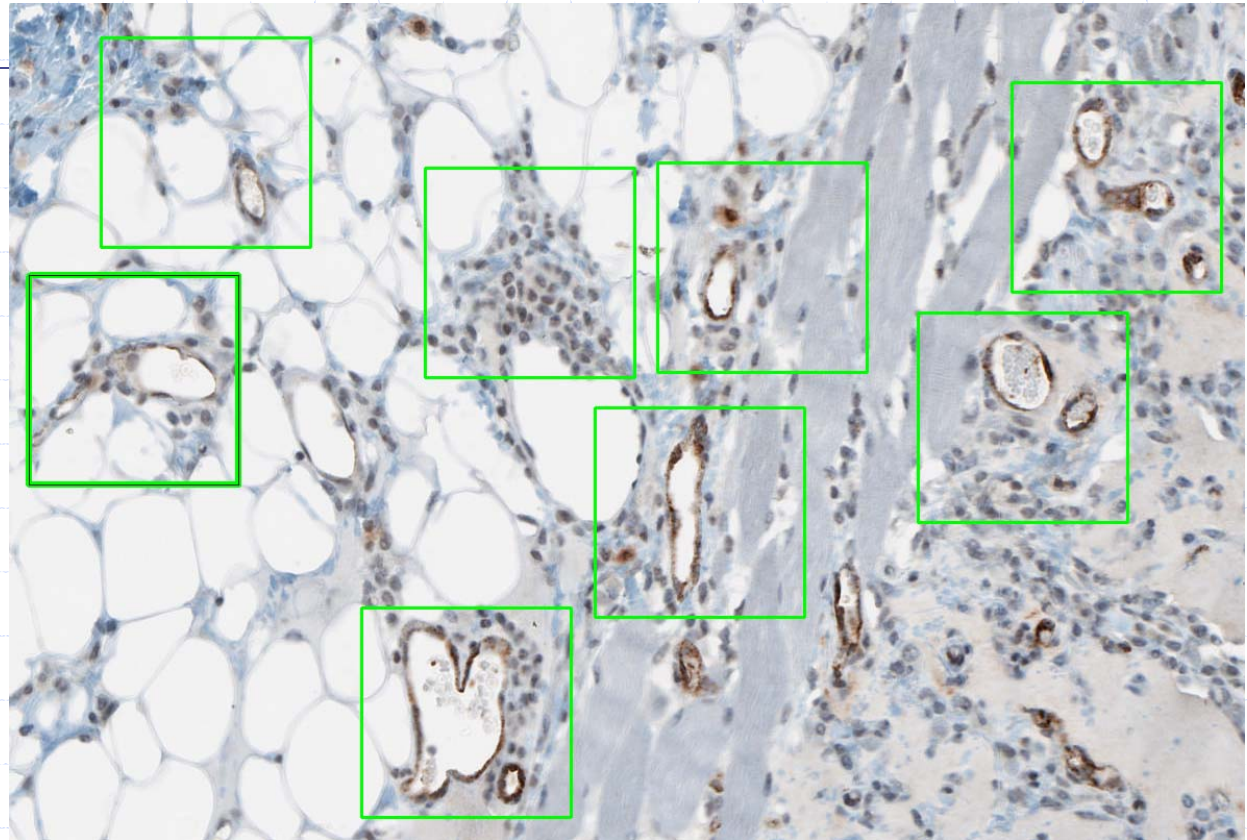
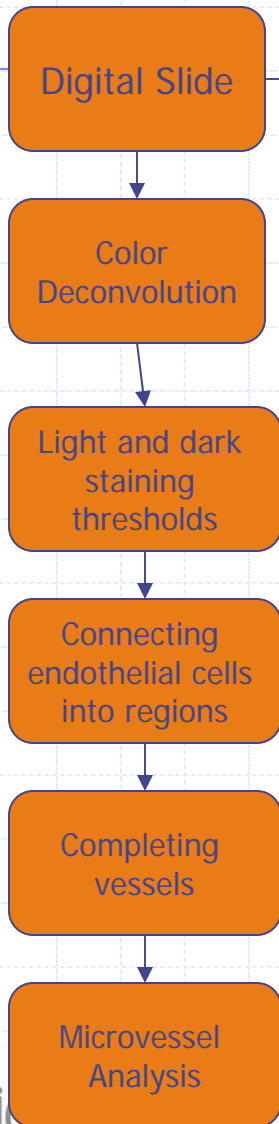


Image Analysis Process



We will walk through the process using an example from mouse skin with injected matrigel solution, and Factor VIII staining
(courtesy Reg Valdez, Novartis NIBR)

Matrigel plug assay for modeling of angiogenesis

Angiogenesis (2005) 8:327-338
DOI 10.1007/s10456-005-9022-9

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A bone-derived mixture of TGF β -superfamily members forms a more mature vascular network than bFGF or TGF- β 2 *in vivo*

Mark Roedersheimer^{1,2}, James West¹, William Huffer³, Julie Harral¹ & James Benedict²

¹CVP Research Lab, University of Colorado, Health Science Center, Denver, Colorado, USA; ²Sulzer Biologics Inc., Wheat Ridge, Colorado, USA; ³Department of Pathology, University of Colorado, Health Sciences Center, Denver, Colorado, USA

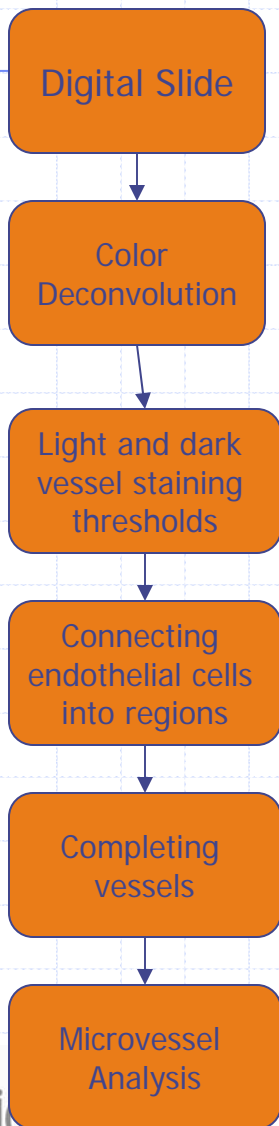
HEMOSTASIS, THROMBOSIS, AND VASCULAR BIOLOGY

Dosage-dependent requirement of BMP type II receptor for maintenance of vascular integrity

Dong Liu,¹ Jian Wang,¹ Bernd Kinzel,² Matthias Müller,² Xiaohong Mao,¹ Reginald Valdez,¹ Yongxing Liu,¹ and En Li³

¹Developmental and Molecular Pathways, Novartis Institutes for Biomedical Research, Cambridge, MA; ²Developmental and Molecular Pathways, Novartis Institutes for Biomedical Research, Basel, Switzerland; and ³Models of Disease Center, Novartis Institutes for Biomedical Research, Cambridge, MA

Color Deconvolution

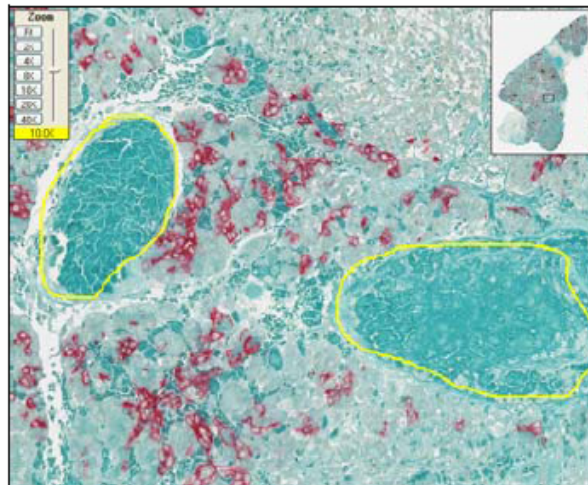


- Separates out the stain contribution from the endothelial stain (e.g. CD31, Factor VIII), and only uses this color for subsequent analysis
- Default setting is DAB as endothelial stain and hemotoxylin as background stain 1, with background stain 2 = 0

Endothelial Stain - Red Component	0.268
Endothelial Stain - Green Component	0.57
Endothelial Stain - Blue Component	0.776
Background Stain 1 - Red Component	0.68
Background Stain 1 - Green Component	0.57
Background Stain 1 - Blue Component	0.44
Background Stain 2 - Red Component	0
Background Stain 2 - Green Component	0
Background Stain 2 - Blue Component	0

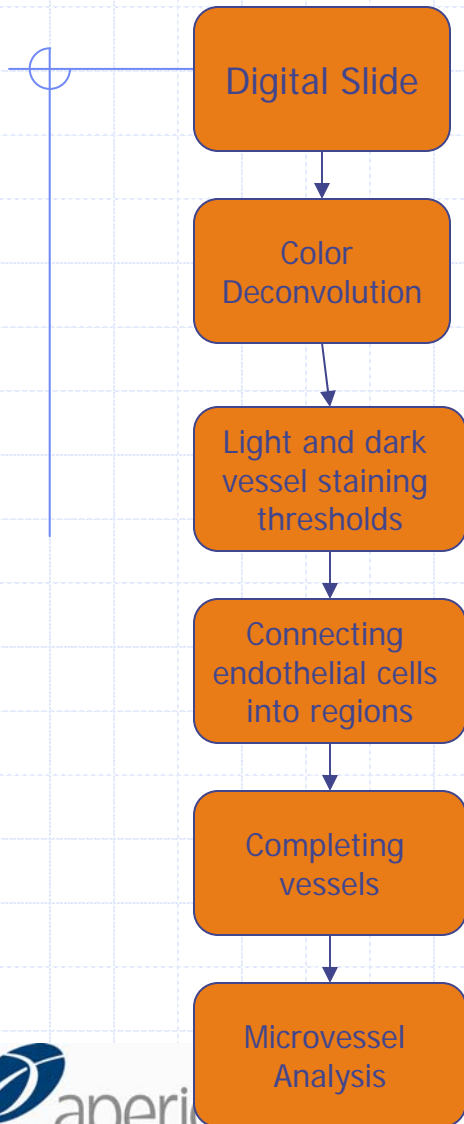
Selecting RGB values for the stains

- Pick an area of LIGHT staining of only one color, run algorithm and look at the average RGB ODs
- Or do this on a single stain control slide
- Darker, overstained areas should be avoided
- Shouldn't need to be adjusted very often (minor color shifts won't impact results substantially)

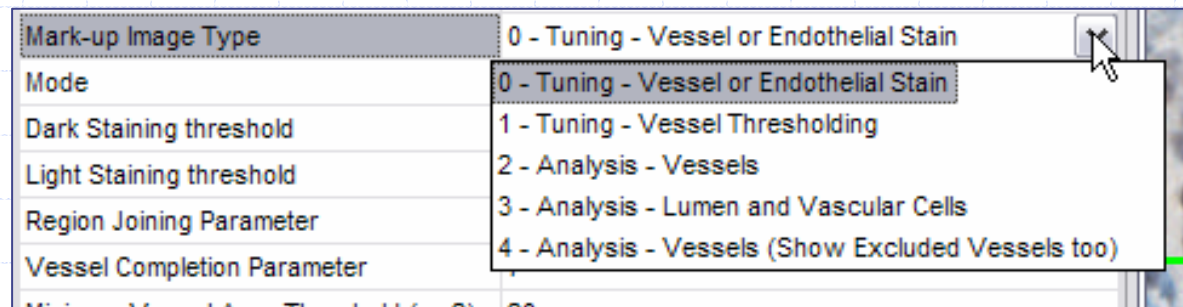


Algorithm	Color Deconvolution
Version	8.001
Average Positive Intensity	152.492
Percent Weak Positive	25.0493
Percent Medium Positive	74.7004
Percent Strong Positive	0.110772
Percent Negative	0.139525
Percent Total Positive	99.8605
Average Weak Positive Intensity	191.002
Average Medium Positive Intensity	139.66
Average Strong Positive Intensity	97.3206
Total Stained Area (mm ²)	0.0733236
Score (0-300)	174.762
Average Red OD	0.925687
Average Green OD	0.266027
Average Blue OD	0.26895

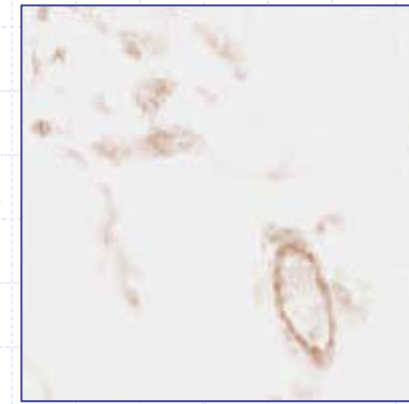
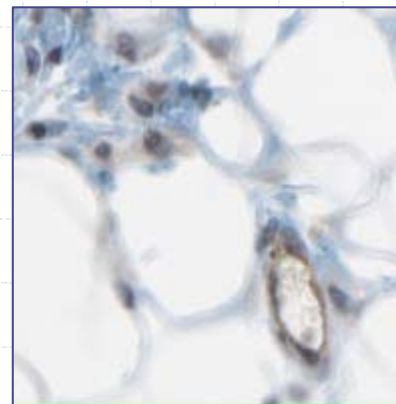
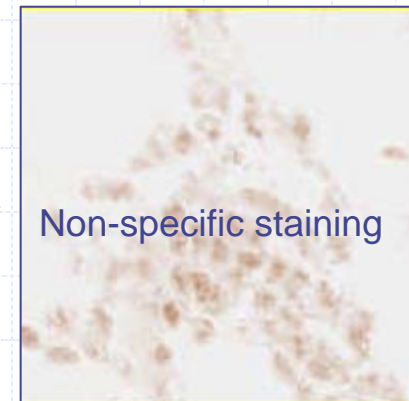
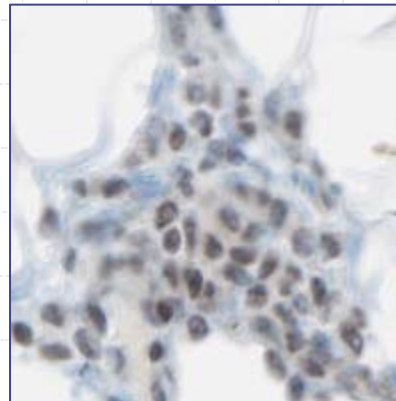
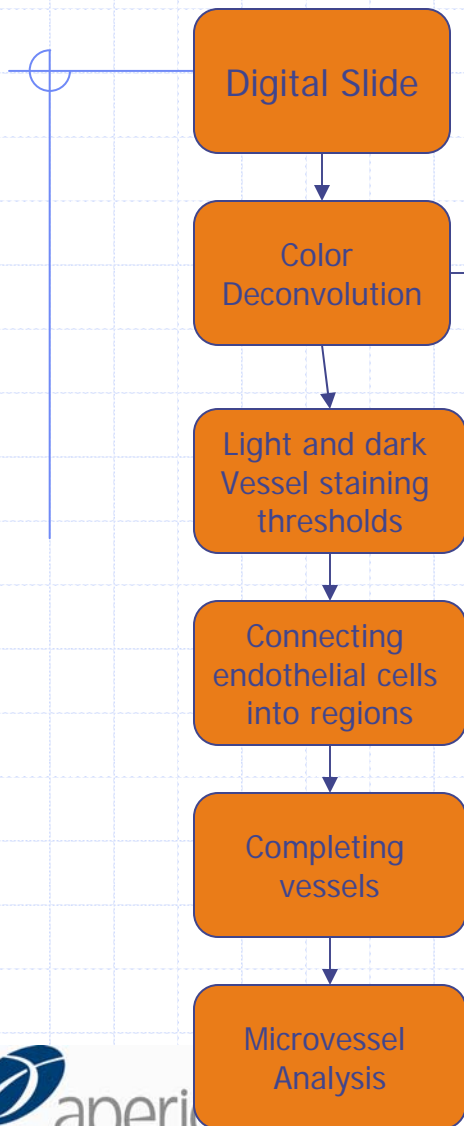
Color Deconvolution - Tuning



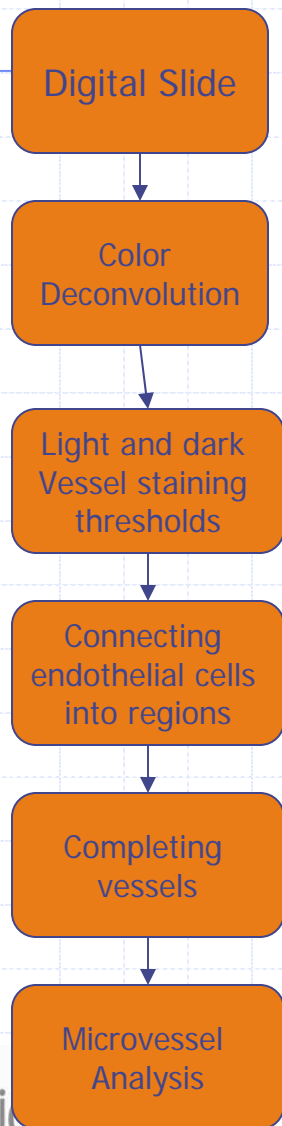
- Set the Mark-Up Image Type to:
0 – Tuning – Vessel or Endothelial Stain
- This is a quick approach to checking the RGB values, doesn't calculate statistics or find vessels, very fast to run



Color Deconvolution



Vessel staining thresholding

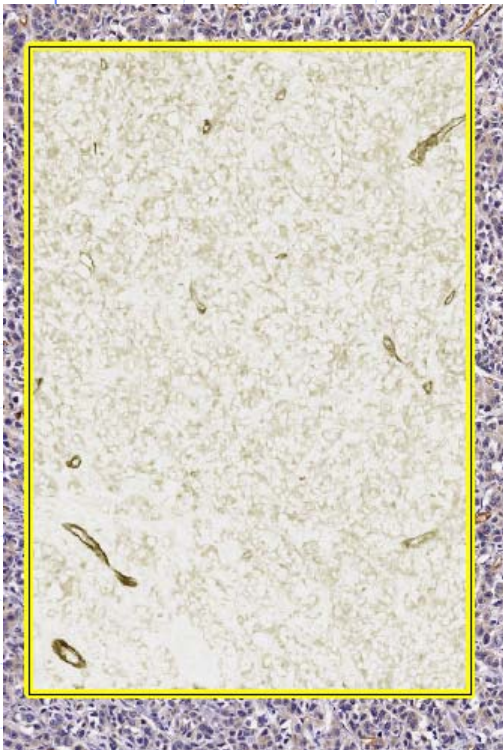


- This step will identify endothelial cells
- Uses a novel approach employing two things simultaneously:
 - Dark staining threshold: all vessels must contain some region that stain darker than this threshold
 - Light staining threshold: all vessels must contain LARGE regions that stain darker than this threshold
 - Eliminates single point staining (e.g. false stain uptake by non-endothelial cells)

Dark Staining threshold	161
Light Staining threshold	210

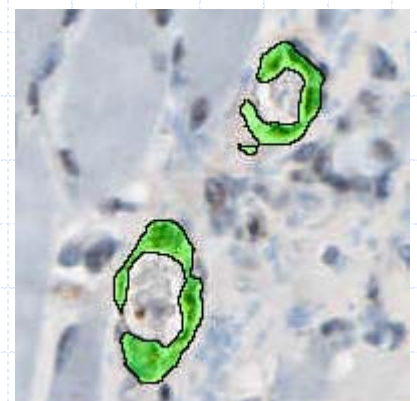
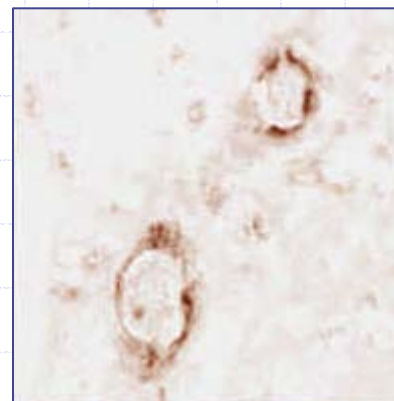
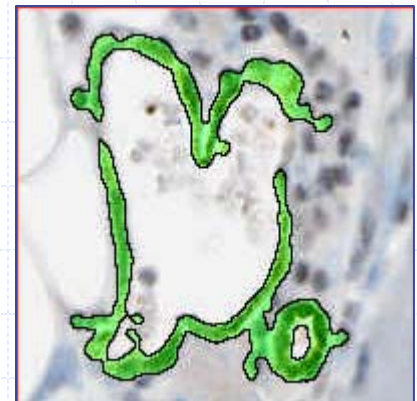
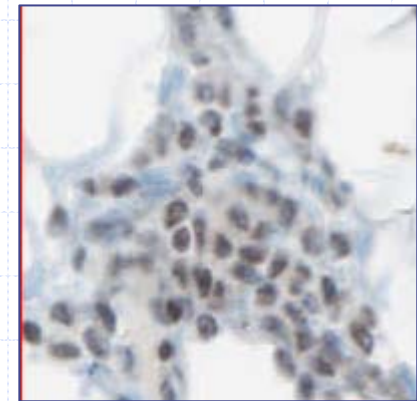
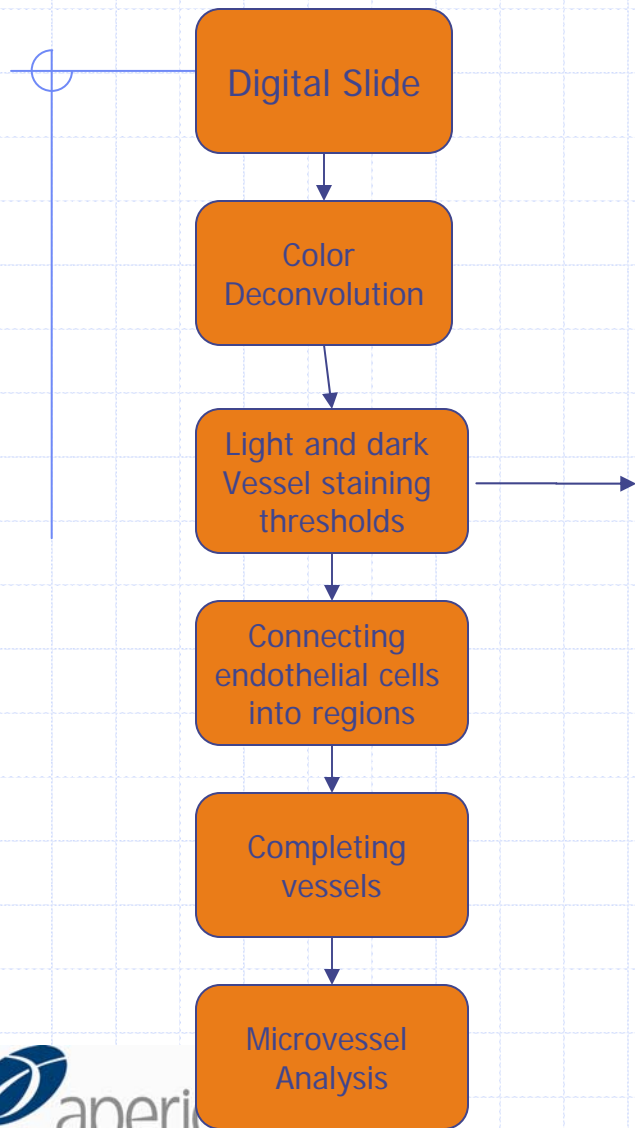
- Darkest value = 0, Lightest = 255

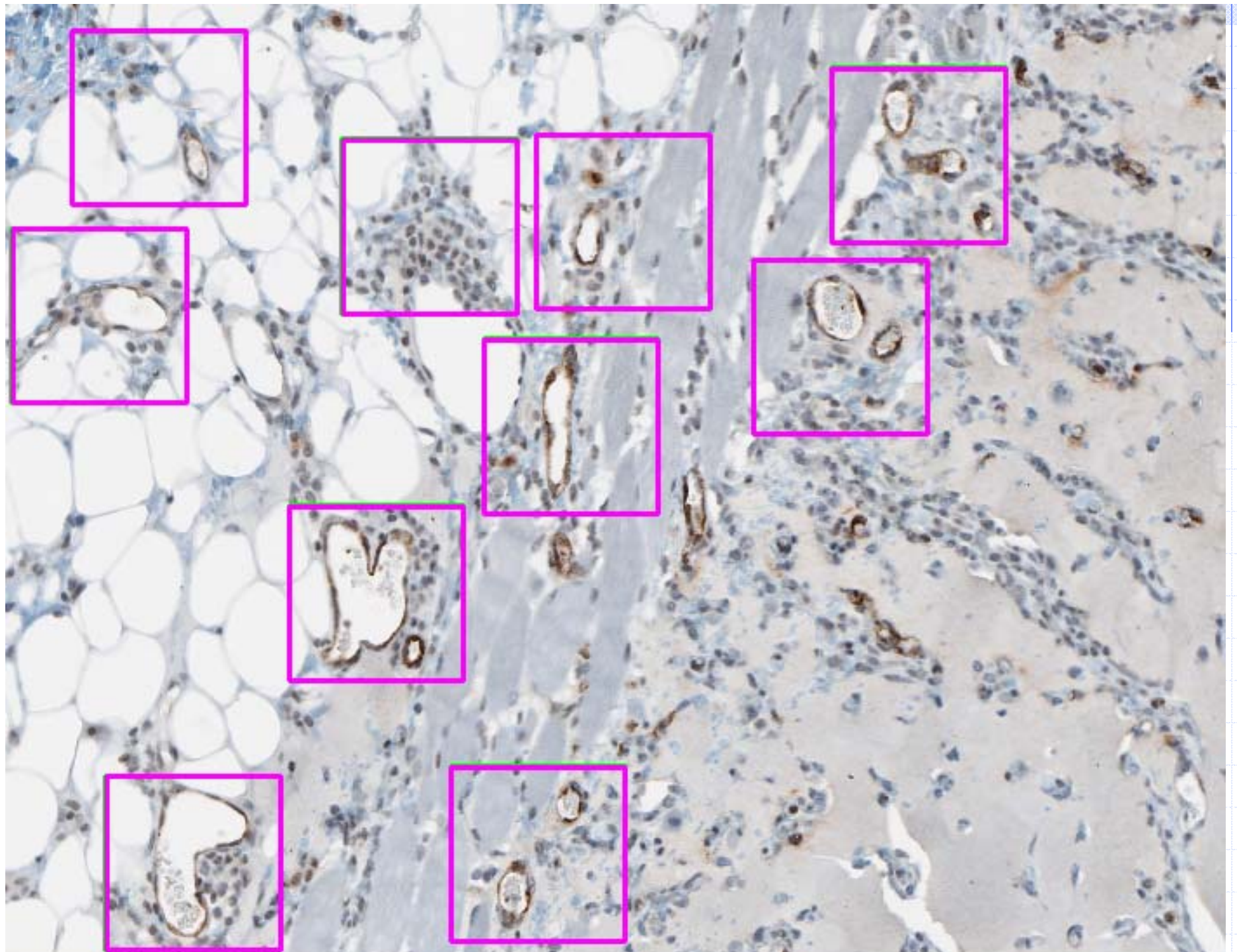
Vessel staining thresholding

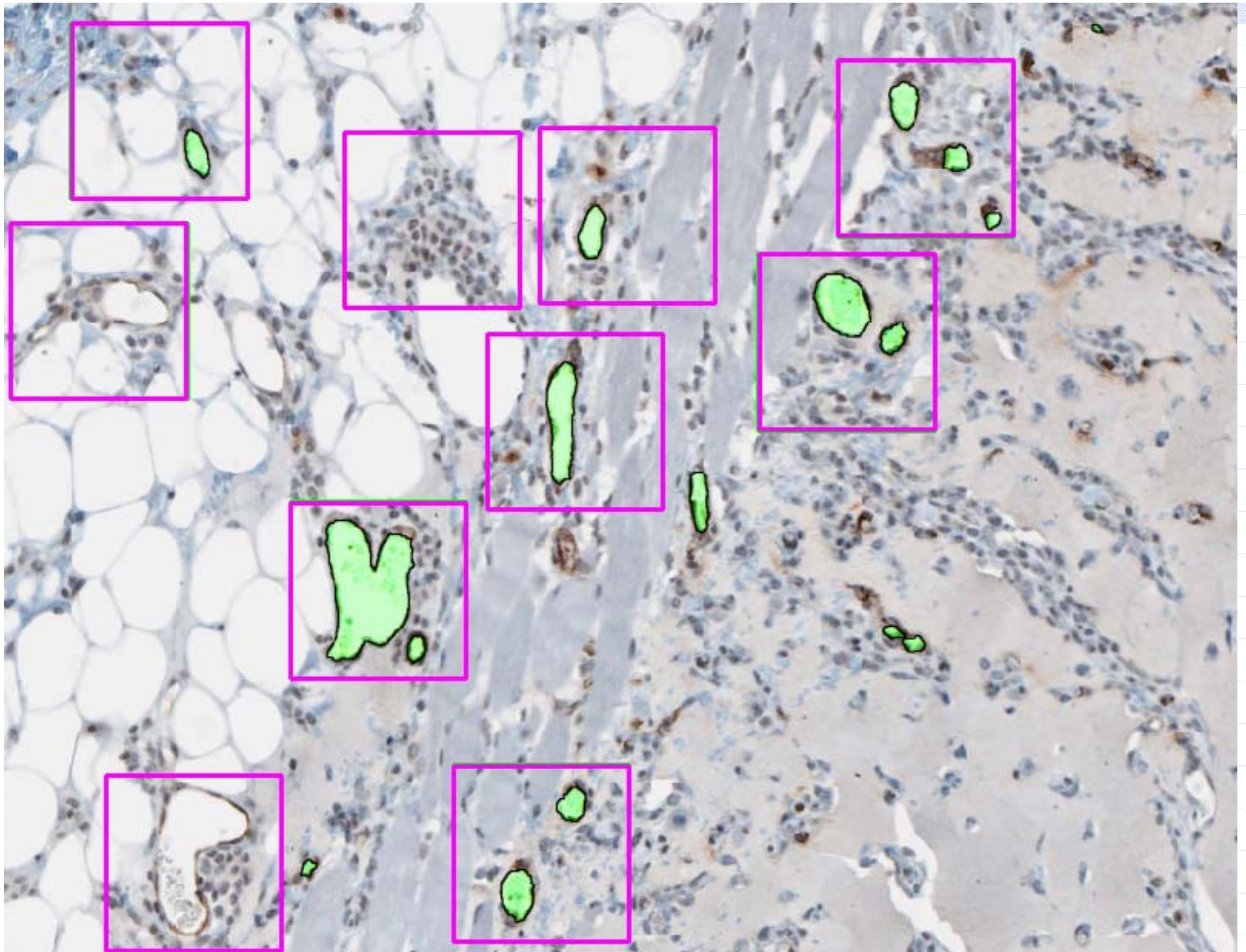


- Think of this in a biology context:
 - Typically have specific staining and non-specific background staining)
 - Dark staining threshold: all vessels must contain some region that stain darker than this threshold
 - Light staining threshold: all vessels must contain LARGE regions that stain darker than this threshold
 - Dark staining ~ specific staining
 - Light staining ~ non-specific staining

Vessel staining thresholding







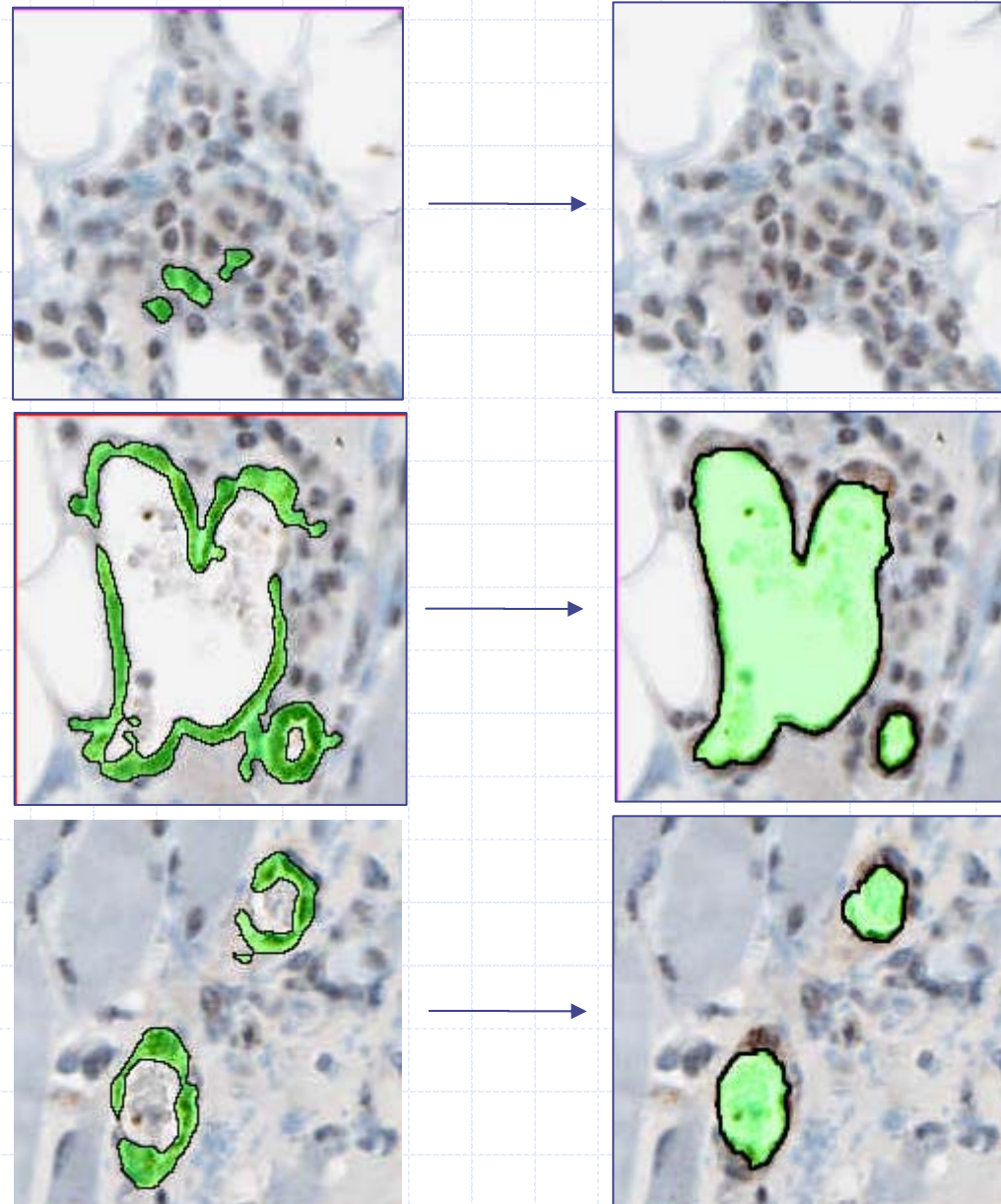
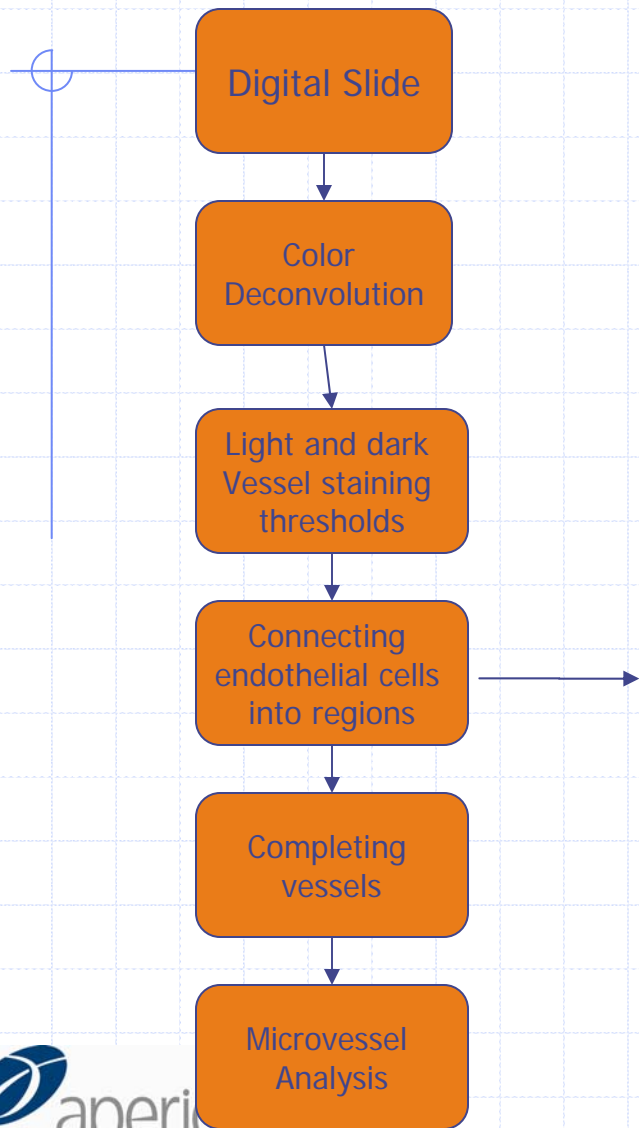
Vessel staining thresholding - tuning

Dark Staining threshold	161
Light Staining threshold	210

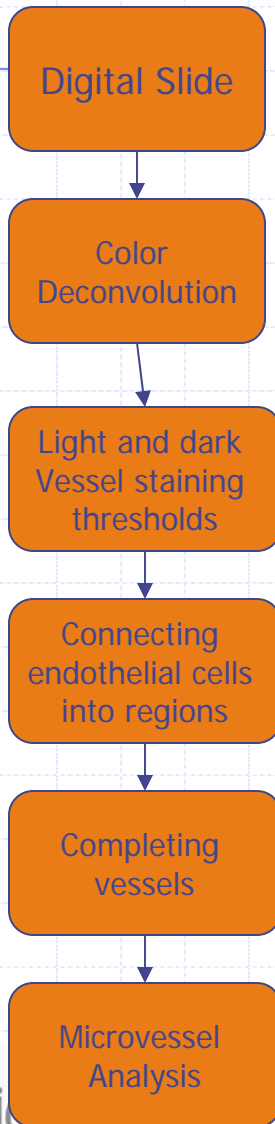
1. Start with both thresholds the same ($d=210, l=210$)
This is the now the same as color deconvolution algorithm
2. Lower the dark staining to a local optimum ($d=160, l=210$)
3. Adjust the light staining threshold to ensure that nearly all endothelial cells are detected, and a minimal number of false stained cells

It is okay at this point to have a few false stained cells, the next step can eliminate these (looks for lumen)

Connecting endothelial cells into regions



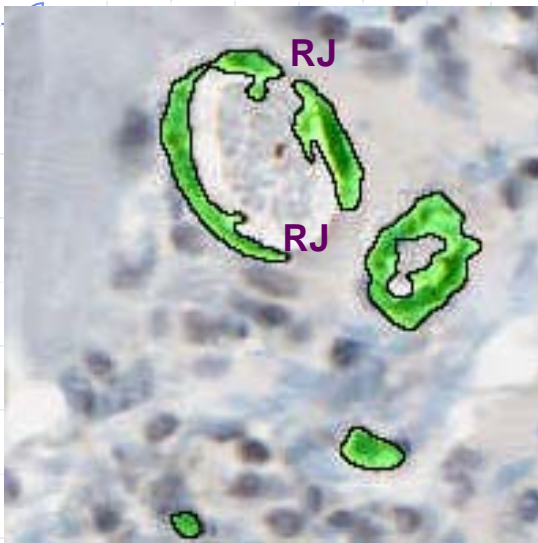
Connecting cells into regions



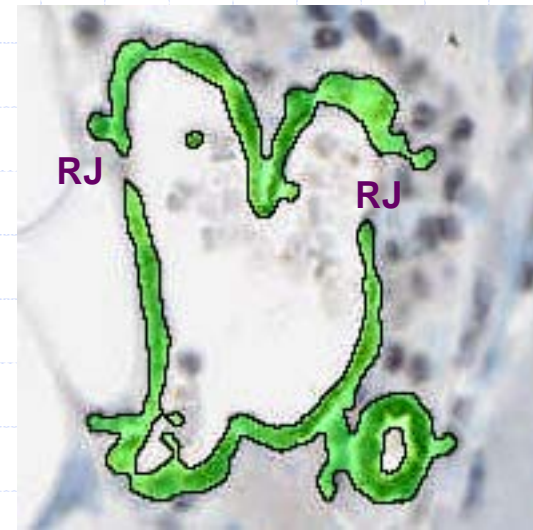
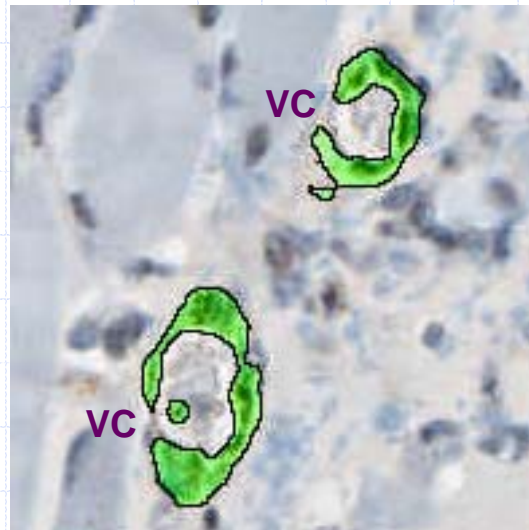
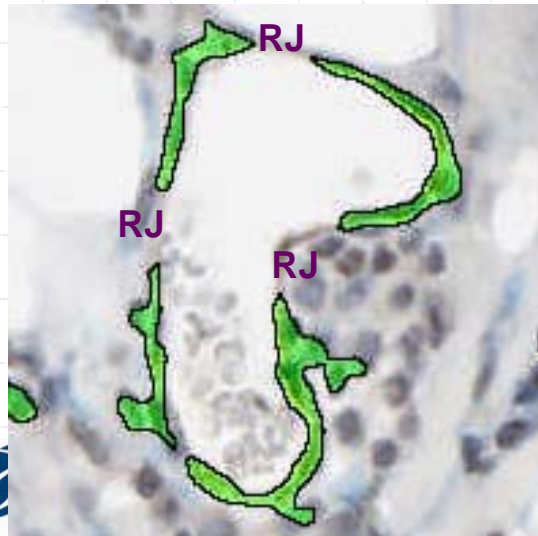
- This step is specifically looking for lumen, and connecting endothelial cells around the lumen together to call it one vessel
- Two things can be set:
 - **Region Joining Parameter:**
Connects broken pieces of vessel walls – all pieces within this distance may be joined to the same vessel
 - **Vessel Completion Parameter:**
Connects incomplete and open vessel walls – incomplete vessel walls with less than this distance between their ends may be completed into one vessel

Region Joining Parameter	6
Vessel Completion Parameter	7

Connecting cells into regions



- Region Joining Parameter: (RJ)
Connects broken pieces of vessel walls – all pieces within this distance may be joined to the same vessel
- Vessel Completion Parameter (VC)
Connects incomplete and open vessel walls – incomplete vessel walls with less than this distance between their ends may be completed into one vessel



Additional screening settings

- Minimum Vessel Area Threshold
- Maximum Vessel Area Threshold
 - Good for dividing neovascular capillaries from older, larger vessels
- Maximum Vessel Wall Thickness

Minimum Vessel Area Threshold (um ²)	30
Maximum Vessel Area Threshold (um ²)	20000
Maximum Vessel Wall Thickness (um)	4

Examples:

Examples

1. Cardiovascular: matrigel plug assay in mouse skin for modeling of angiogenesis with Factor VIII (courtesy Reg Valdez, Novartis NIBR)
2. Oncology: CD31 xenografts
Sensitivity to overstaining?
3. Ophthalmology: Factor VIII stained rodent eyes
4. Hematology: Clinical AML bone marrows & Factor VIII (courtesy Maher Albitar, Quest Diagnostics)
5. Kidney with endothelial stain (courtesy Children's Hospital, Boston)

Further research possibilities

1. Using dual staining with colocalization
 - First stain to identify endothelial cells:
 - Second stain for protein expression in endothelial cells (nuclear, membrane, or cytoplasm)
 - Second stain for further differentiating endothelial progenitor cells (e.g. CD34, CD133)
 - Using a second stain to further assist in better identifying endothelial cells (e.g. in bone marrows, hematoxylin is absorbed more strongly by megakaryocytes than endothelial cells, can use this to help distinguish megakaryocytes from endothelial cells with dual thresholding colocalization algorithm)

Acknowledgements

Customers:

Drs. Reg Valdez, Martina Schinke, Jim Deeds and many others at Novartis

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Aperio:

Allen Olson

