Image analysis in IHC – overview, considerations and applications

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NordiQC workshop
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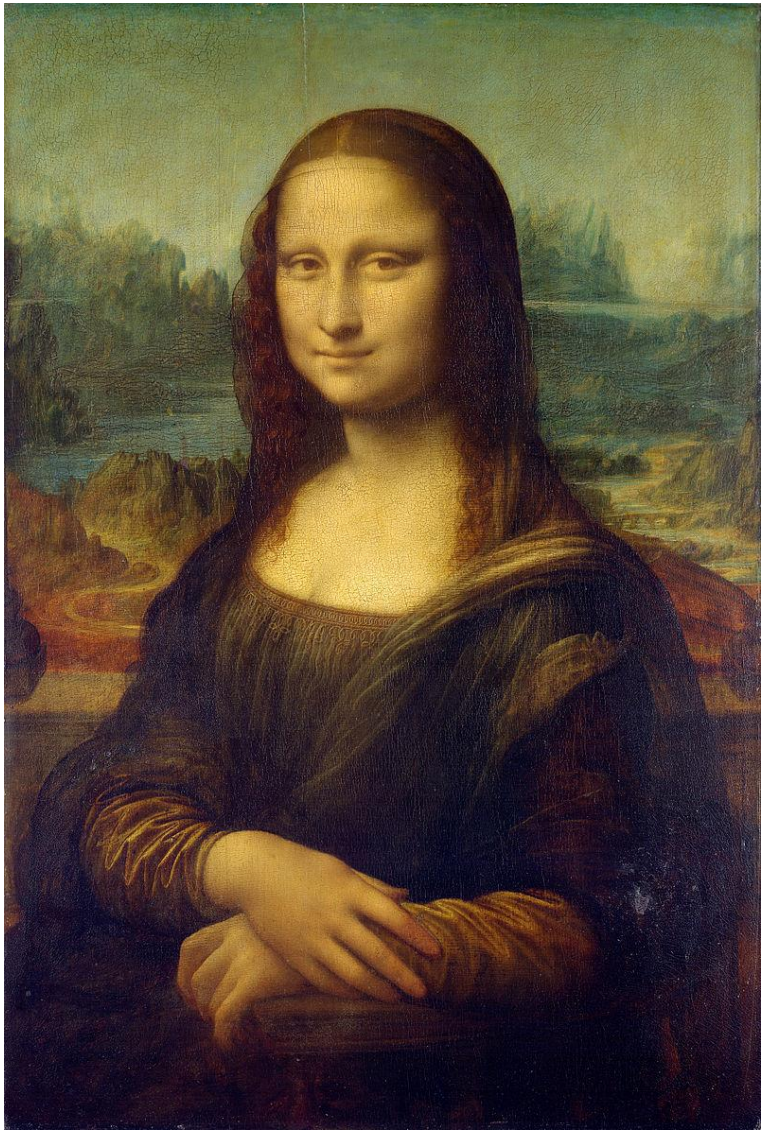
Aalborg, Denmark

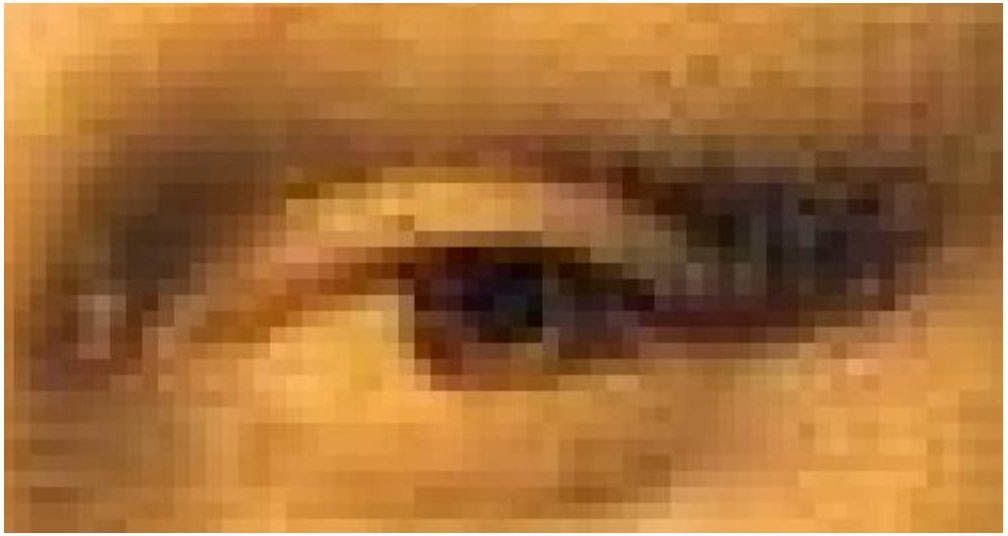
NordiQC

Outline

- Theory
- Image Analysis
- Programs
- PhD-project
  - Virtual Double Staining
  - Validation
- (Image analysis experiment in NordiQC)

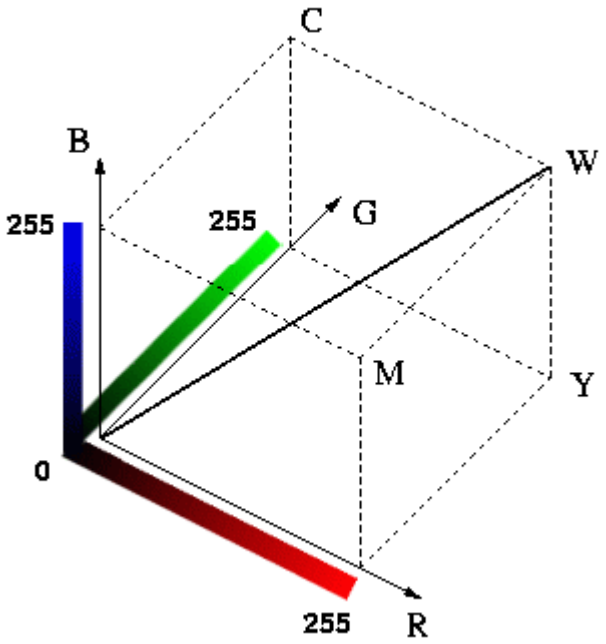
Pixels

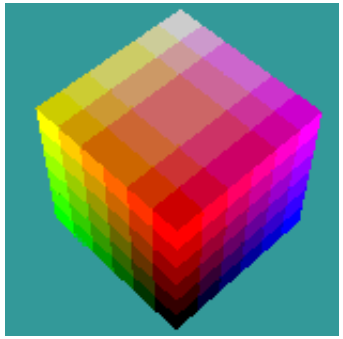




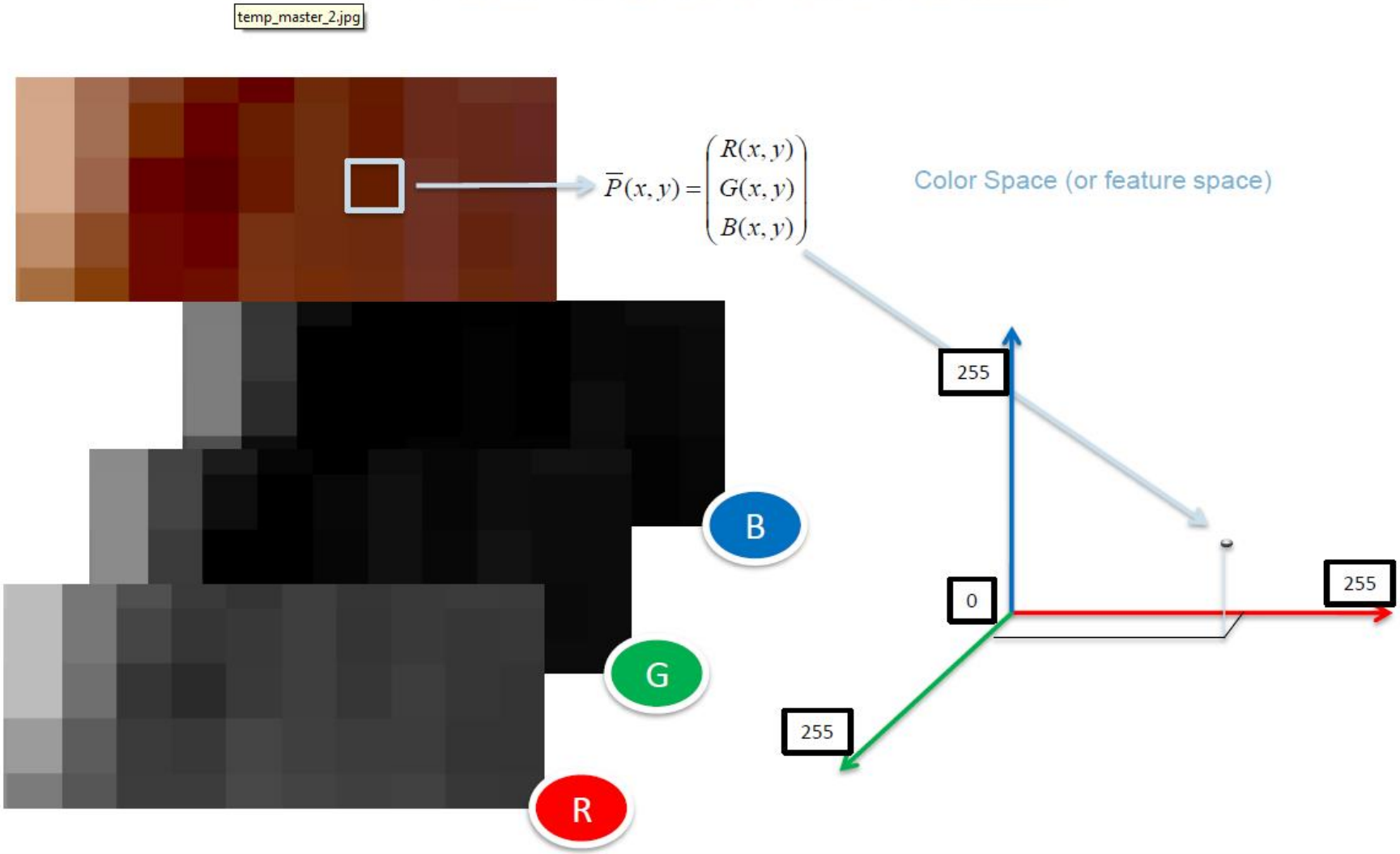
RGB colour model

- Additive colour model
- Red, green and blue light
- System to encode representation of colour





A FEW WORDS ON PIXELS



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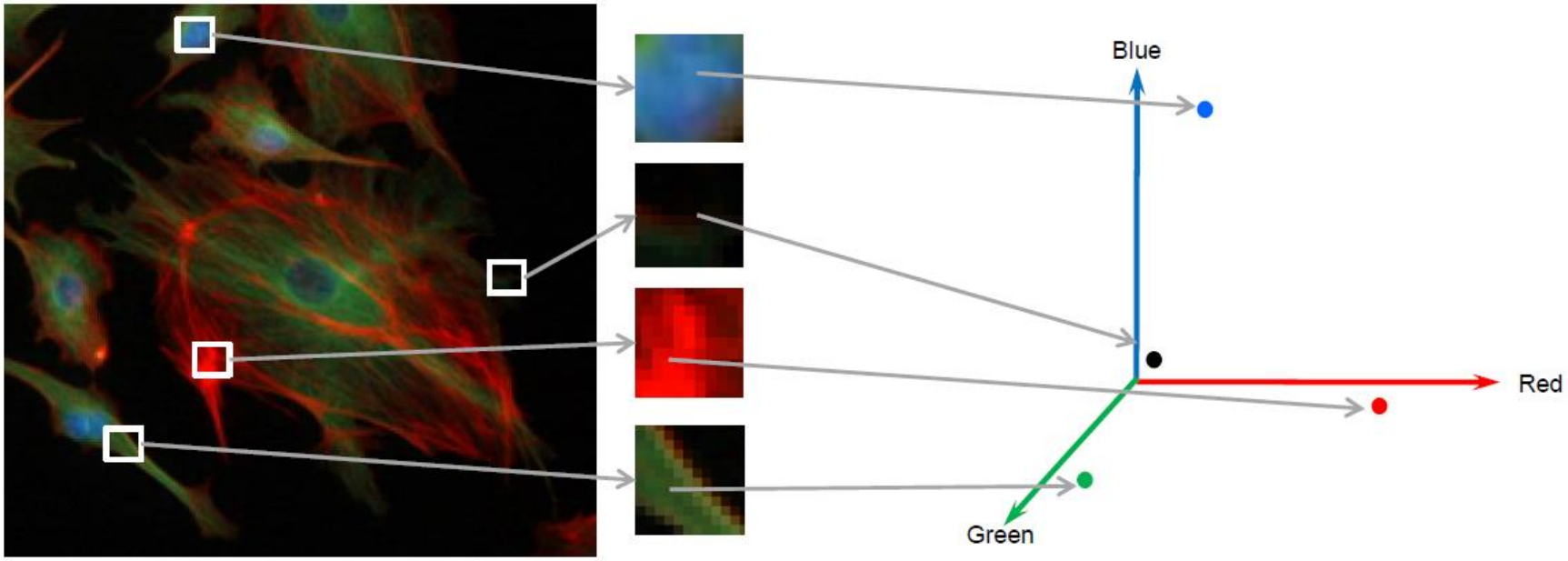
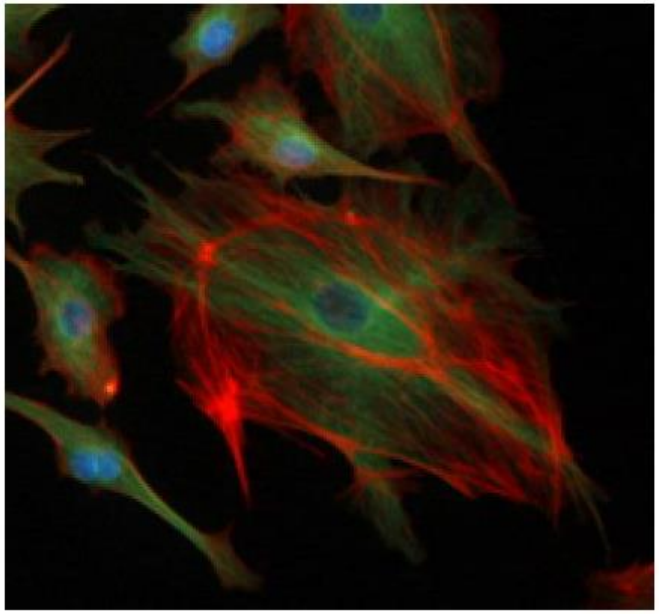
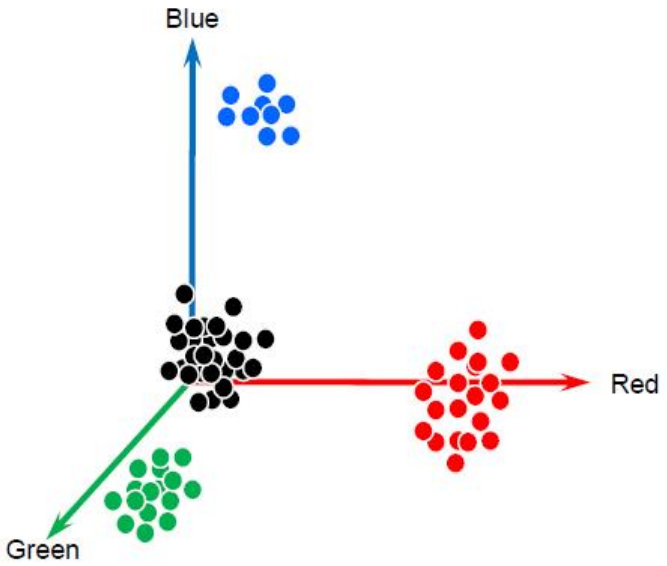


- Each pixel in the image has a combination of red, green and blue intensity values.
- The three axes in color space correspond to R, G, and B values.
- A red pixel will have a high red value and low green and blue values

A FEW WORDS ON PIXELS

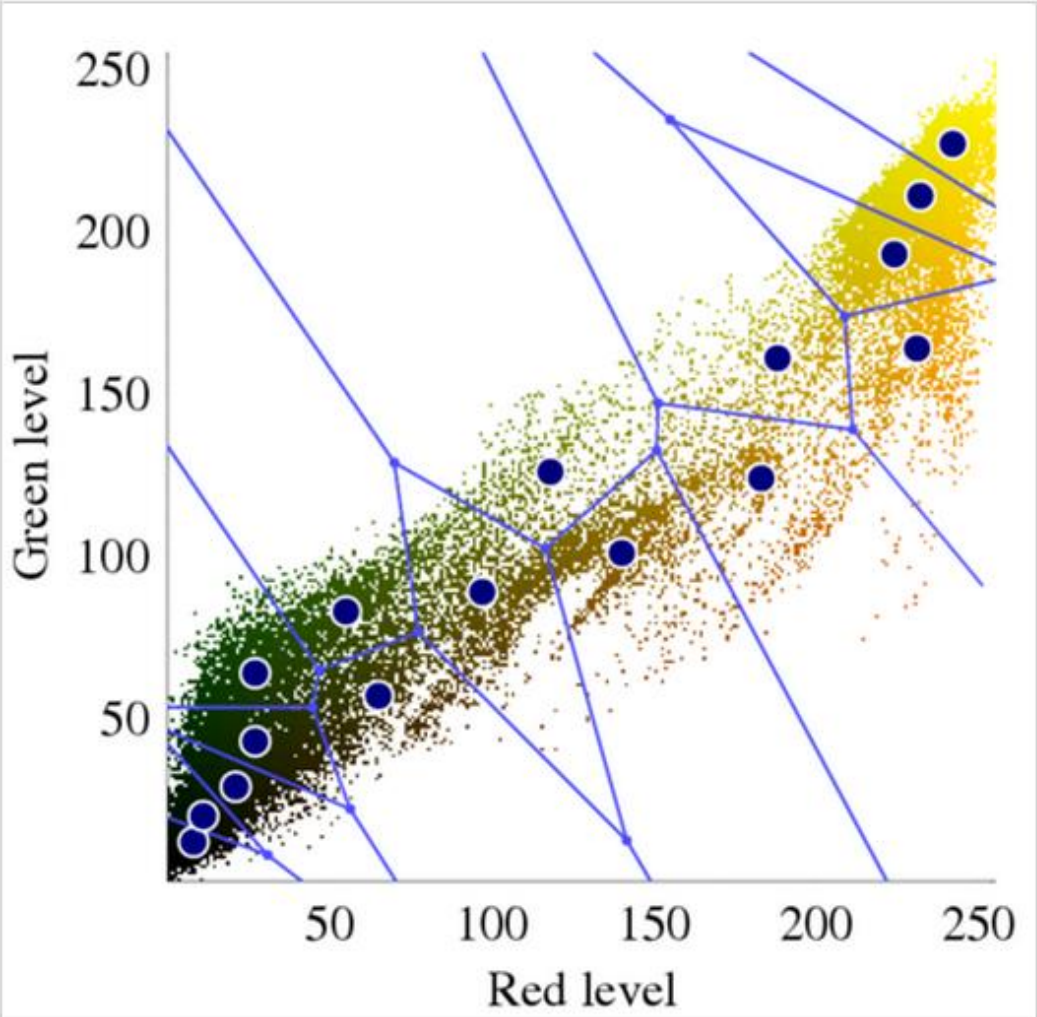
Grouping in color space





- Pixels which have similar colors will be closely grouped in color space

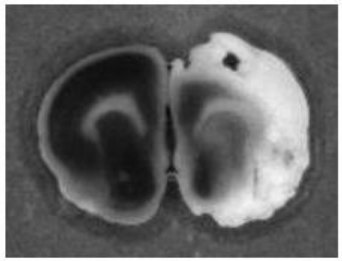
A small photograph that has had its blue channel removed. This means all of its pixel colors lie in a two-dimensional plane in the color cube.

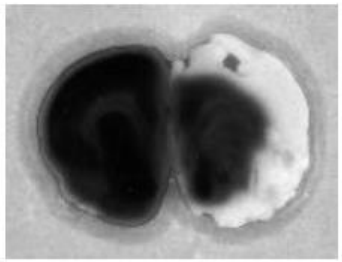


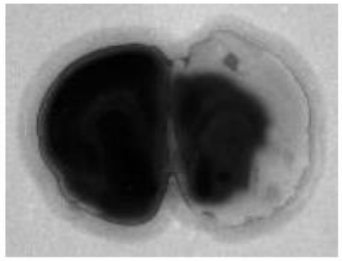
The color space of the photograph to the left, along with a 16-color optimized palette produced by Photoshop. The Voronoi regions of each palette entry are shown.

Color Models

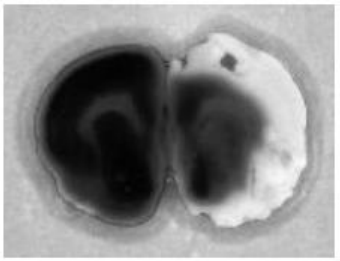
R,G,B

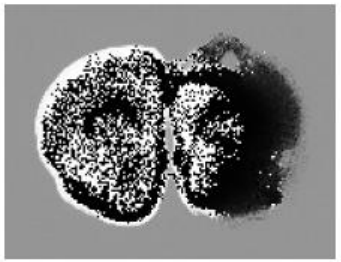


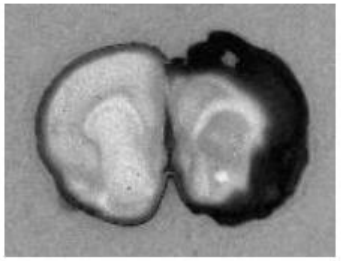




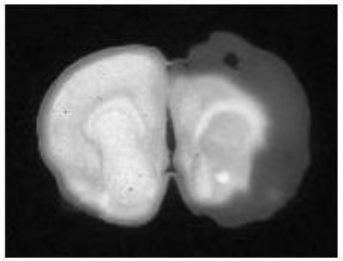
I,H,S

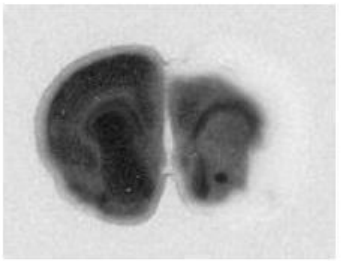


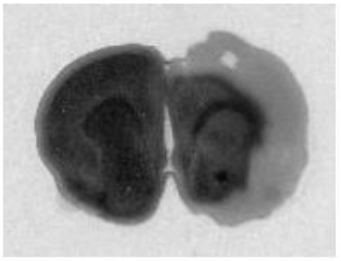




r,g,b







Software

- ImageJ (http://imagej.nih.gov/ij/): Open-source, FREE, platform-independent, large community, Requires programming-skills
- VIS (http://www.visiopharm.com/): fully developed apps, expensive, database-handling of data and images, scanner independent
- Definiens
- Aperio
- PathXL

SOME COMMON TERMS

IMAGING

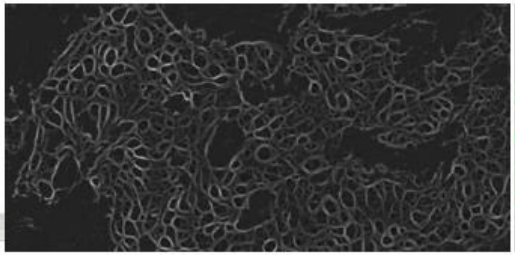
Imaging deals with the acquisition of images

IMAGE PROCESSING

Image processing deals with transforming the image from one form into another form which is more convenient: Enhancing structures, suppressing noise, ...

IMAGE ANALYSIS

Image analysis deals with extraction of (quantitative) data from acquired images



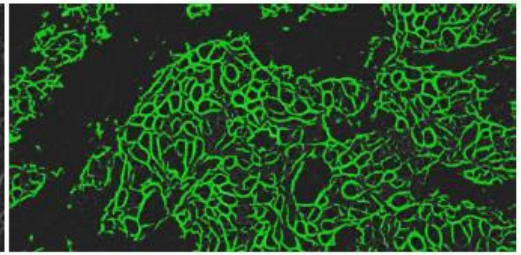
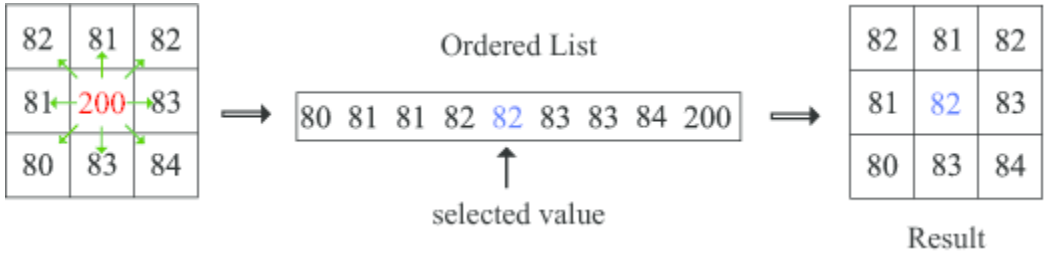
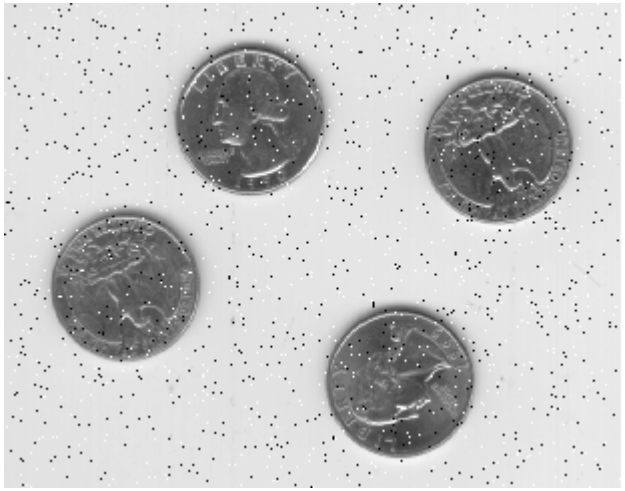


Image analysis

- Selection of filters
- Preprocessing
  - Noise filtering, enhancement
- Classification / Segmentation
- Post processing
- Report of quantitative results

Noise Filtering





Edge Enhancement

Standard deviation filter

Edge Enhancement

Standard deviation filter

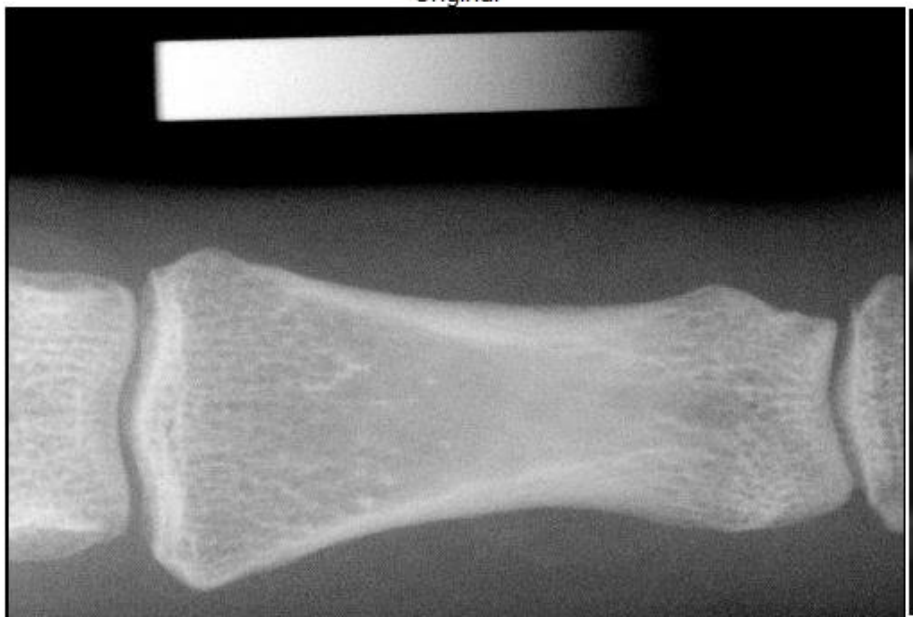
100	100	100
100	100	100
100	100	100

-> 0

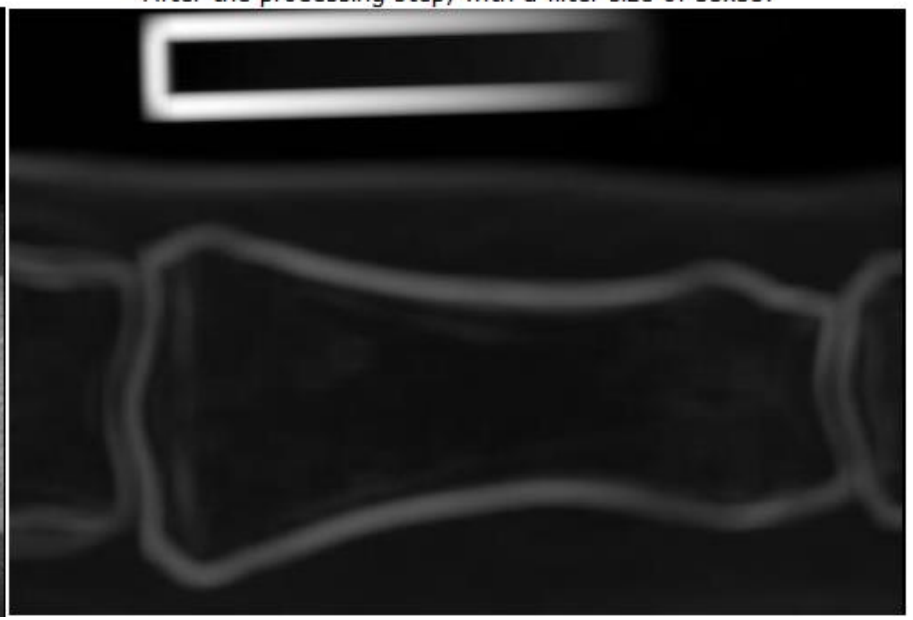
200	198	7
255	132	3
150	150	2

-> 96

Original



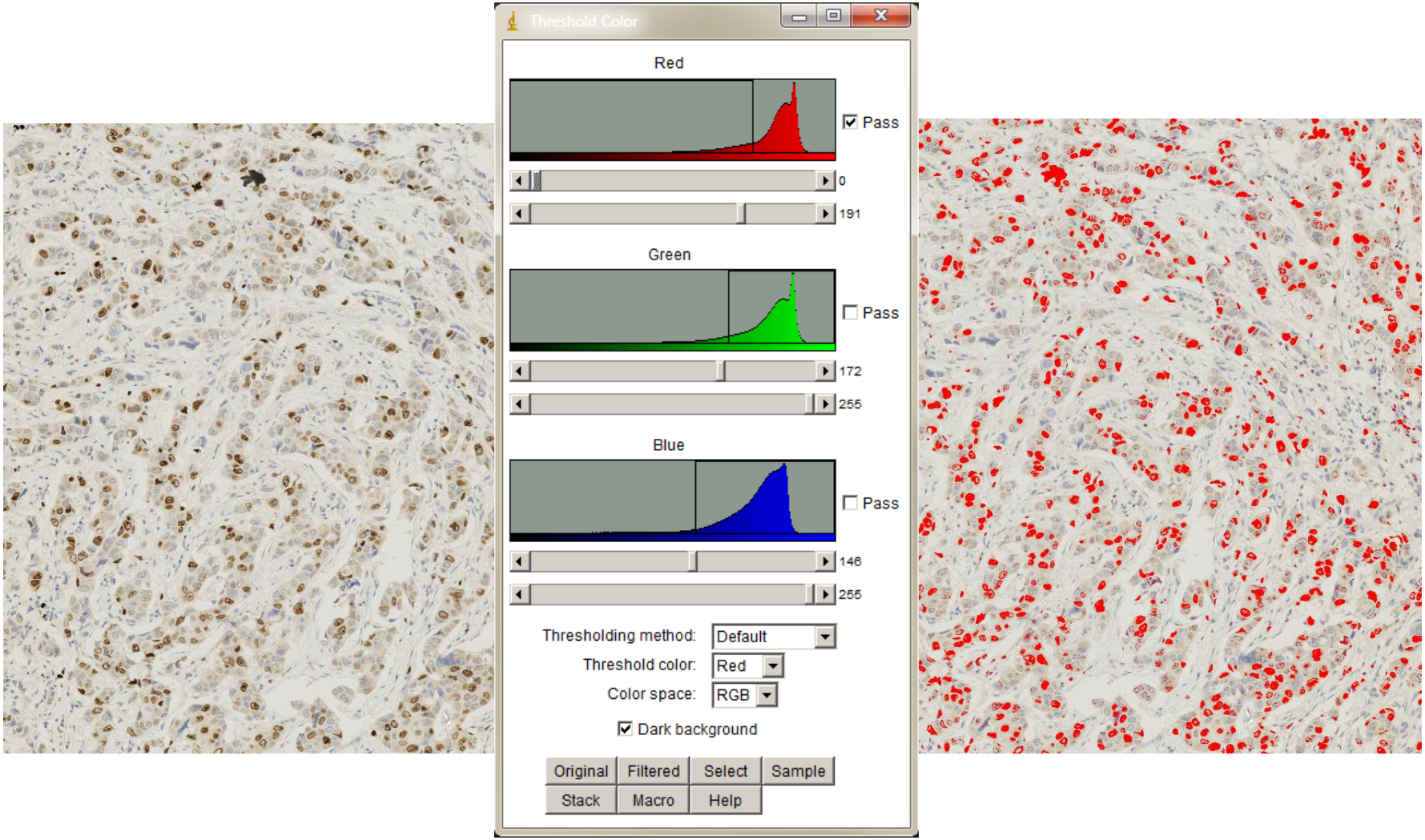
After the processing step, with a filter size of 35x35.



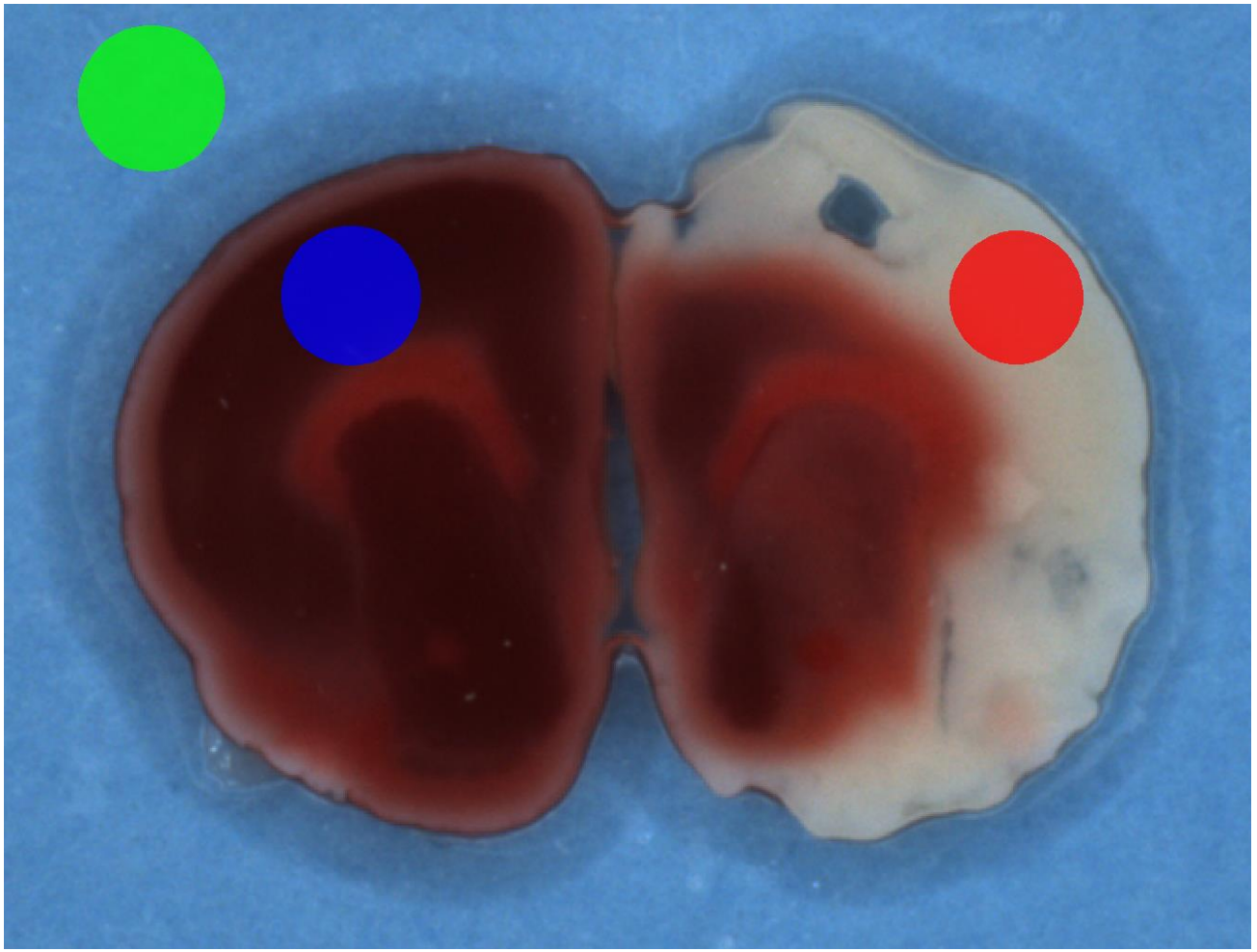
Classification / segmentation

- Algorithms that group every pixels according to defined criteria
- Can be unsupervised or supervised
  - Simple: based on threshold
  - Complex: several thresholds, probabilistic (Bayesian), model-fitting (K-means), texture

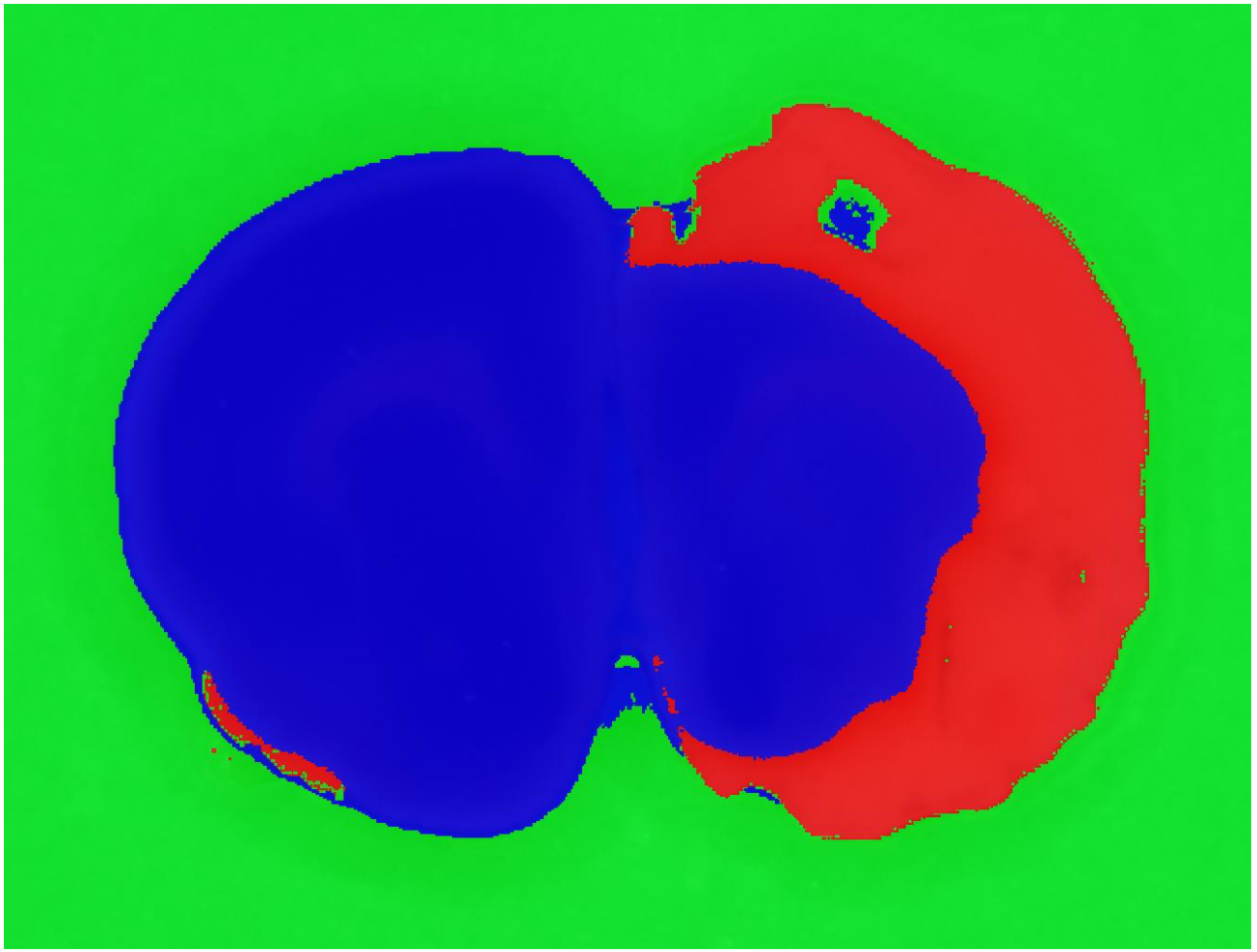
Threshold


Threshold Color
Red
Pass
0
191
Green
Pass
172
255
Blue
Pass
146
255
Thresholding method:
Default
Threshold color:
Red
Color space:
RGB
Dark background
Original
Filtered
Select
Sample
Stack
Macro
Help

Bayesian

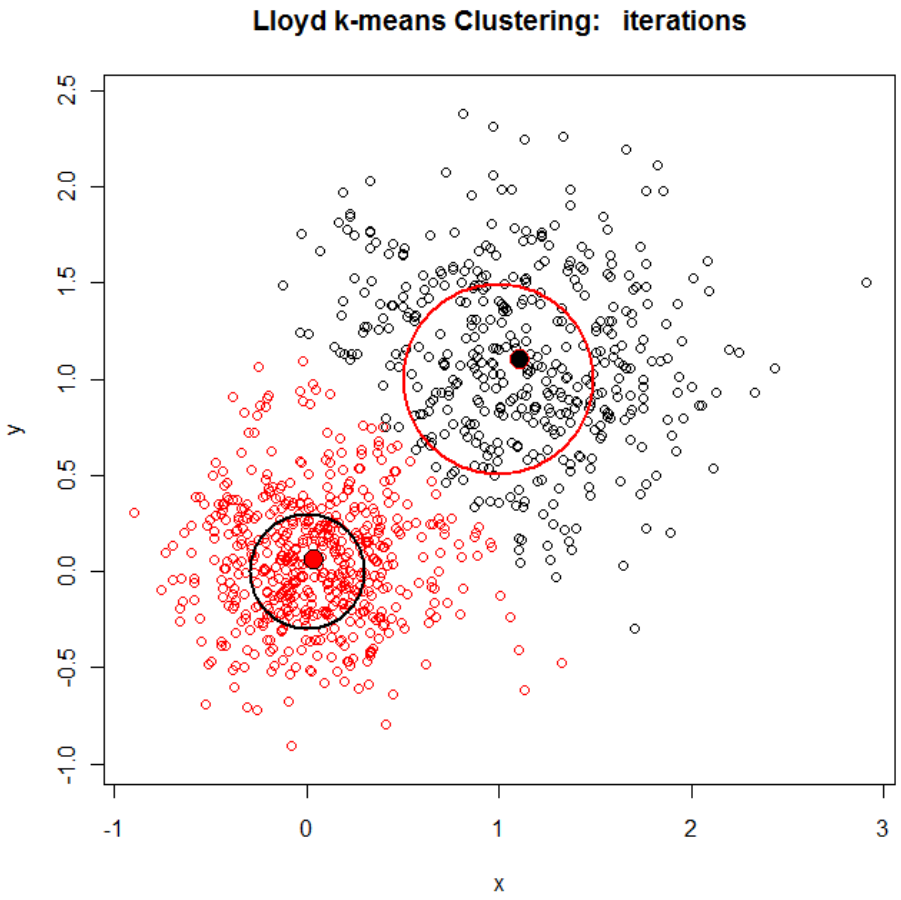


Bayesian

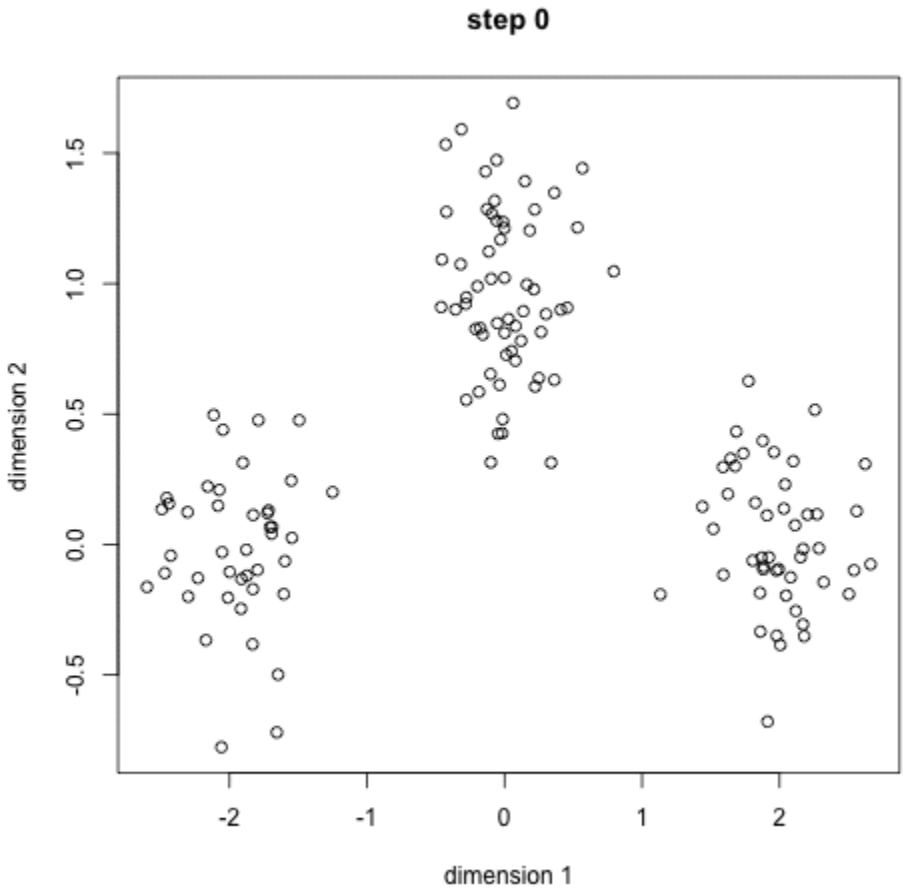


K-means

- Clustering algorithm
- Manually select number of categories (K)
- Randomly select K points (center of groups)
- Assign all point to category according to euclidian distance to center
- Calculate new center
- Repeat as needed

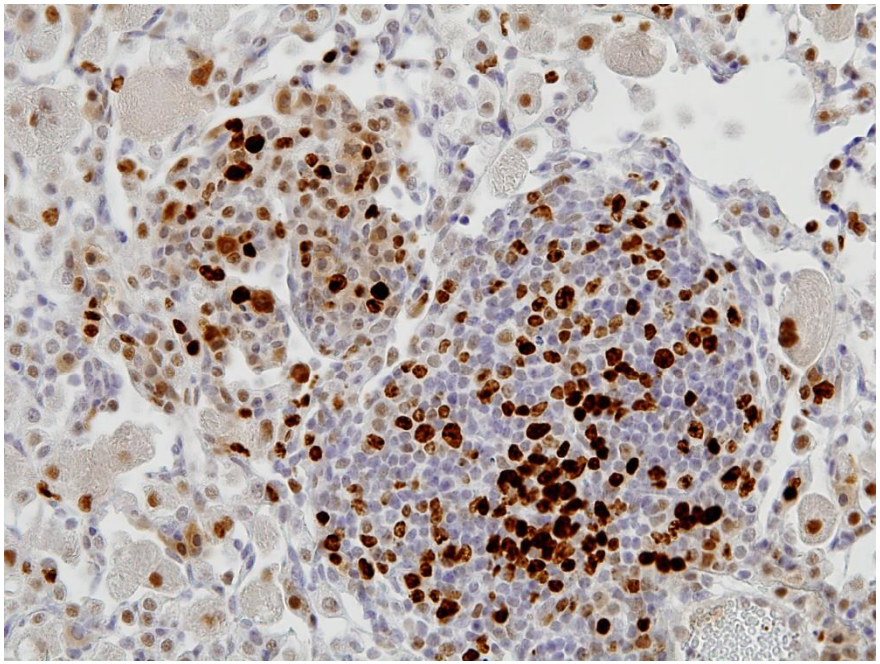


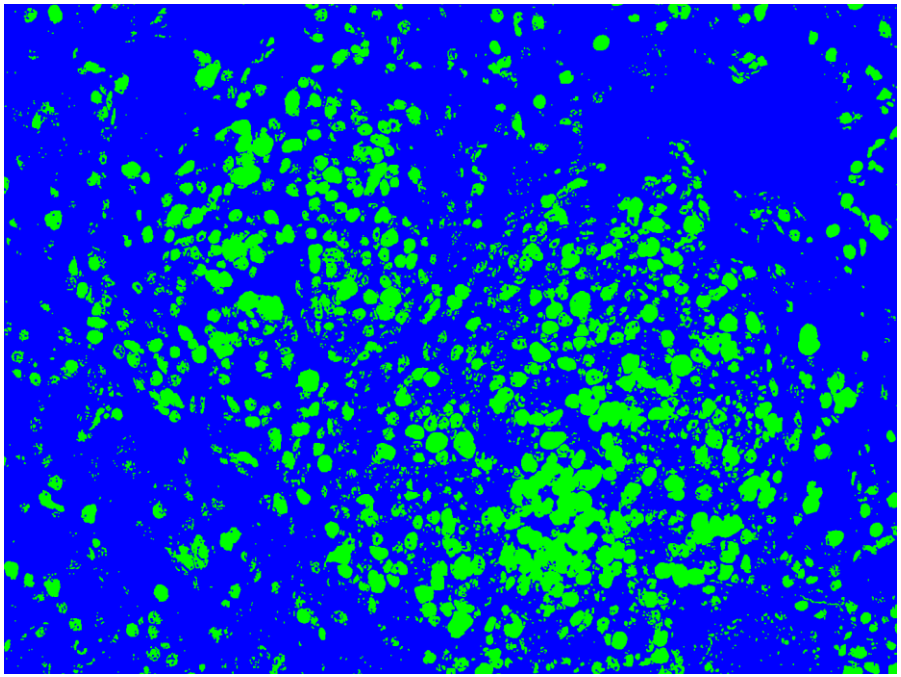
K-means



K-means

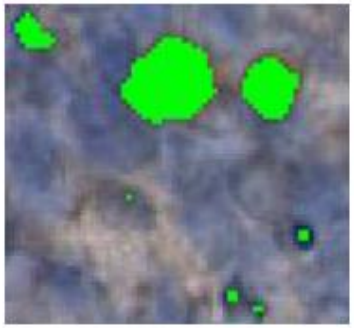
K-means

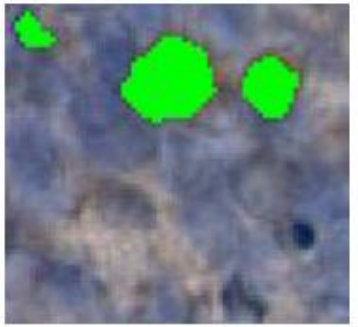




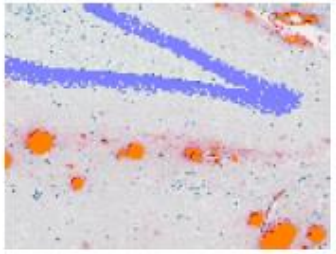
Post processing

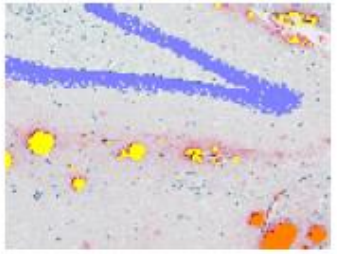
Clean-up / Noise removal: elimination of small or large objects



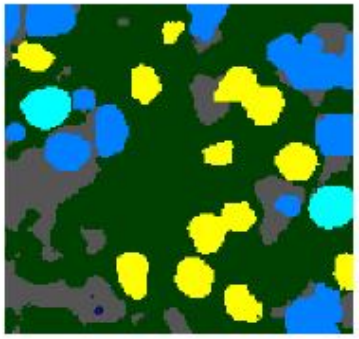


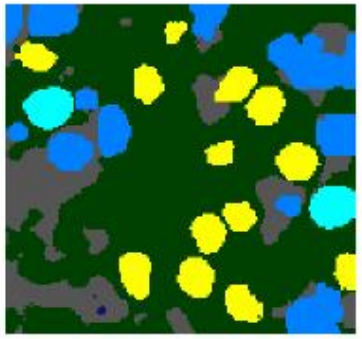
Discriminate objects based on distance to other objects



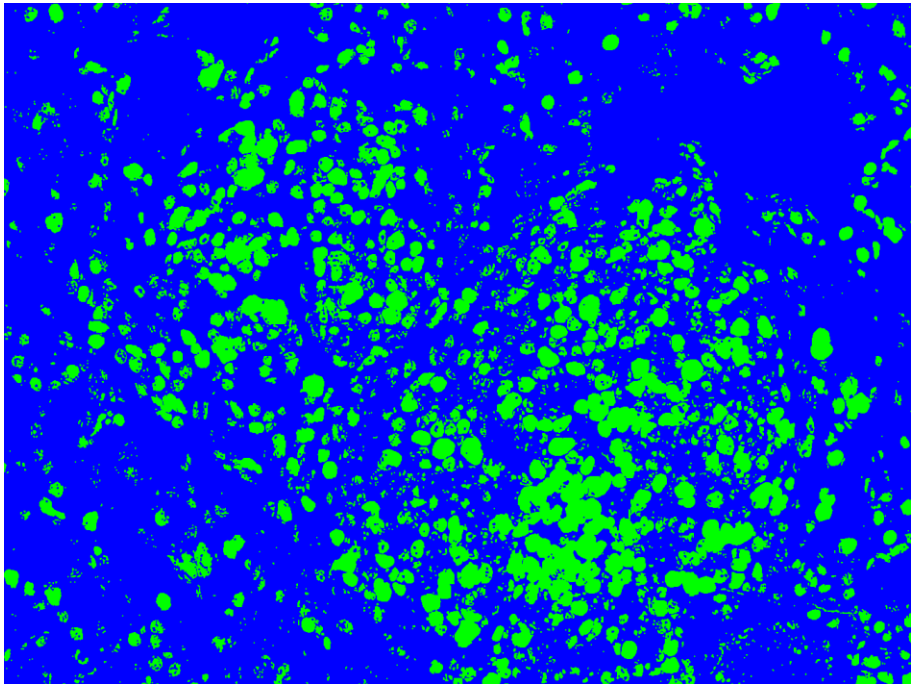


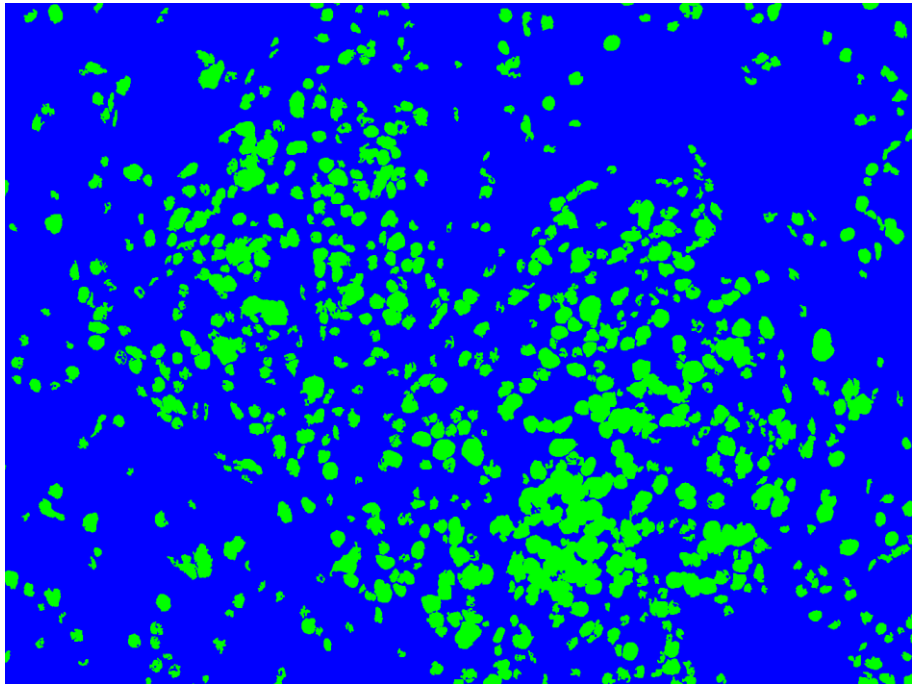
Separate objects, change based on shape or surroundings, erode, dilate, open, close, skeletonize, mark maxima, ...





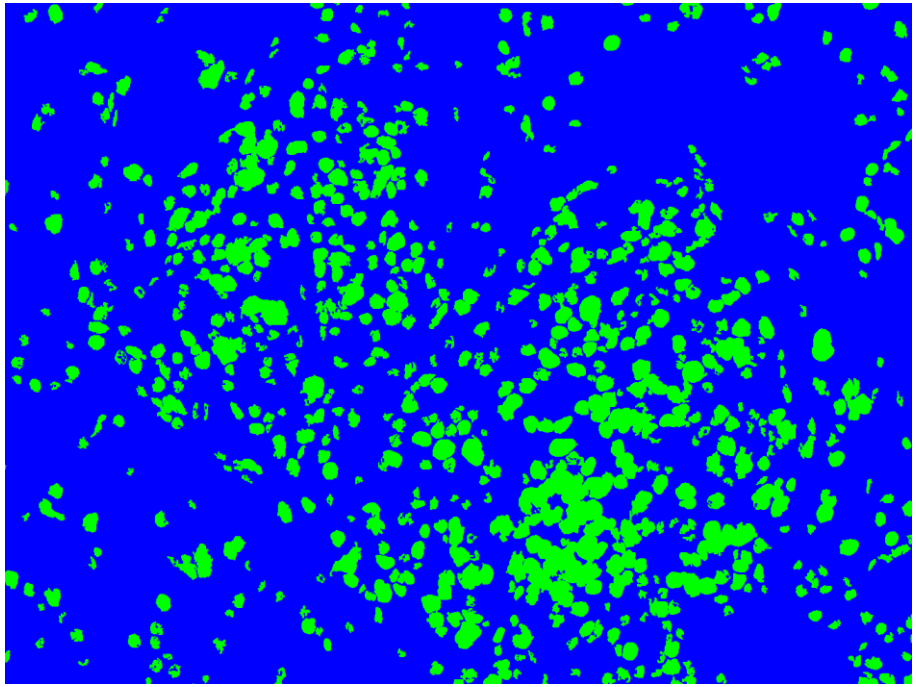
Post processing

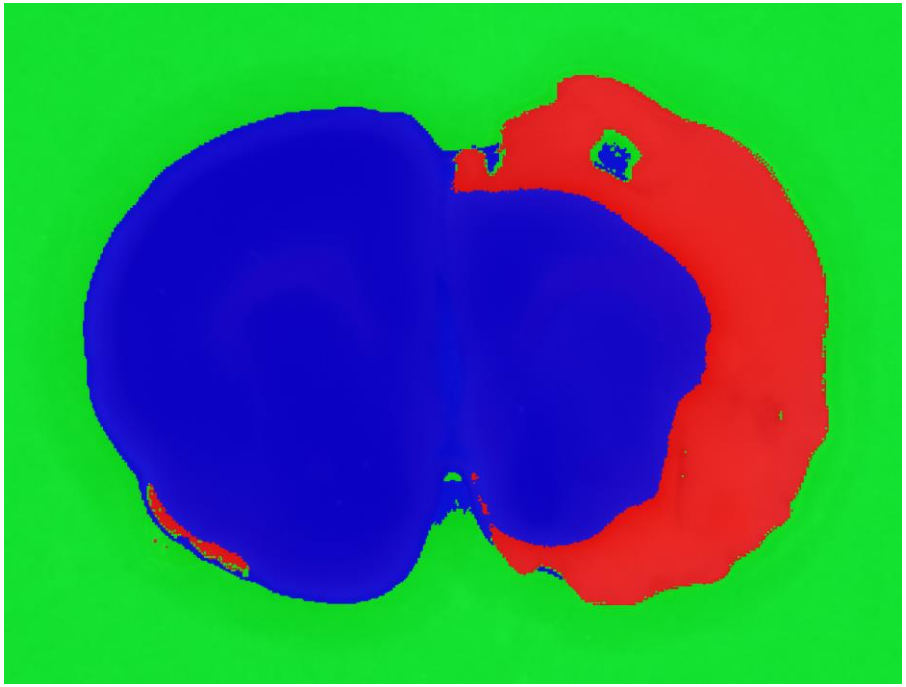




Post-processing:
Small green area, replaced by blue
Small blue area, replaced by green

Report of quantitative results



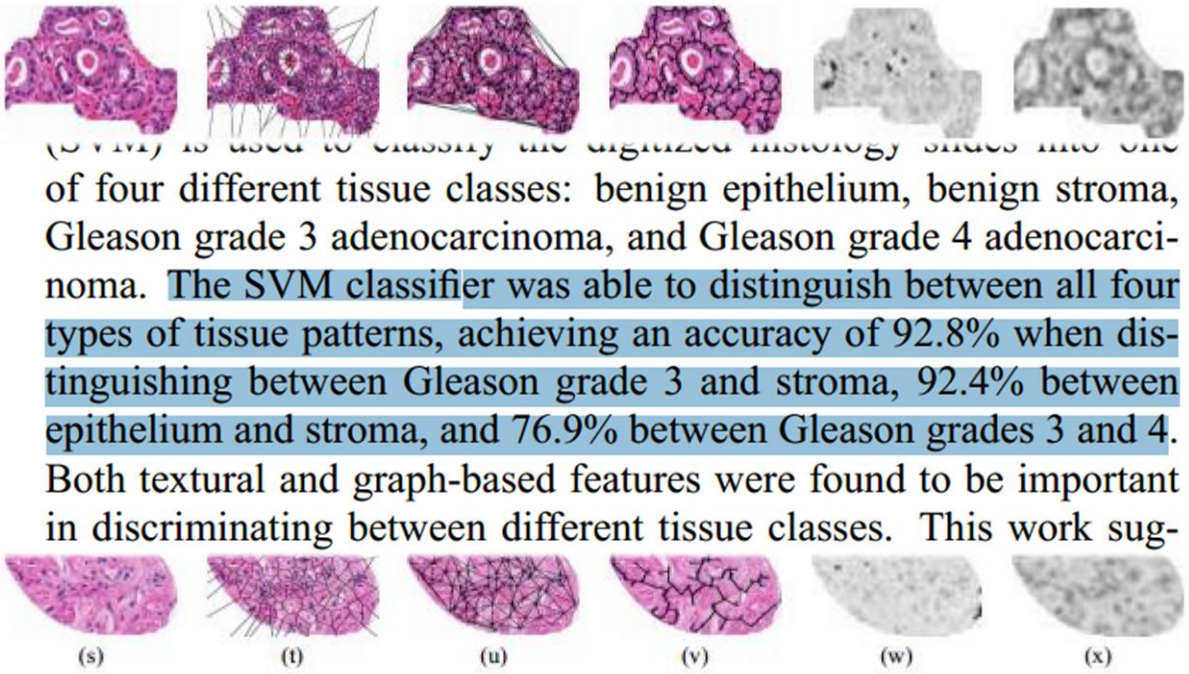


COUNT:
Typical number or fraction of objects

AREA:
Area of each category

Advanced algorithms

AUTOMATED GRADING OF PROSTATE CANCER USING ARCHITECTURAL AND TEXTURAL IMAGE FEATURES



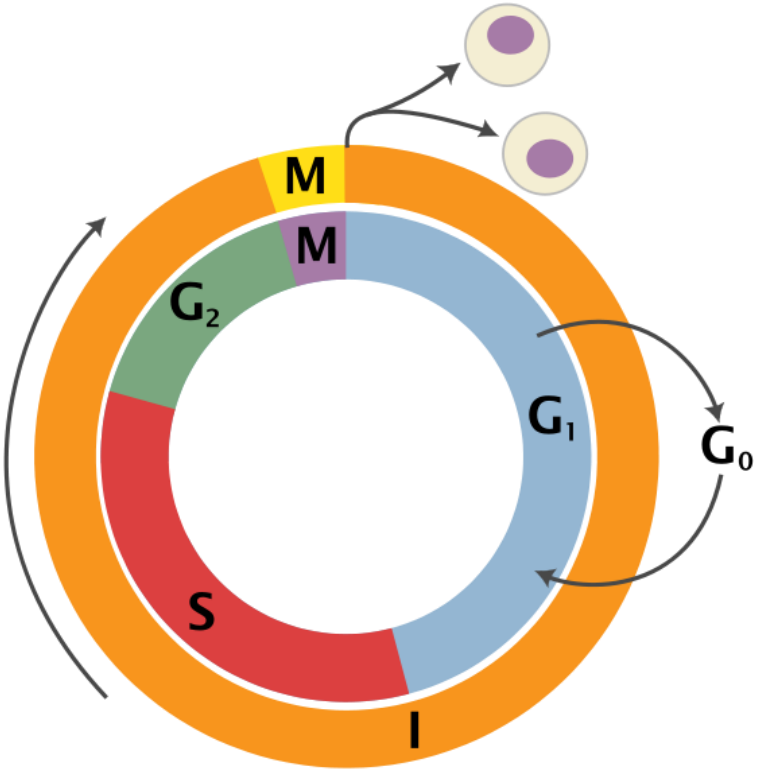
of four different tissue classes: benign epithelium, benign stroma, Gleason grade 3 adenocarcinoma, and Gleason grade 4 adenocarcinoma. The SVM classifier was able to distinguish between all four types of tissue patterns, achieving an accuracy of 92.8% when distinguishing between Gleason grade 3 and stroma, 92.4% between epithelium and stroma, and 76.9% between Gleason grades 3 and 4. Both textural and graph-based features were found to be important in discriminating between different tissue classes. This work sug-

Fig. 3. Comparison of ((a)-(f)) Gleason grade 3 tissue, ((g)-(l)) grade 4 tissue, ((m)-(r)) benign epithelium, and ((s)-(x)) benign stroma. Superimposed on ((a), (g), (m), (s)) the original images are ((b), (h), (n), (t)) the Voronoi diagram, ((c), (i), (o), (u)) the Delaunay triangulation, ((d), (j), (p), (v)) the minimum spanning trees, ((e), (k), (q), (w)) pixel entropy texture feature, and ((f), (l), (r), (x)) Gabor filter ($s = 3, \theta = \frac{5\pi}{8}$).

Ki67

- Ki67 expressed in dividing cells (G1, S, G2 and M phase)
- Ki67 not expressed in resting cells (G0)
- Used to calculate proliferation index (number of positive cell / total number of cells)
- ”Rule of thumb”:

  Higher Ki67 proliferation index means more malignant tumour



Ki67

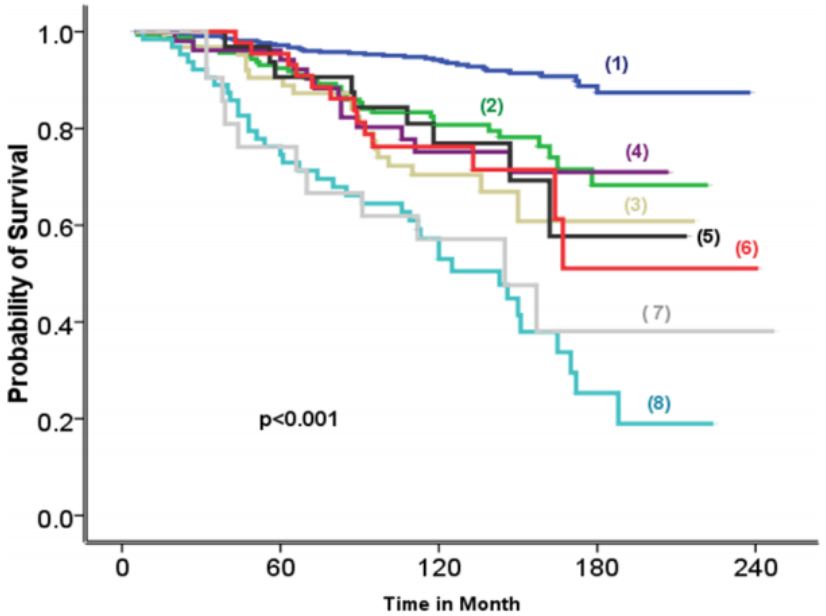


Figure 2 Association between BCSS and Ki-67LI expressed in 10% increments (10% each.) However, tumors showing 50% to 69% and 80% to 100% Ki-67LI were considered as two groups, as the number in each 10% subgroup was small). Labels 1 through 8 represent patients' subsets based on tumor Ki-67LI, where 1 is 0 to 9%; 2 is 10% to 19%; 3 is 20% to 29%; 4 is 30% to 39%; 5 is 40% to 49%; 6 is 50% to 69%; 7 is 70% to 79%; and 8 is 80% to 100%.

Breast cancer

Virtual Double Staining Digital Image analysis – Ki67

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17th Dako User meeting, April 2015, Copenhagen

Disclosures: none

NordiQC

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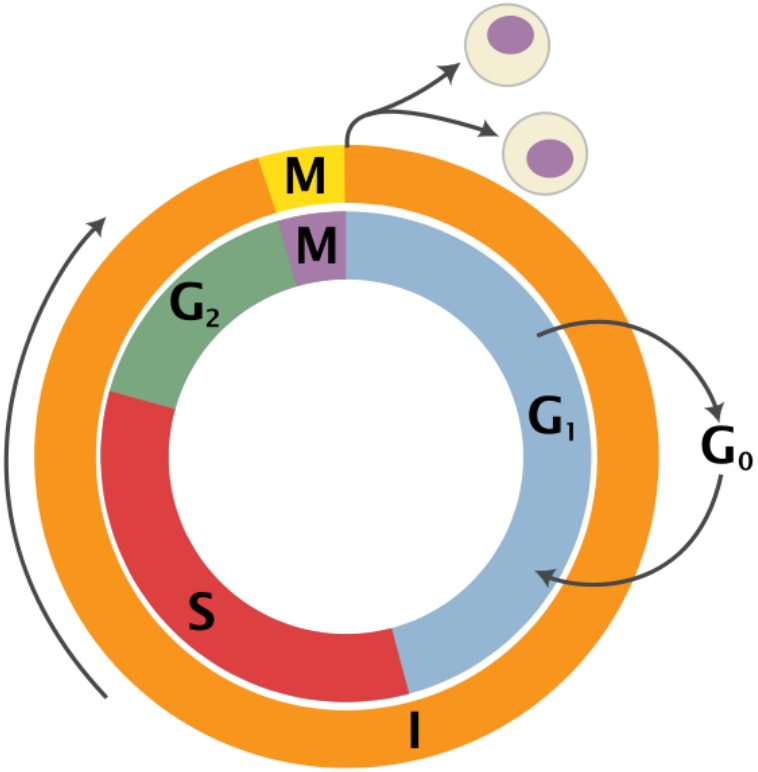
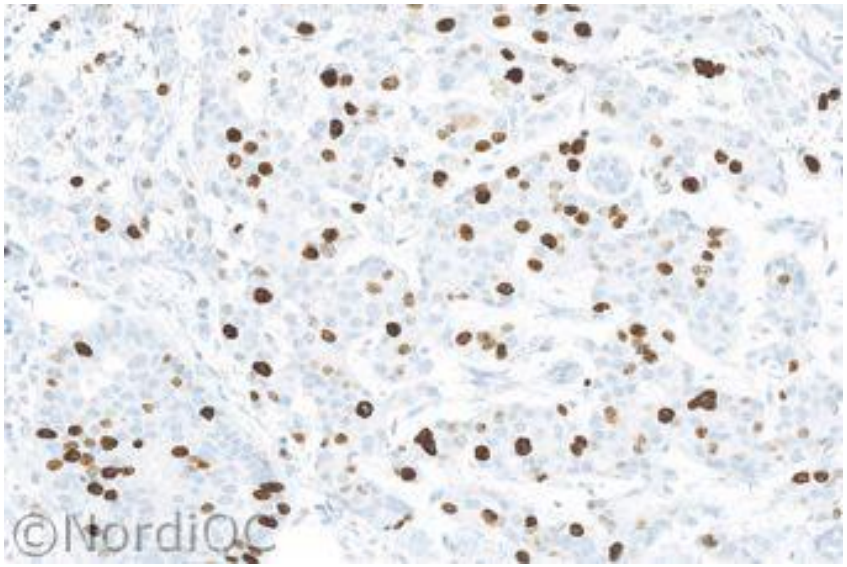
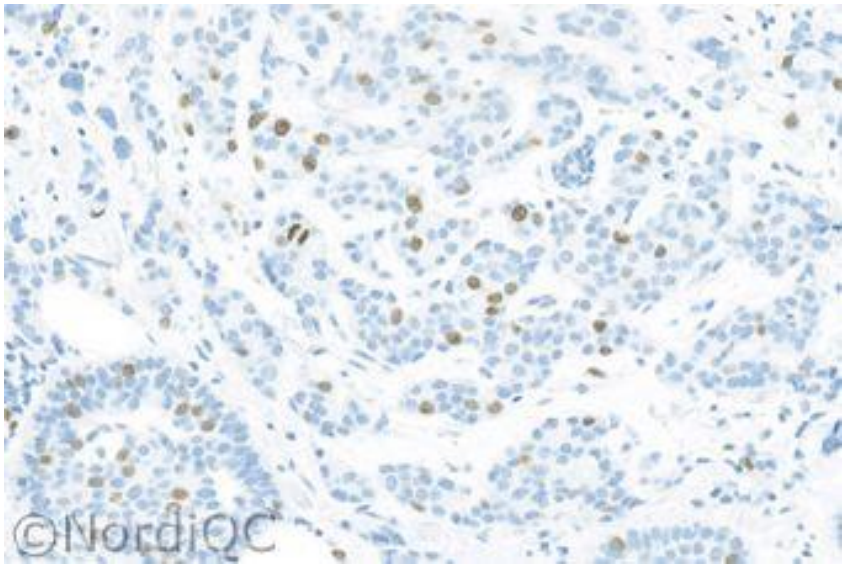


Ki67 – why is it important?

- Breast cancer:
  - Both a prognostic and predictive marker
  - Cut-off points have been suggested
- Neuroendocrine tumours
  - Grading

Ki67 – why staining quality is important





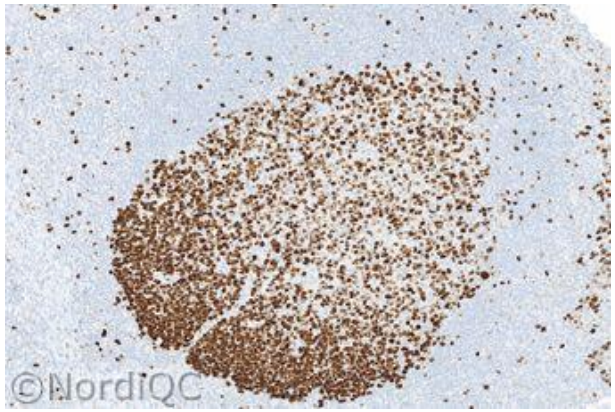
Ki67 - NordiQC

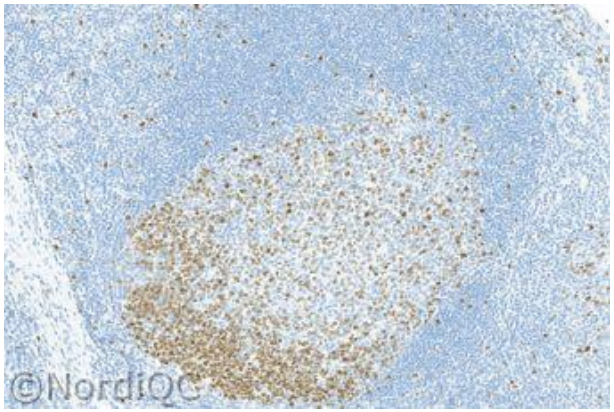
Performance in 4 NordiQC runs

	2001	2007	2009	2012
Participants	42	100	124	229
Sufficient	71%	73%	77%	89%

Performance marks in Run B13 (2012)

	Optimal	Good	Borderline	Poor
Total	166	39	18	6
Proportion	72%	**17%**	**8%**	**3%**



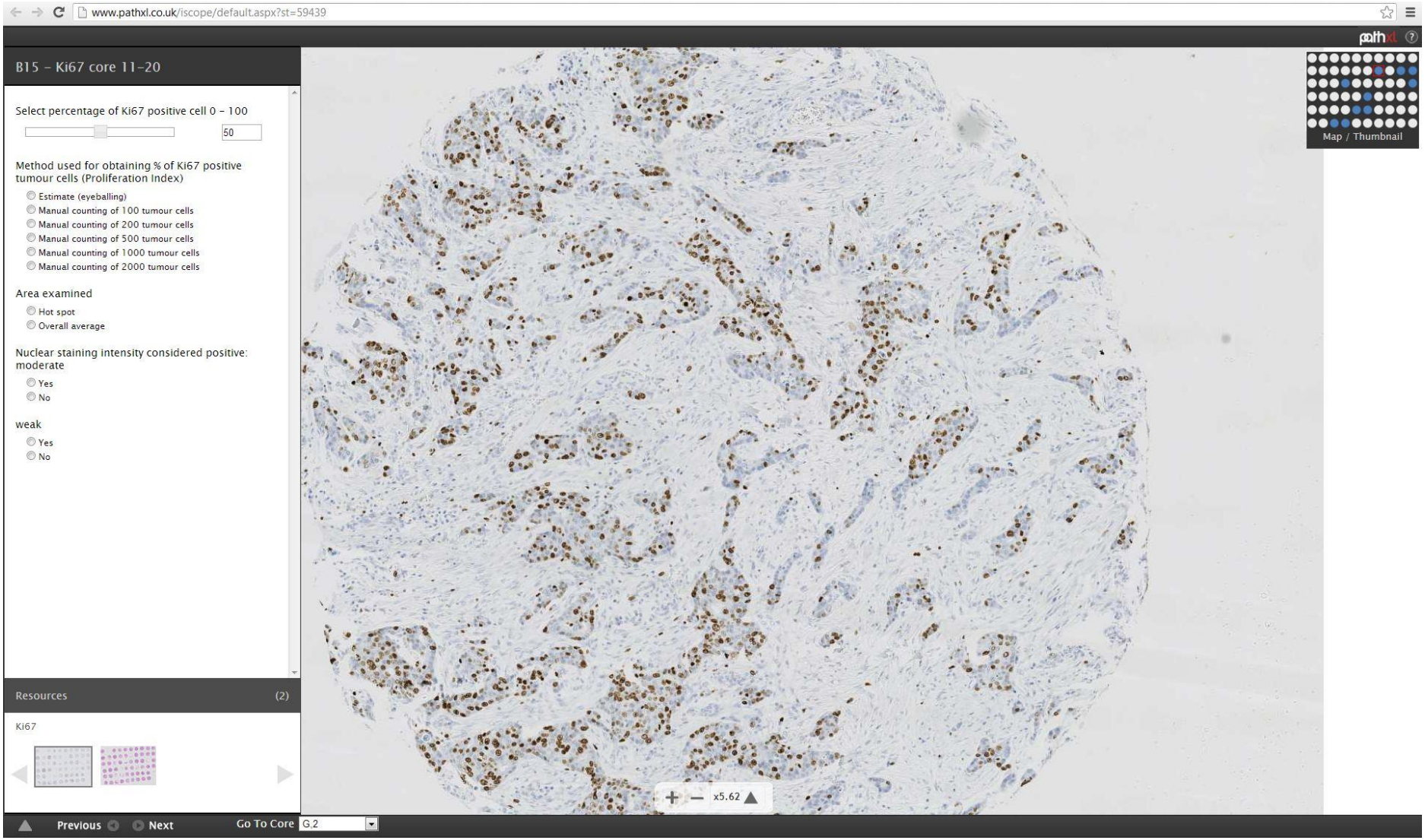


Second NordiQC Ki67 challenge

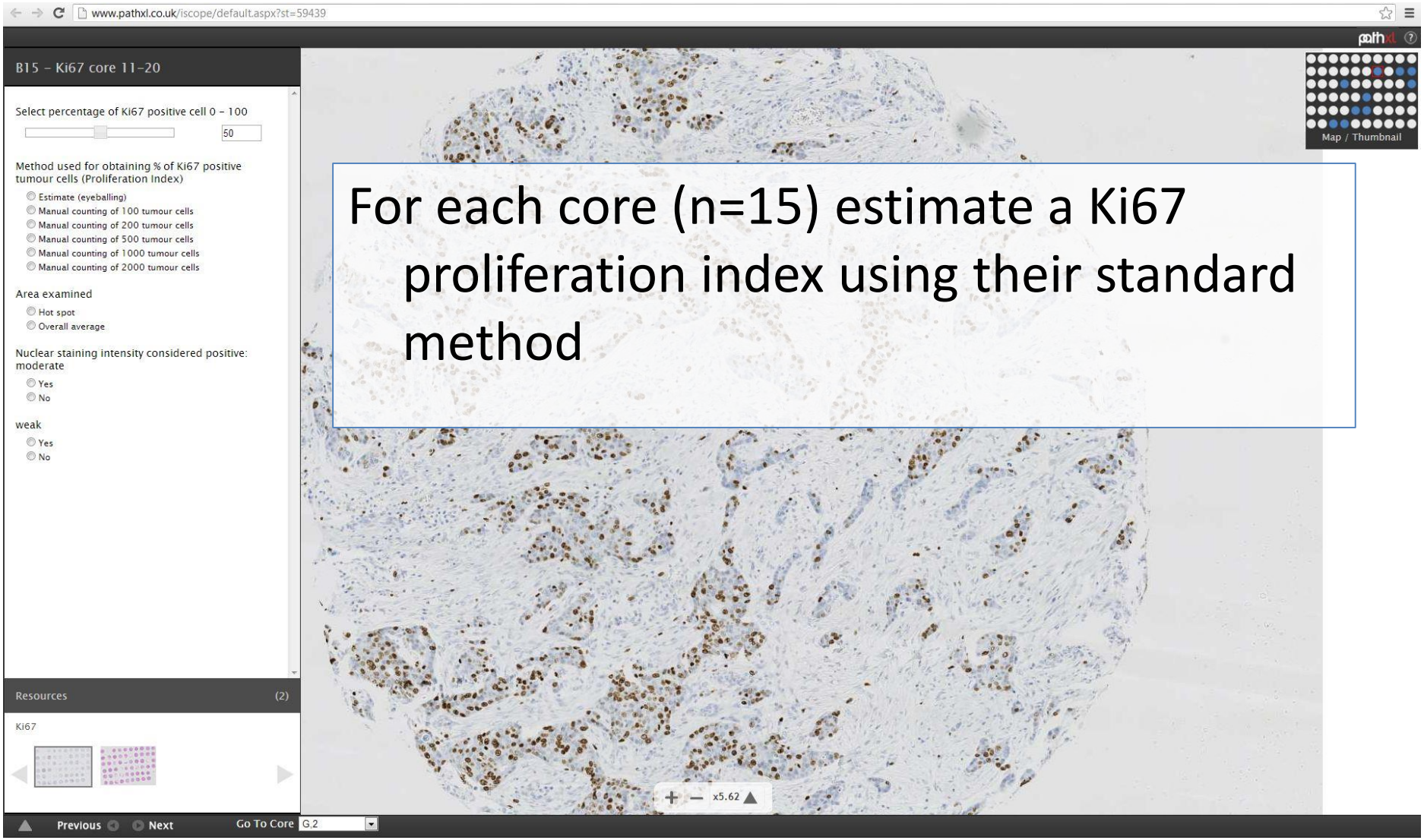
- Objective:
  - Examine current practices for scoring of Ki67 stained breast carcinomas among the NordiQC participants

- 605 laboratories invited to participate

Virtual microscopy



Virtual microscopy



For each core (n=15) estimate a Ki67 proliferation index using their standard method

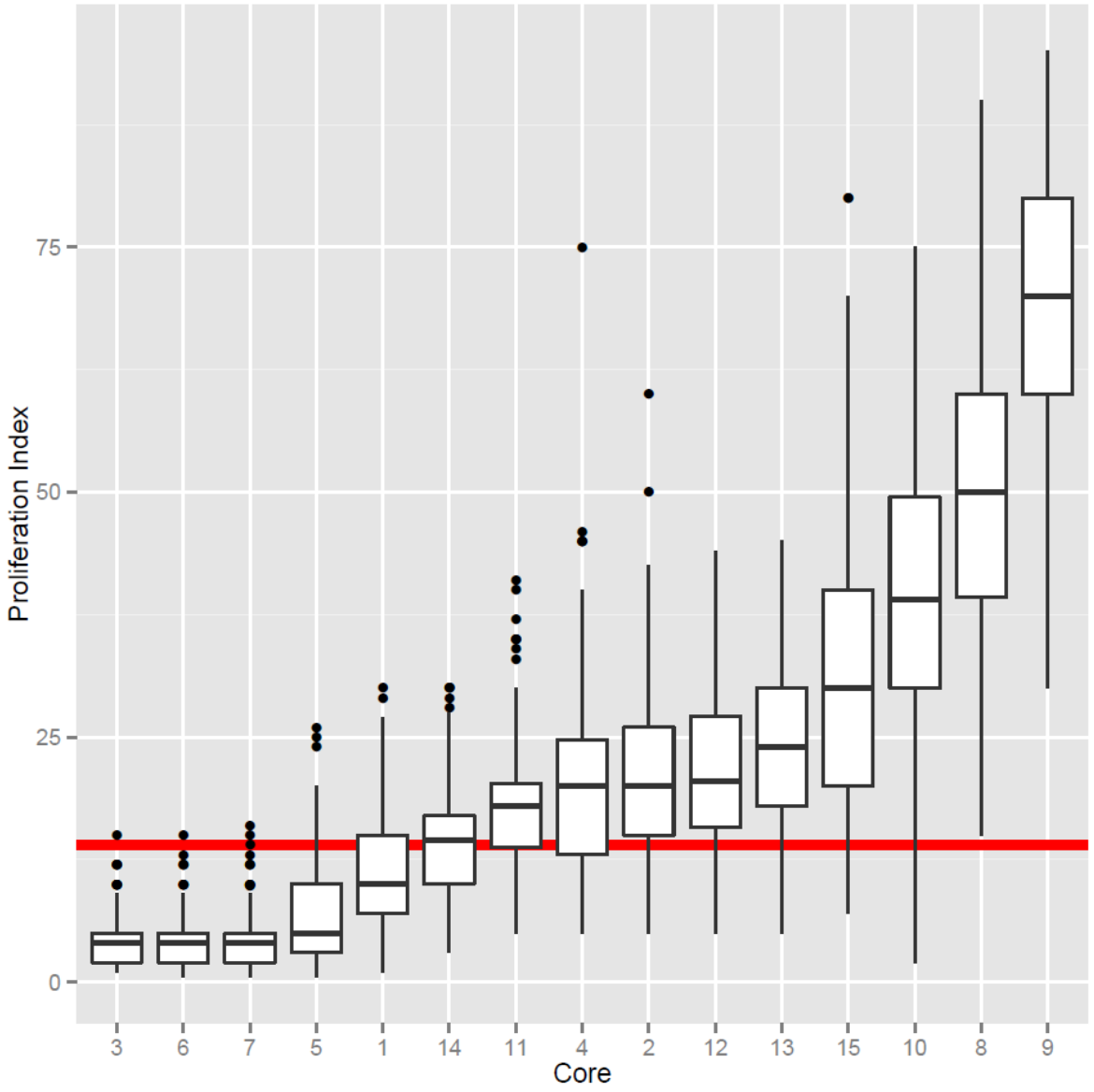
2nd NordiQC Ki67 challenge

Also asked:

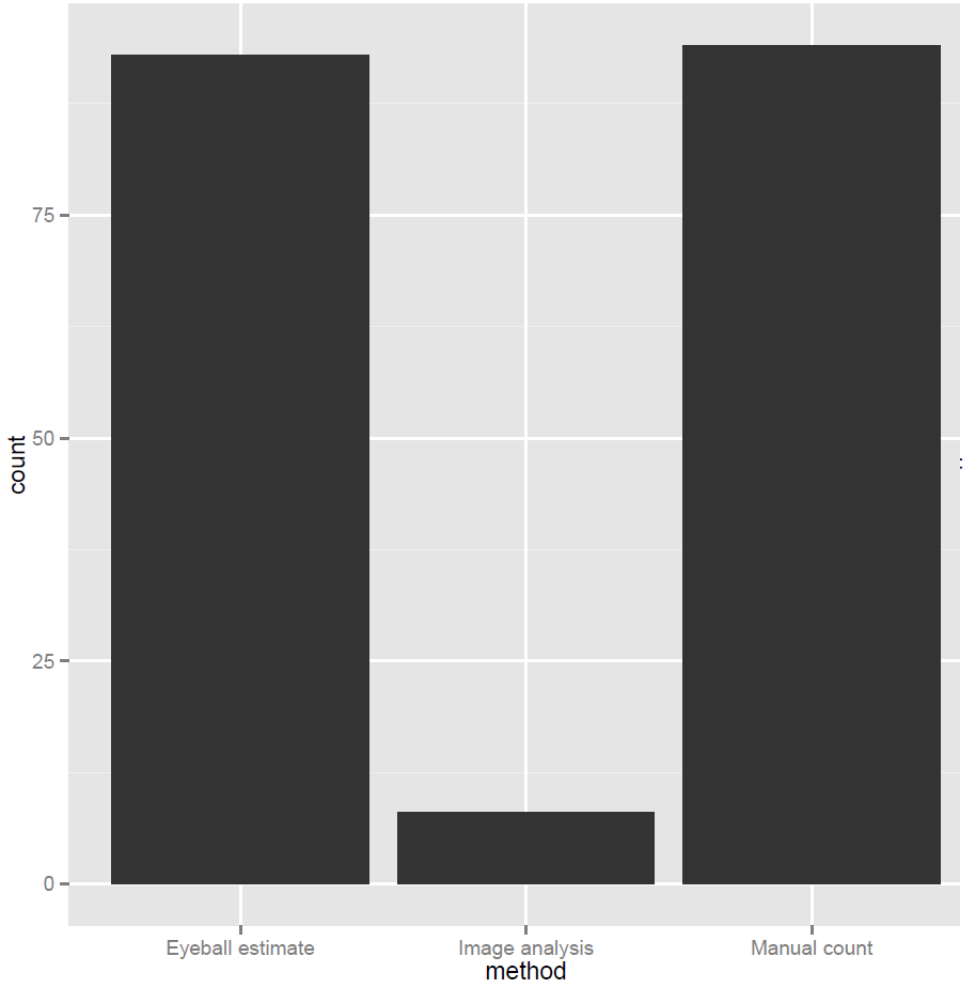
- Job title
- Method used
- Area examined
- Consider moderately stained nuclei as positive
- Consider weakly stained nuclei as positive

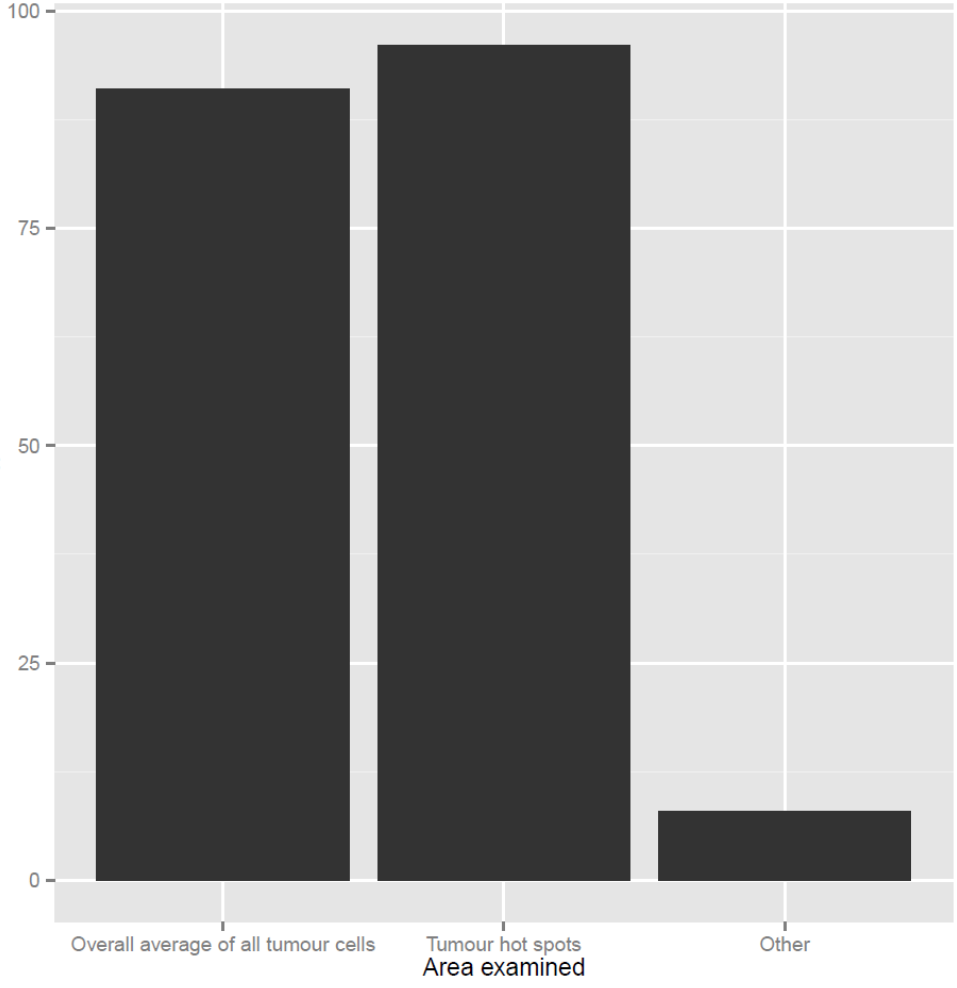
Overall results

n = 204



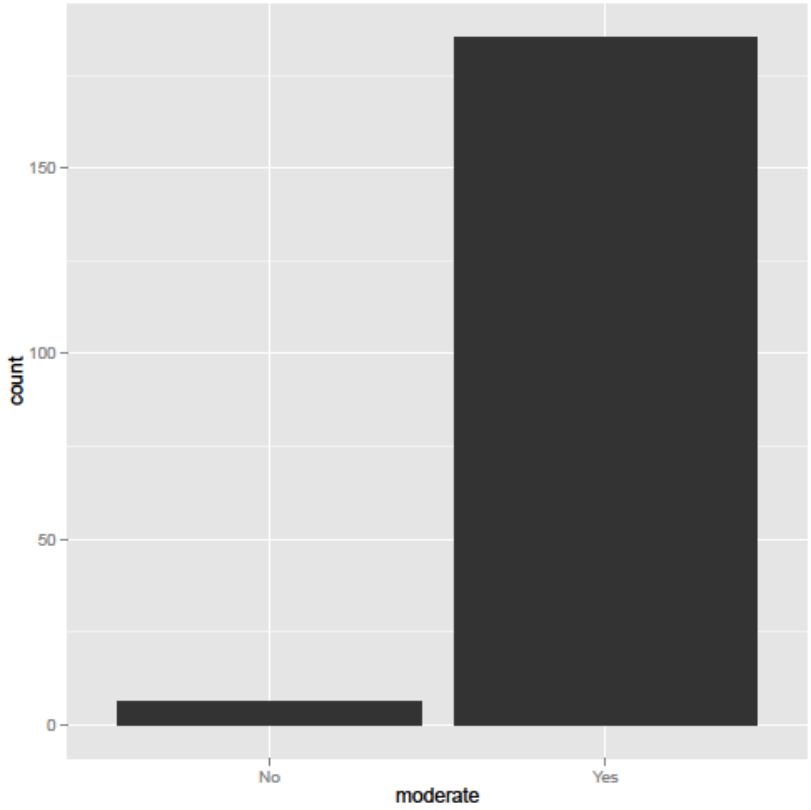
Results

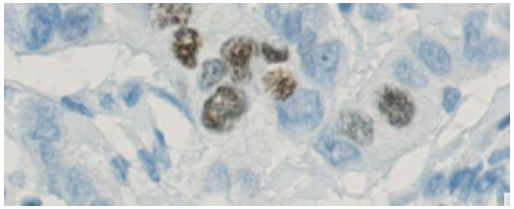

count
75
50
25
0
Eyeball estimate
Image analysis
Manual count
method


100
75
50
25
0
Overall average of all tumour cells
Tumour hot spots
Other
Area examined

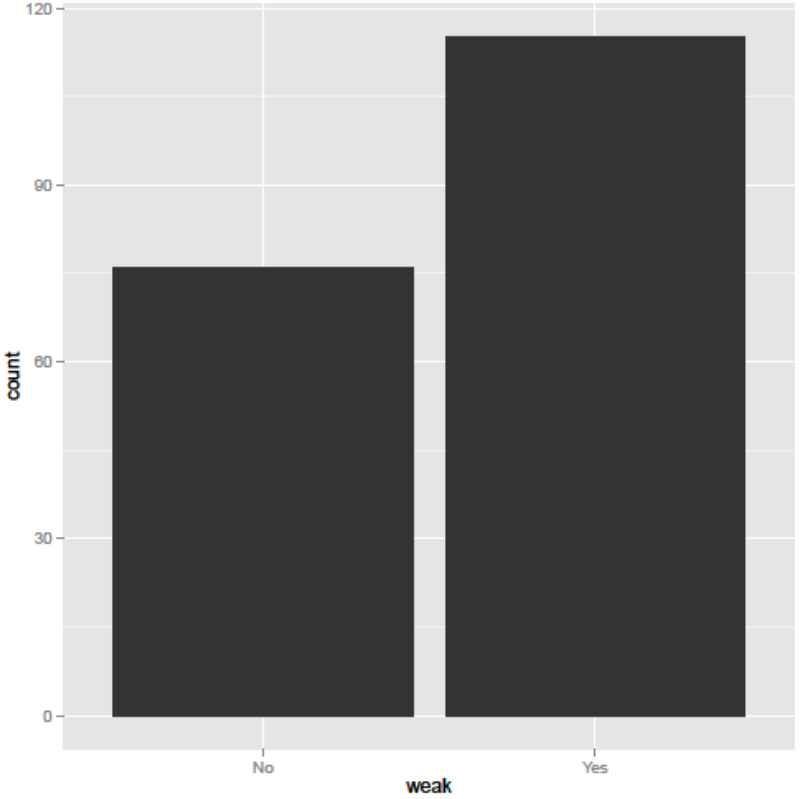
Which cells were considered positive?

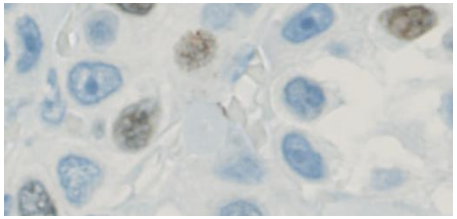
Moderately stained



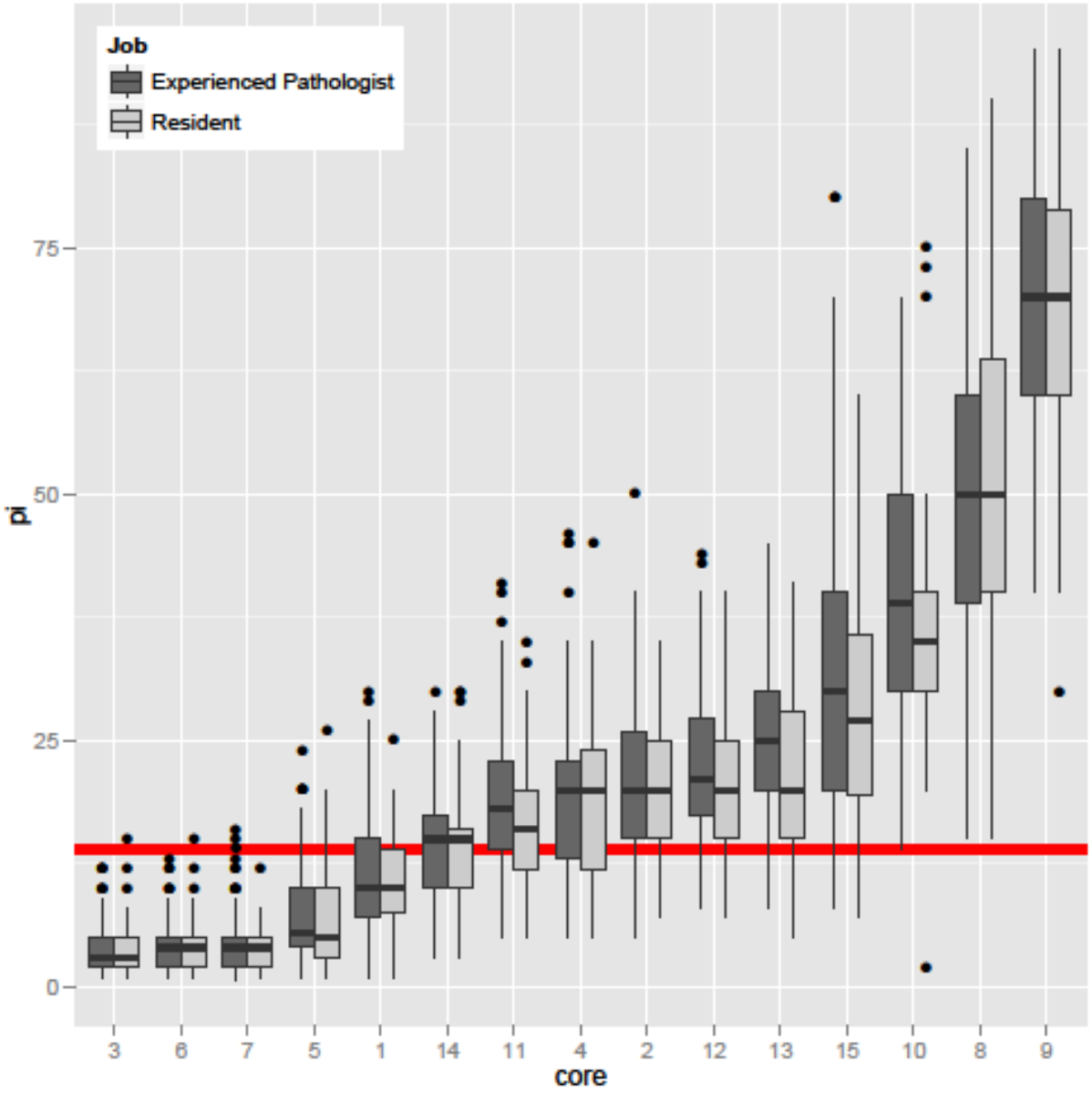


Weakly stained

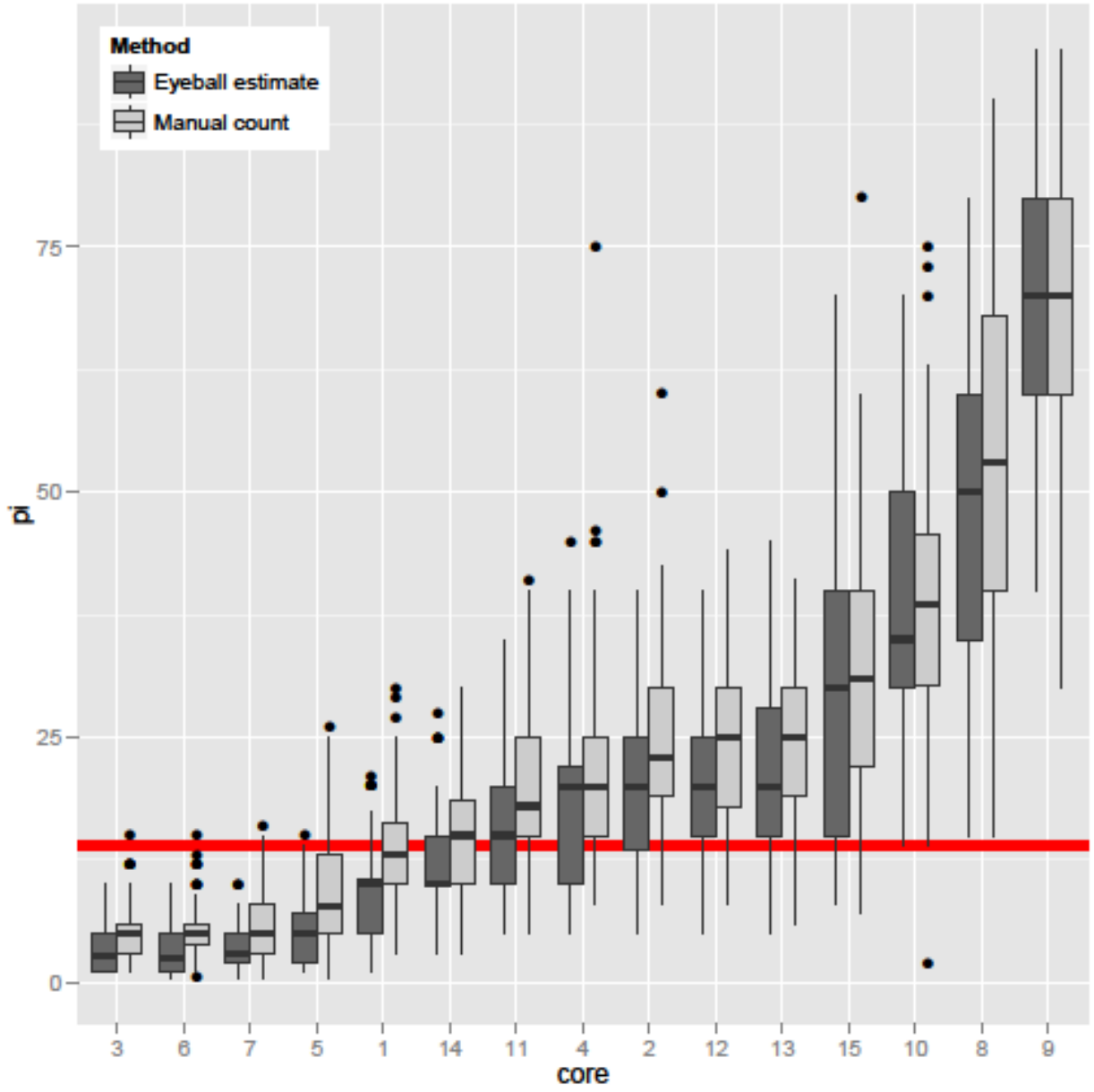




Influence of experience



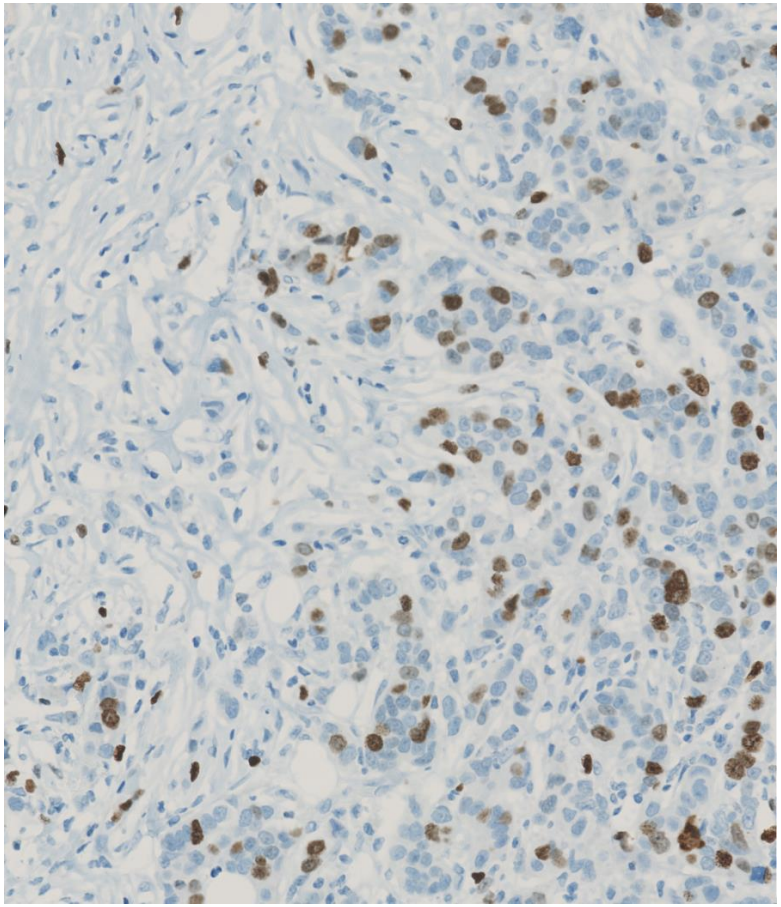
Influence of method



Digital Image Analysis

Criteria

- Identify nuclei
- Distinguish Ki67 positive and negative nuclei
- Exclude non-tumour cells from analysis



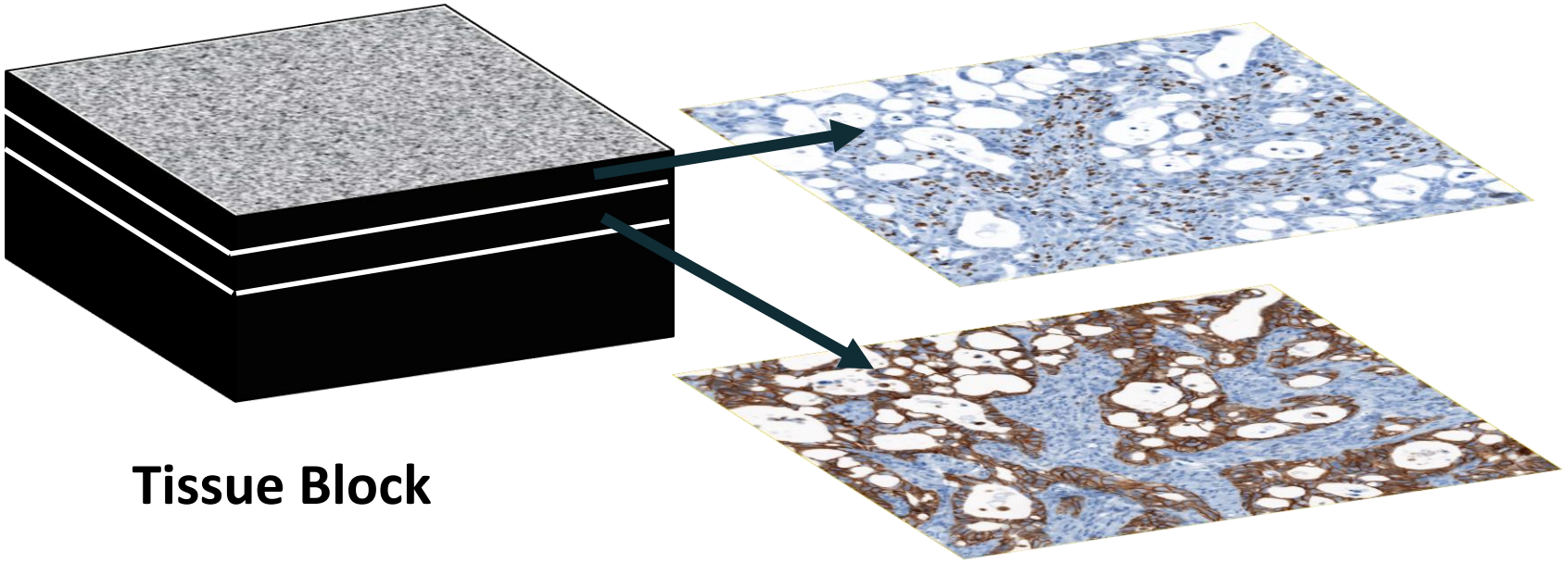
Digital Image Analysis – Ki67

VIRTUAL DOUBLE STAINING (VDS)

Virtuel Double Staining: concept

Cut serial sections (3µm):

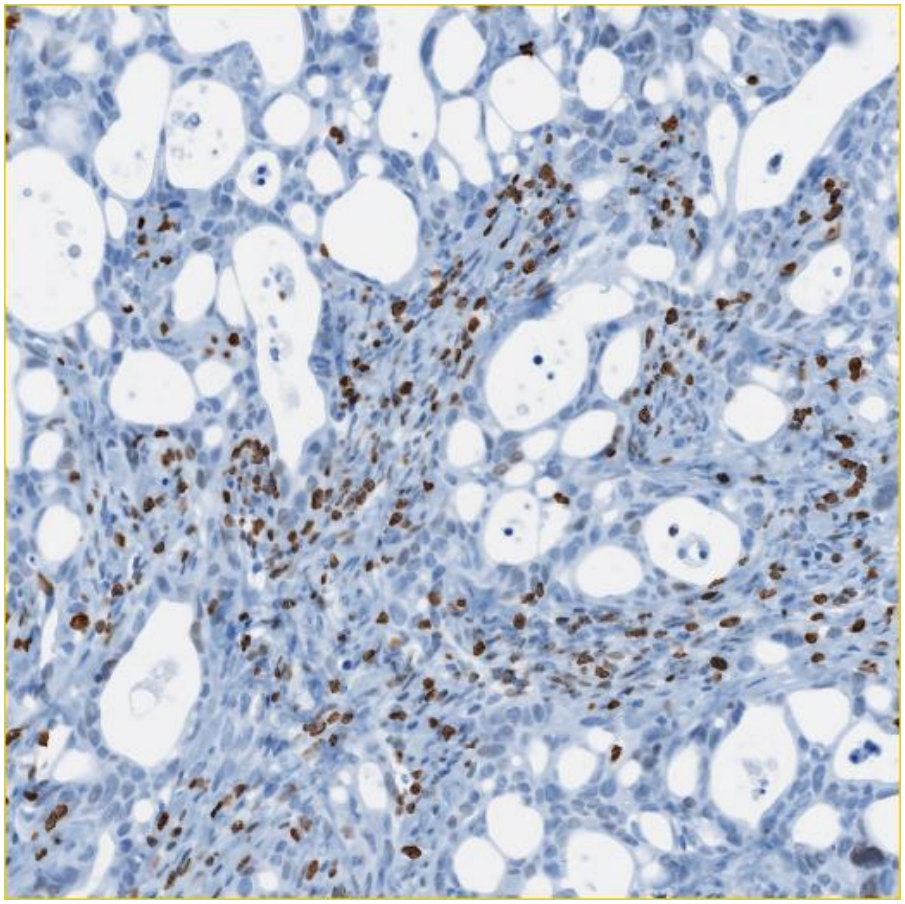
- **Slide stained for Ki67**



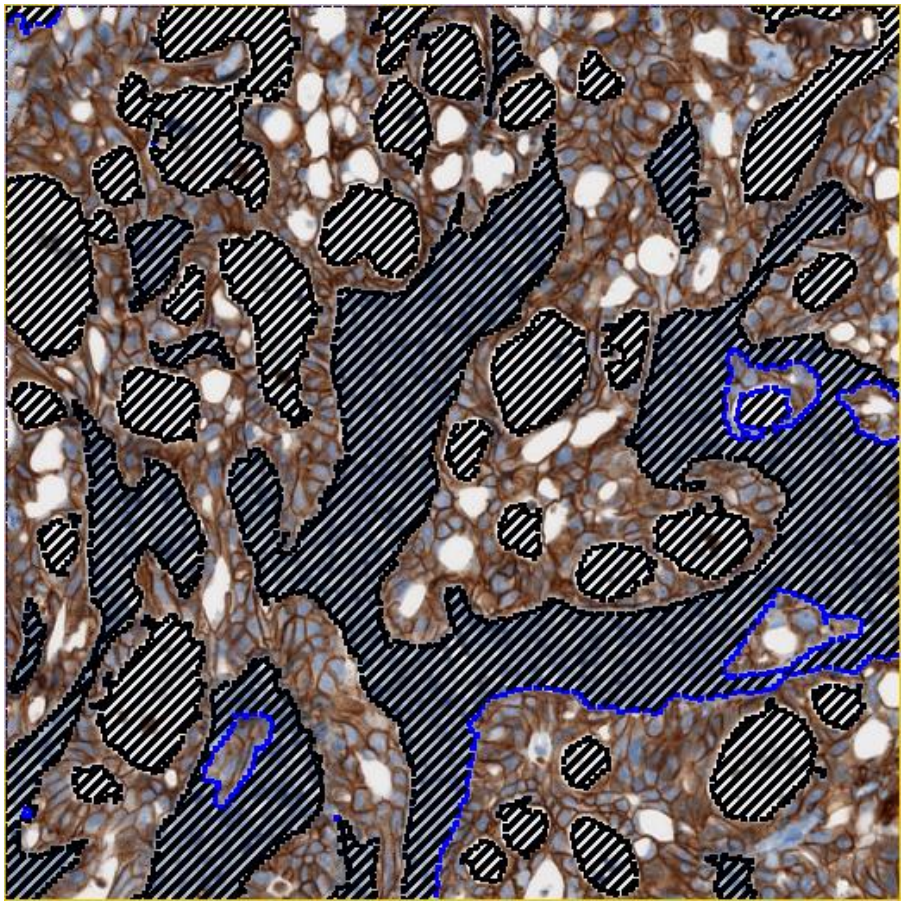
Tissue Block

- **Neighboring slide stained for pancytokeratin**

Image analysis for identification of tumor

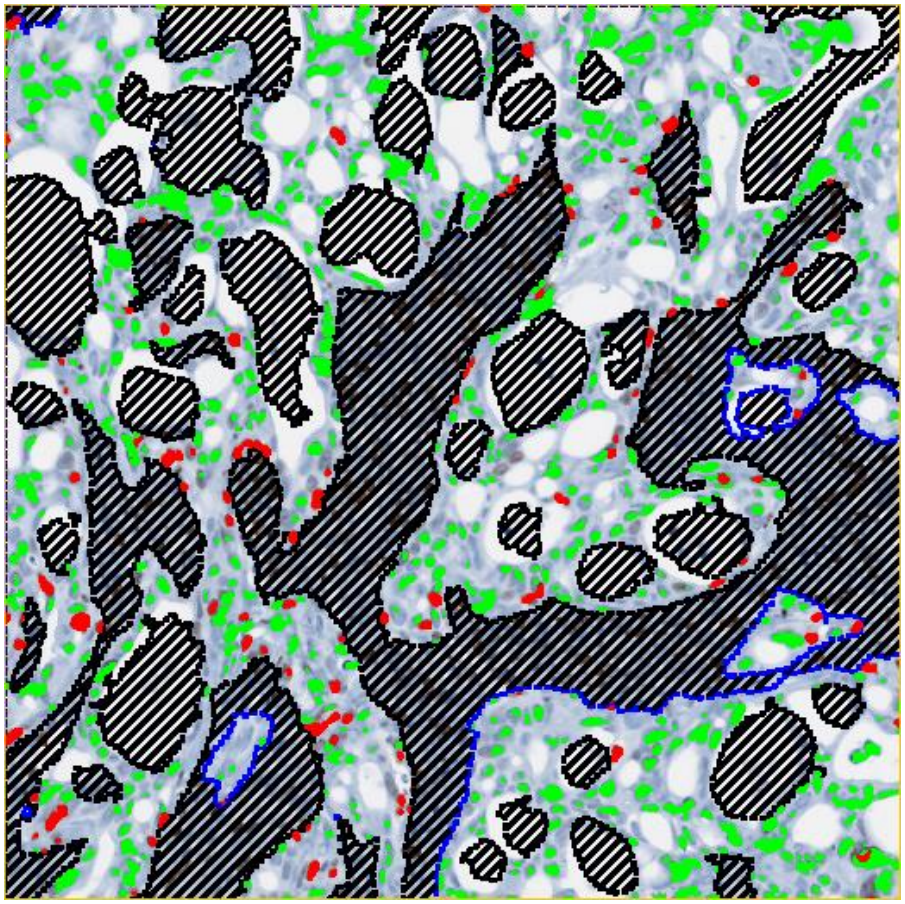


Ki67

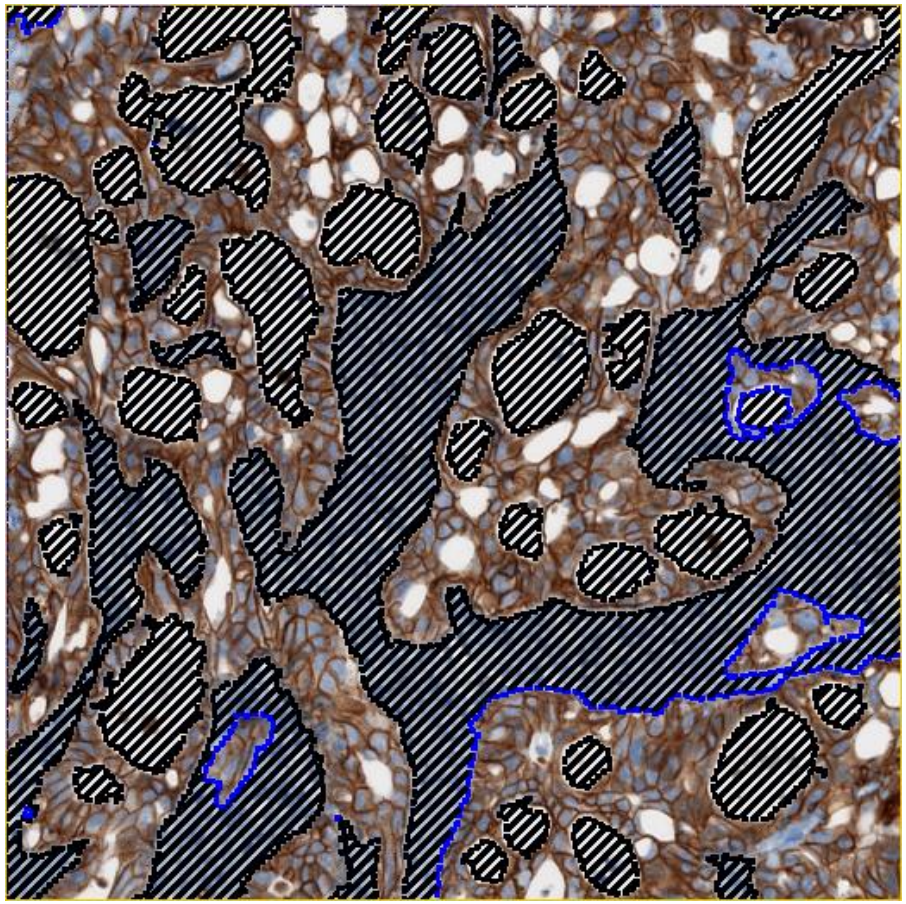


Pancytokeratin

Image analysis for identification of biomarker (Ki67)



Ki67



Pancytokeratin

Digital Image Analysis – Ki67

VALIDATION OF VDS

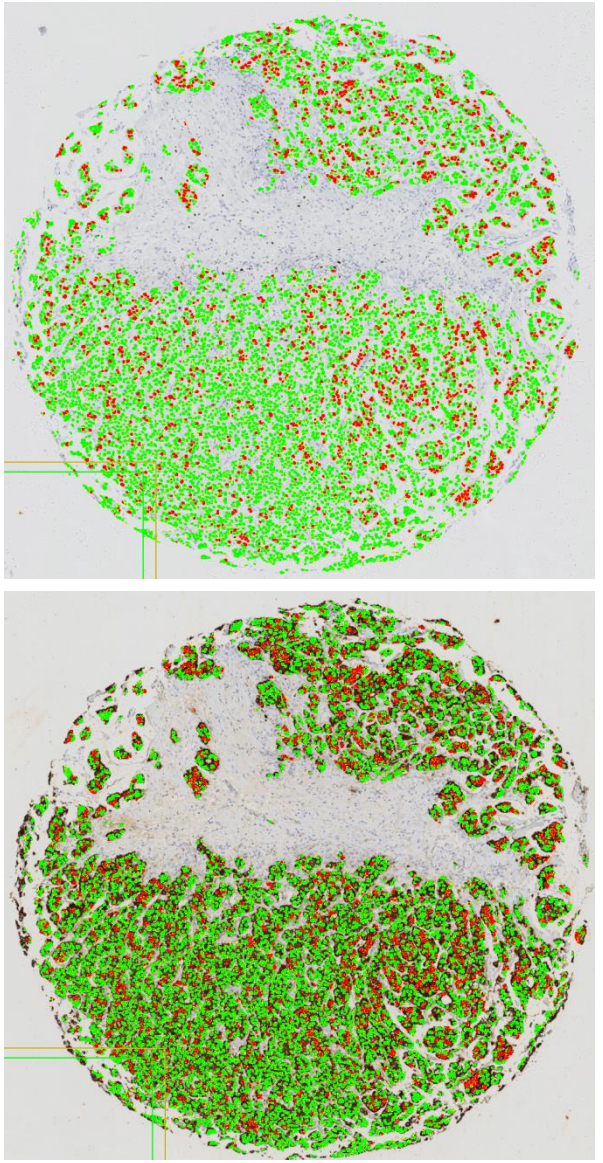
Validation of Virtual Double Staining

- Validation of the Nuclear detection and segmentation (number of positive and negative nuclei)

- Validation of the alignment algorithm
  - Overlap/agreement between slides
  - Sensitivity to distance between slides

Validation of Ki67 counting

- Algorithm was developed by Visiopharm according to sample cases labelled of pathologists
- Identifies nuclei based on form and categorises as ”positive” or ”negative” based on intensity and extension of stain
- Also possible to calculate a Digital H-score based on weakly, moderately and strongly stained nuclei



Validation of Ki67 counting

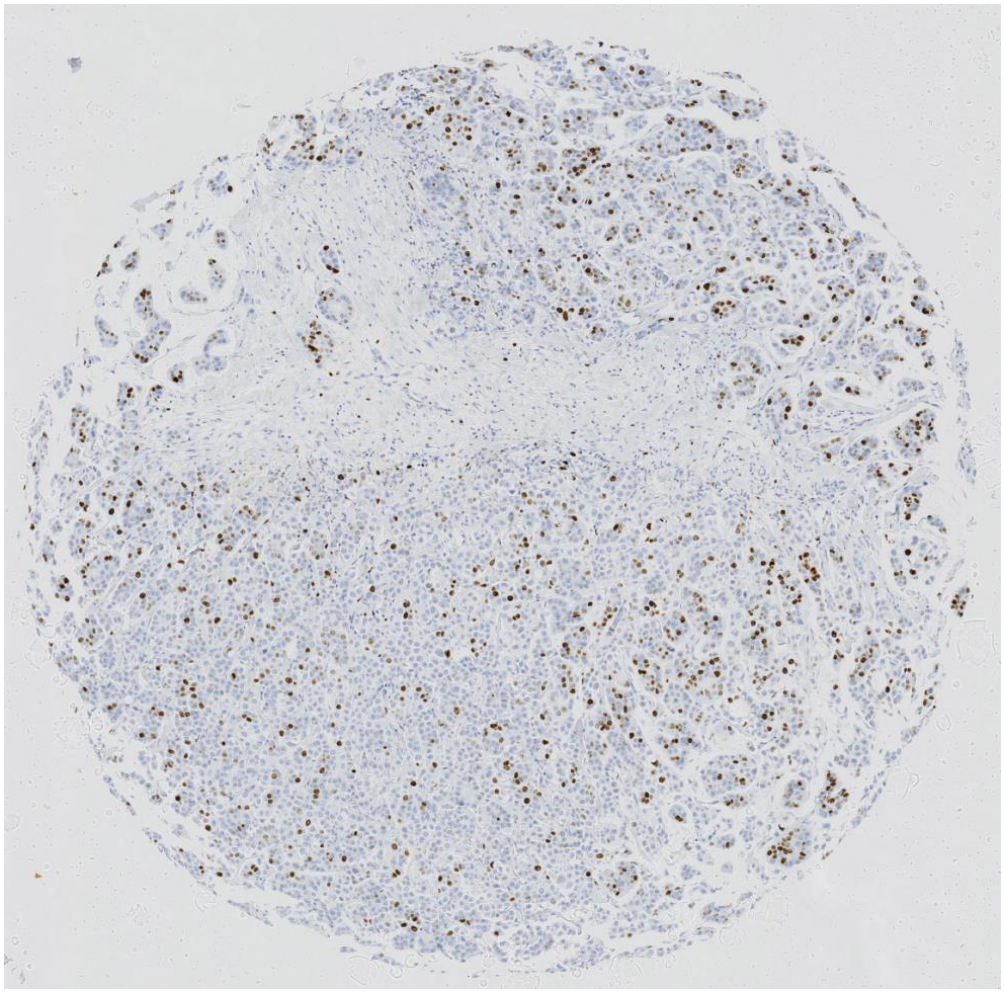
- Comparison of Manual counting of randomly selected areas and Digitial Image Analysis (Virtual Double Staining) on exactly the same areas

- Comparison of Manual counting of randomly selected areas and Digital Image Analysis (Virtual Double Staining)

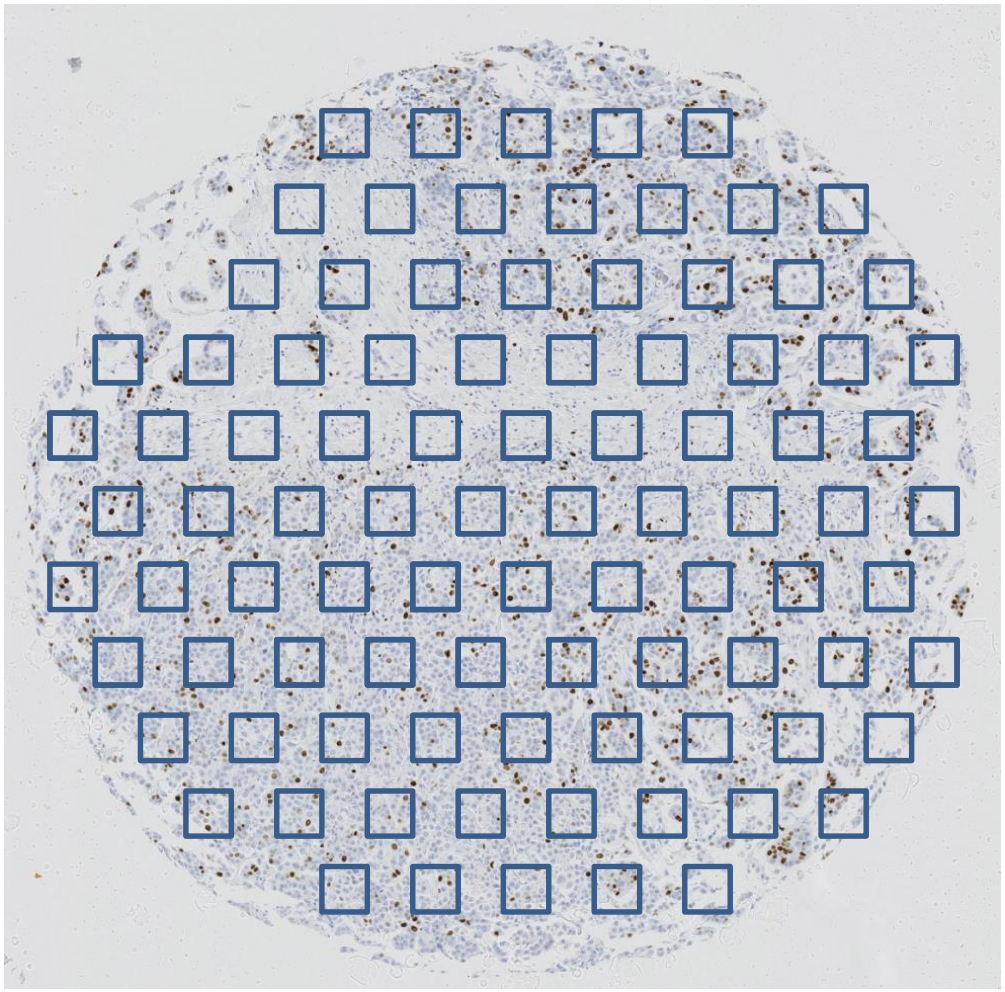
Method

- 3 TMAs containing more than 100 cores of breast carcinomas
- 2 slides were cut from each block, one stained for PCK, one for Ki67
- Areas were sampled from each core using SURS (systematic uniform randomized sampling) for manual counting
- Only a small percentage of total number of cells were counted (200-400)

Systematic Random Sampling

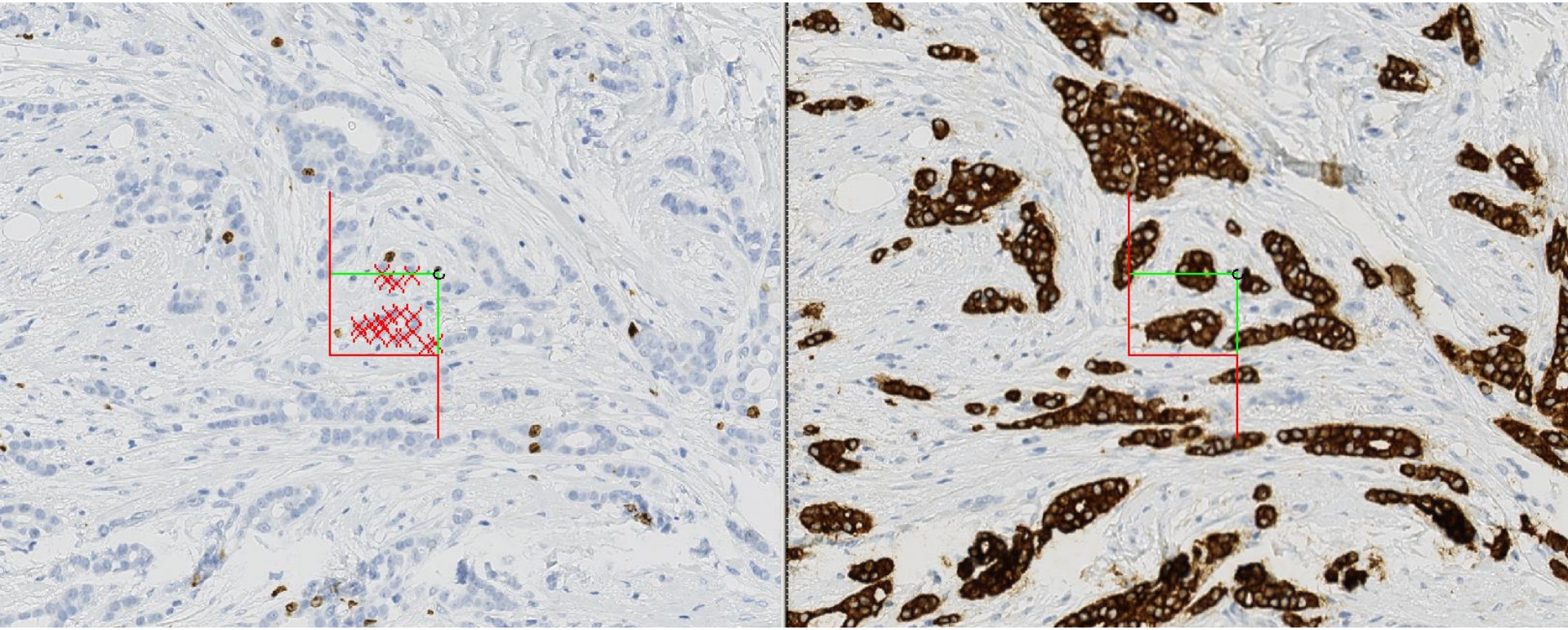


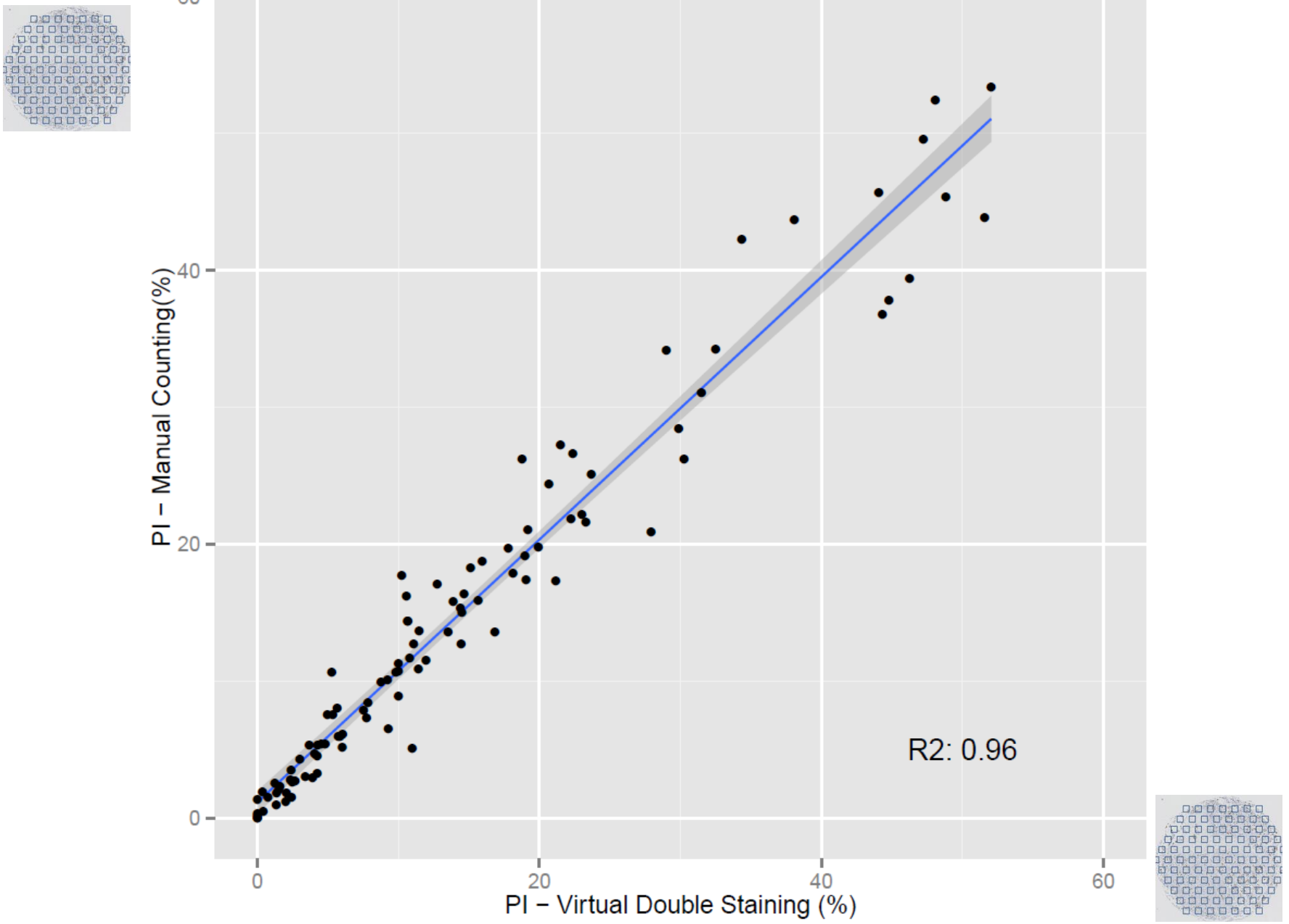
Systematic Random Sampling



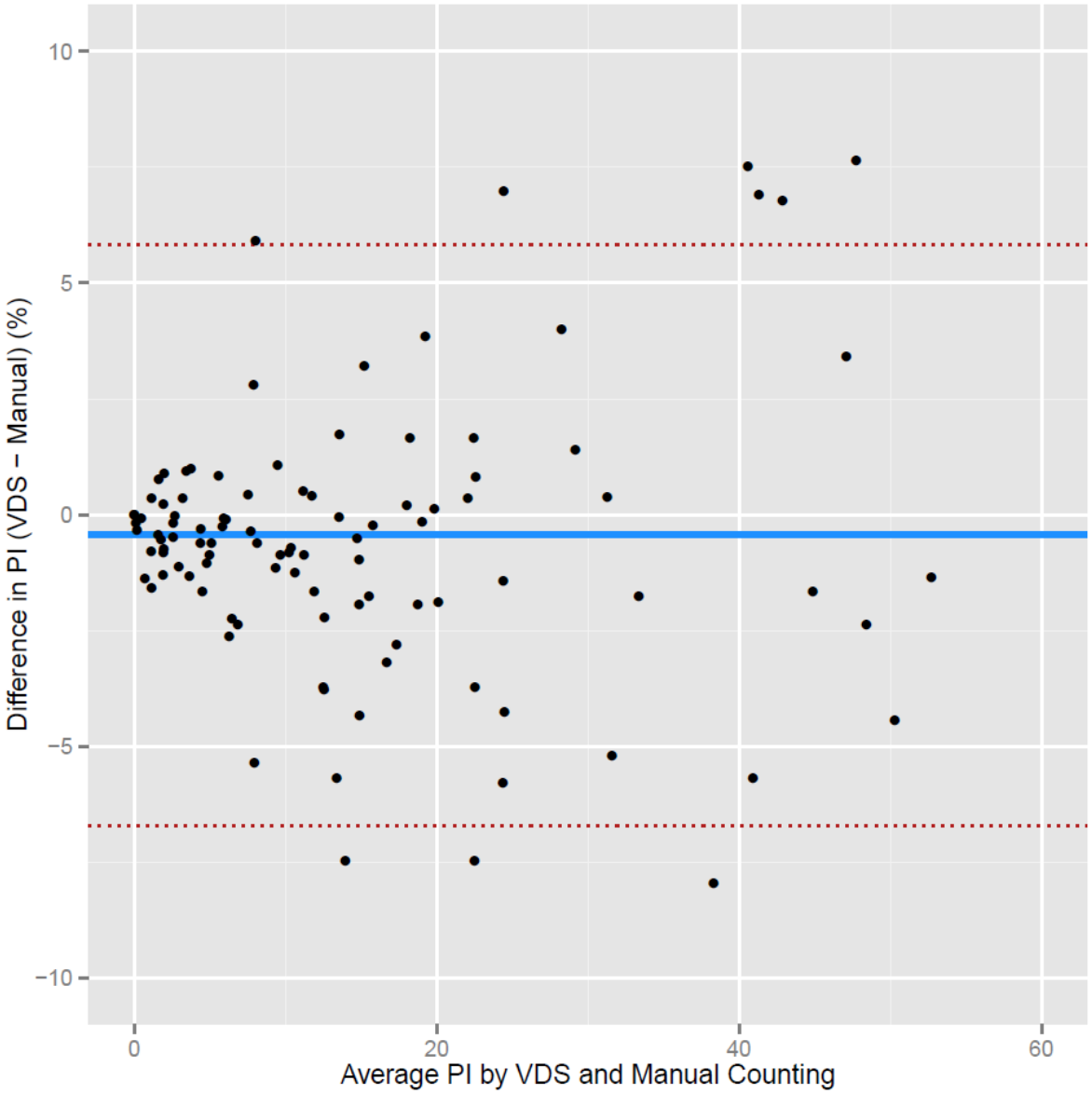
- Grid of frames randomly placed on core
- Positive and negative tumour cells counted manually in each frame
- Each frame extracted as an image for Virtual Double Staining

Stereological counting

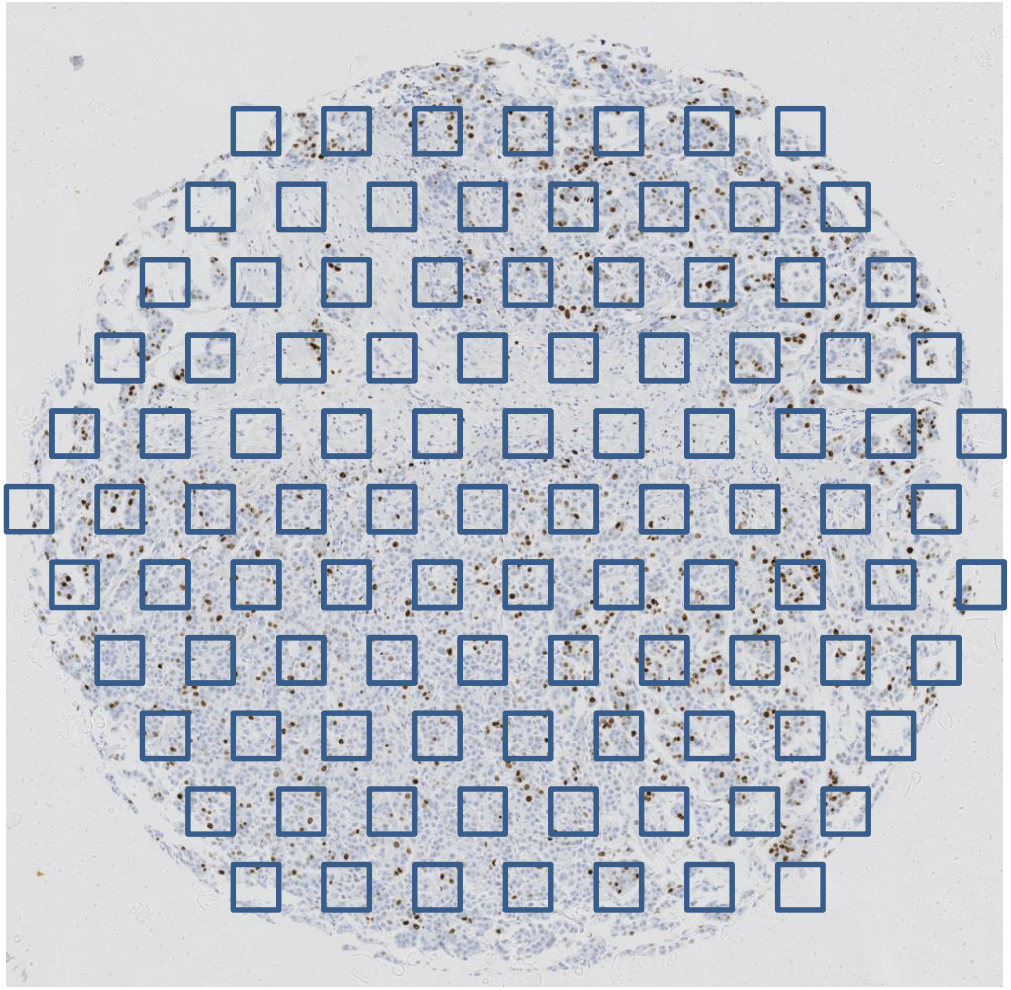



VDS in Sampled Areas
PI – Manual Counting(%)
PI – Virtual Double Staining (%)
0
20
40
60
R2: 0.96

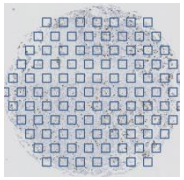
Bland-Altman

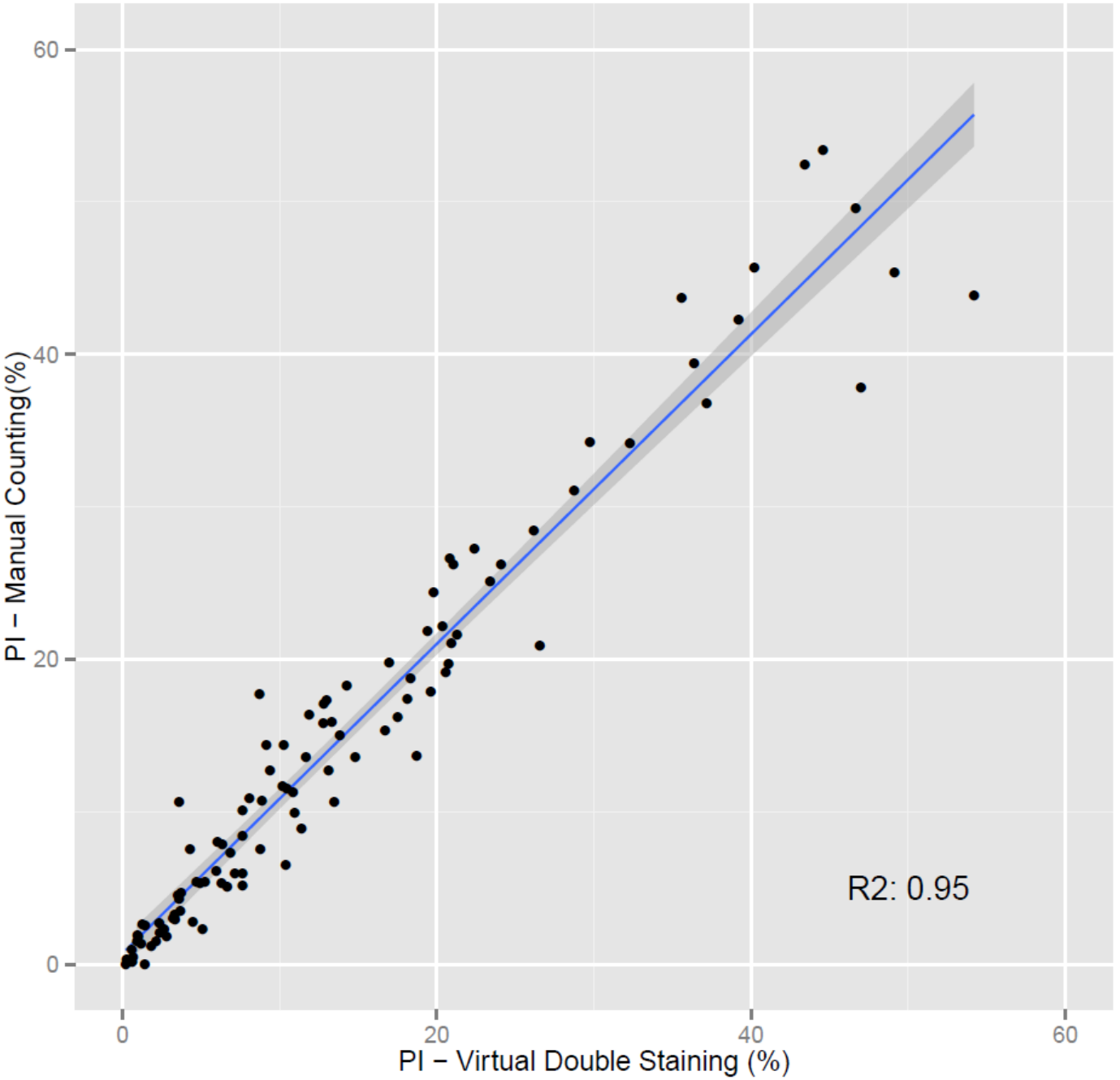


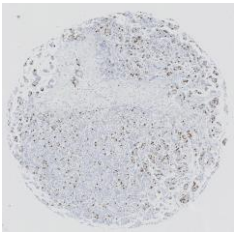
Systematic Random Sampling



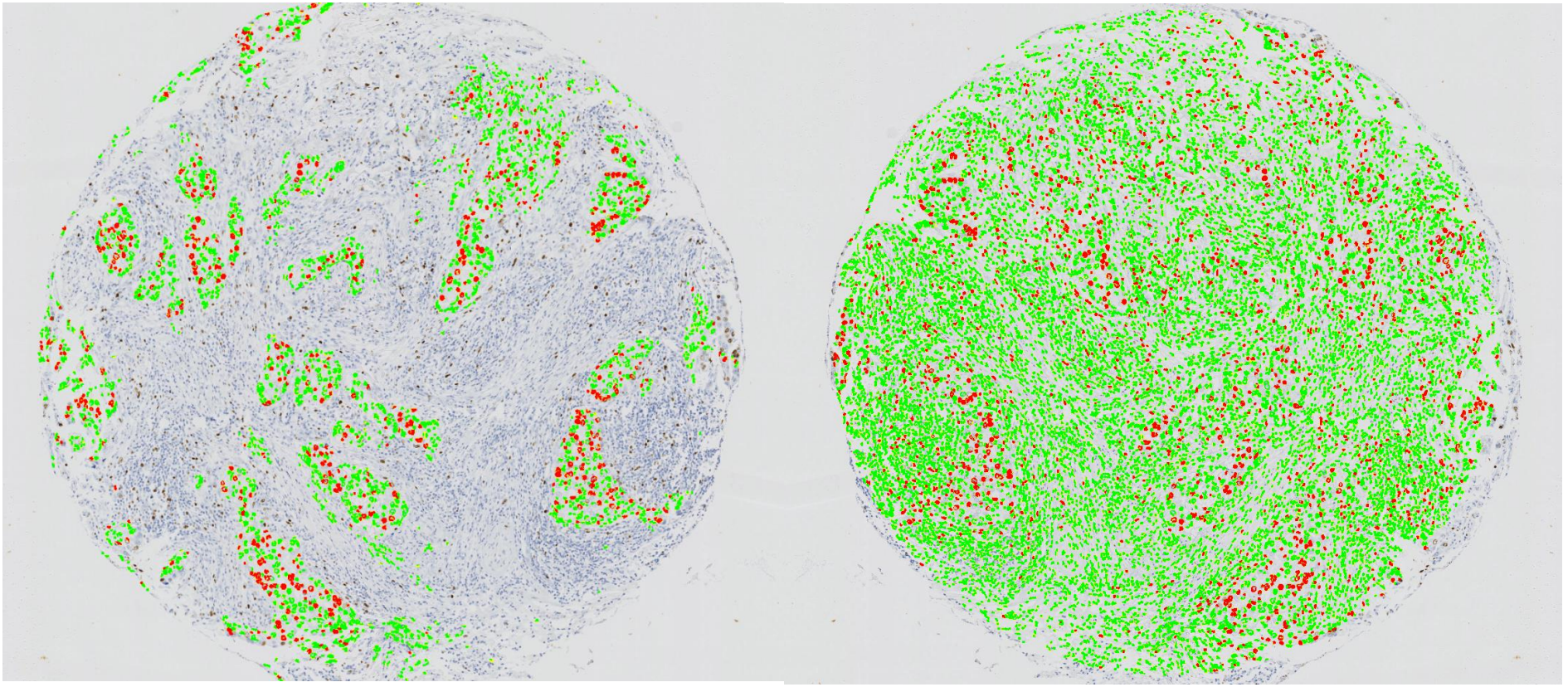
- Manually counted Proliferation Indices (%) were counted in areas selected by Systematic Random Samping
- Therefore, results can be used as an estimate of the whole core



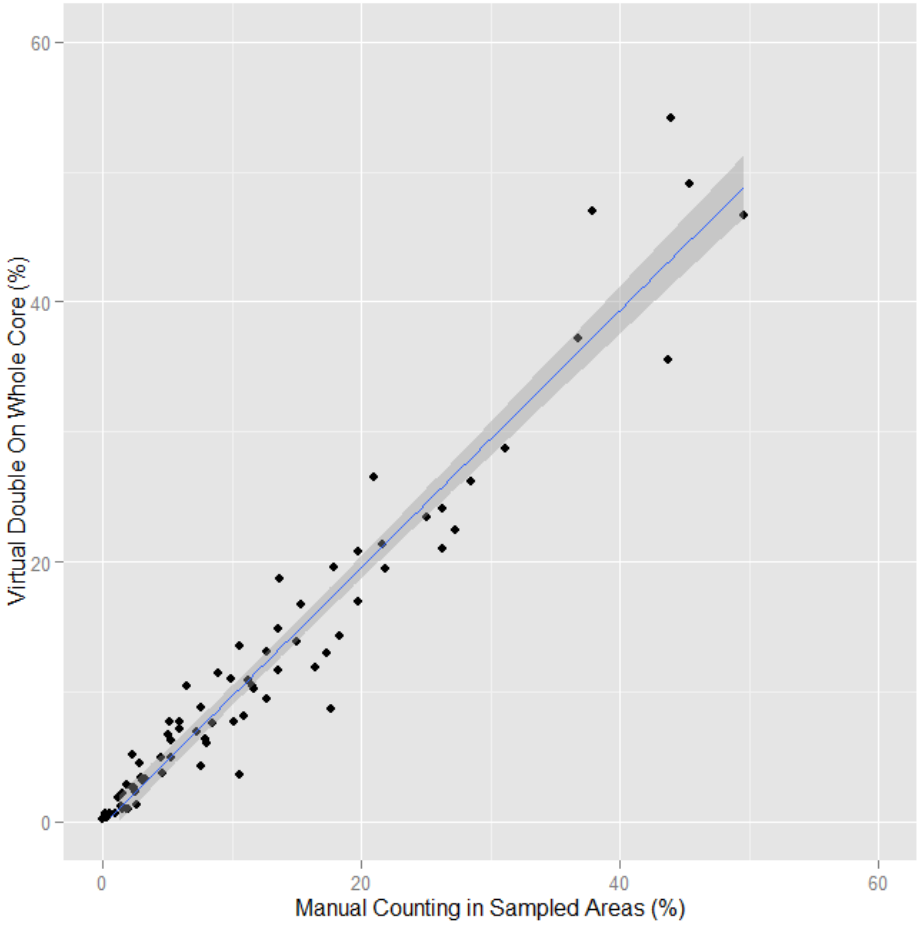

VDS on Whole Core
PI – Manual Counting(%)
PI – Virtual Double Staining (%)
0
20
40
60
R2: 0.95

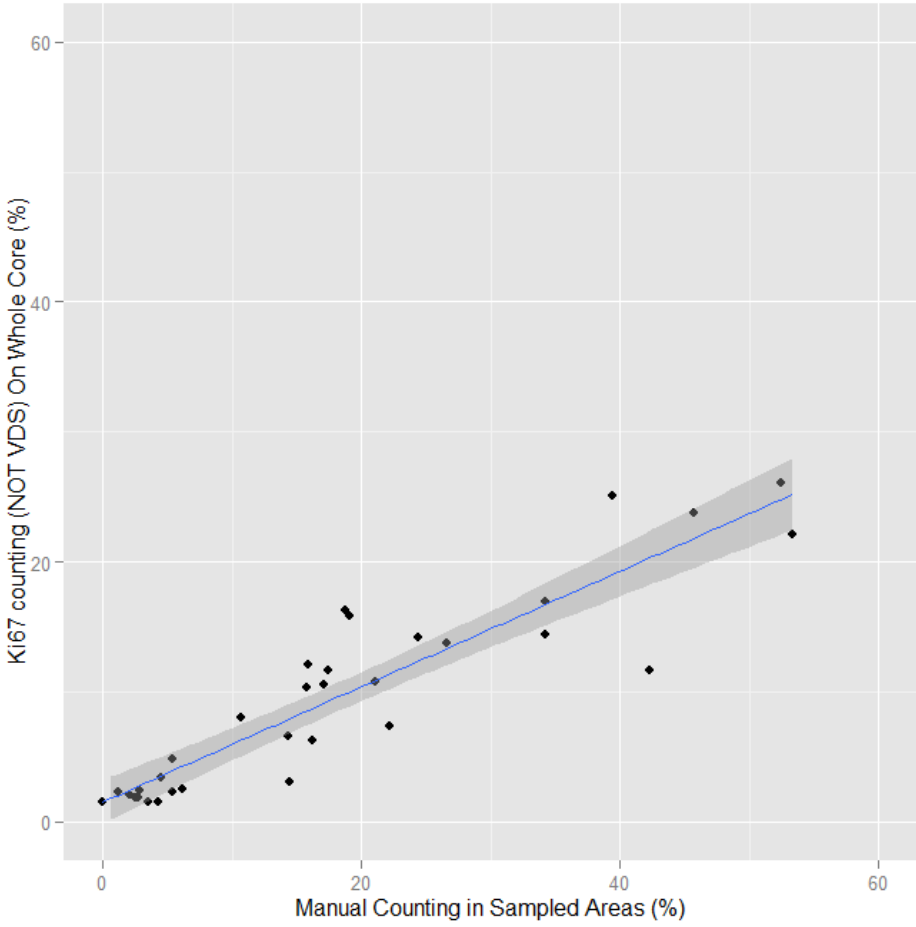


VDS versus Non-VDS

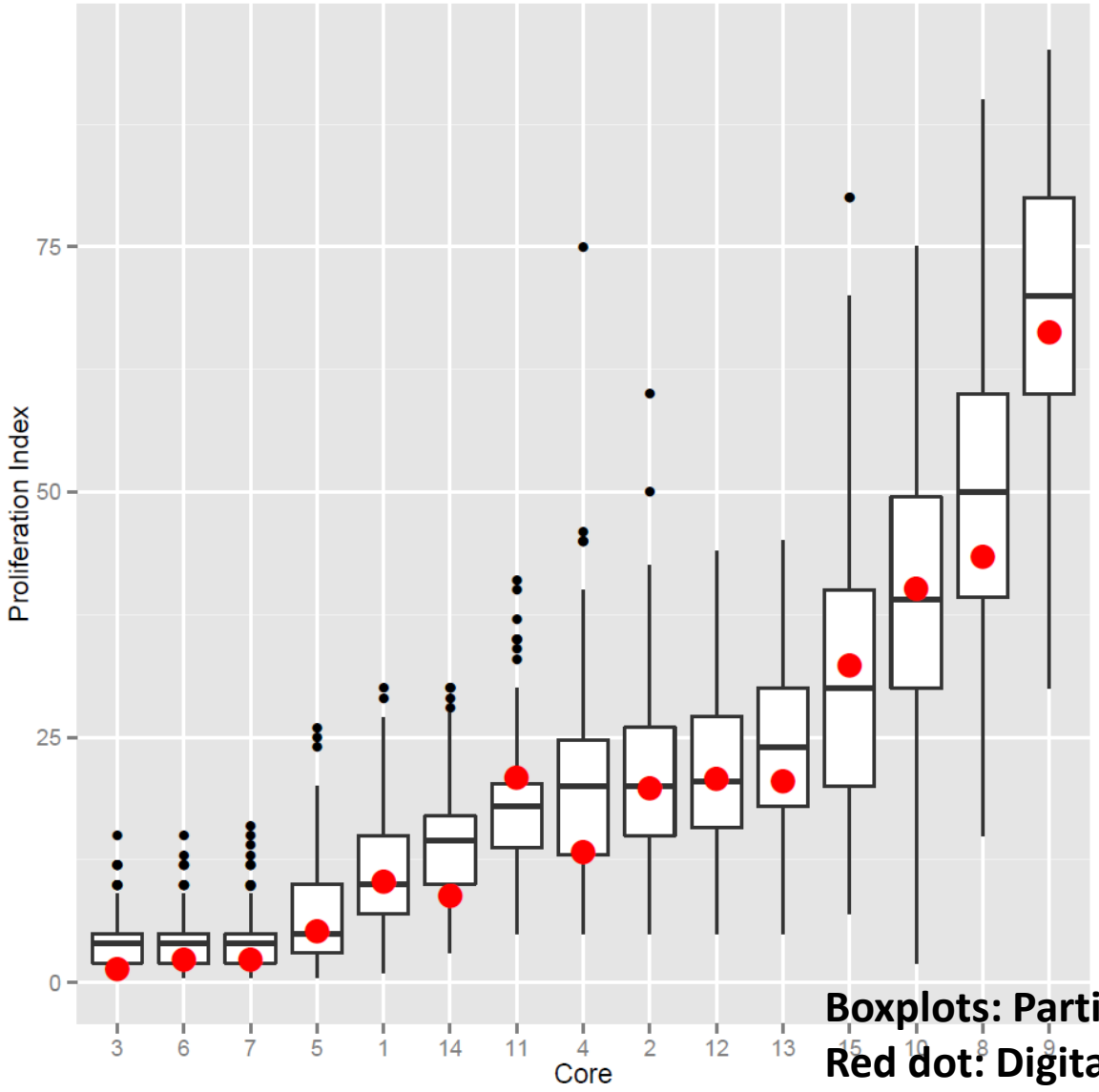


VDS versus Non-VDS





VDS – NordiQC challenge



Boxplots: Participant Ki67 scores
Red dot: Digital Image Analysis

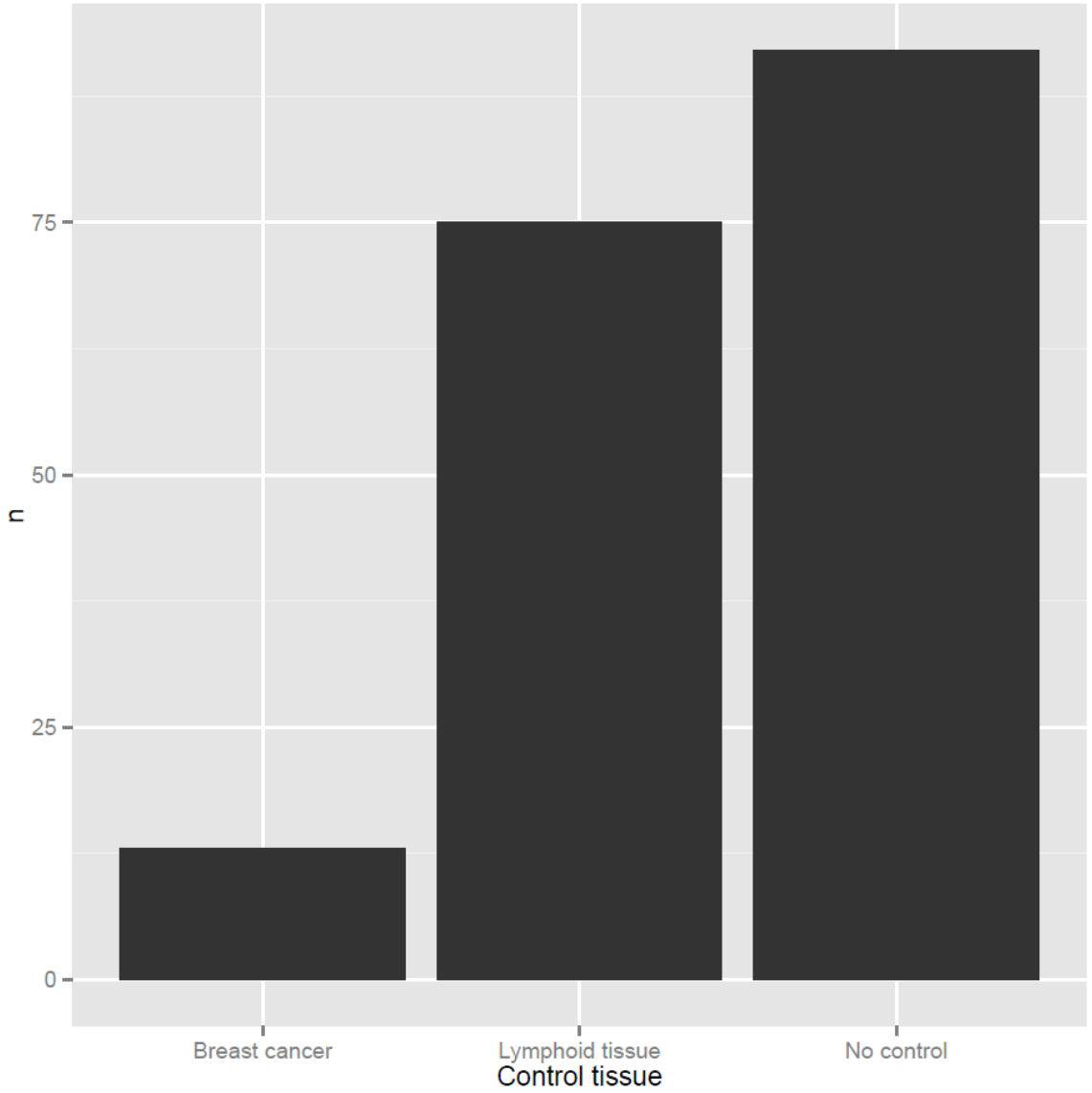
Discussion

- Overall good agreement between neighbouring slides
- Agreement decreases rapidly with distance
- Single cell infiltration can be problematic
- ”Contamination” of tumour areas with non-tumour areas may influence results (decrease Ki67 proliferation index)

Digital Image Analysis – Ki67

CONTROLS

Controls among NordiQC-participants



Ki67 in lymphoid tissue

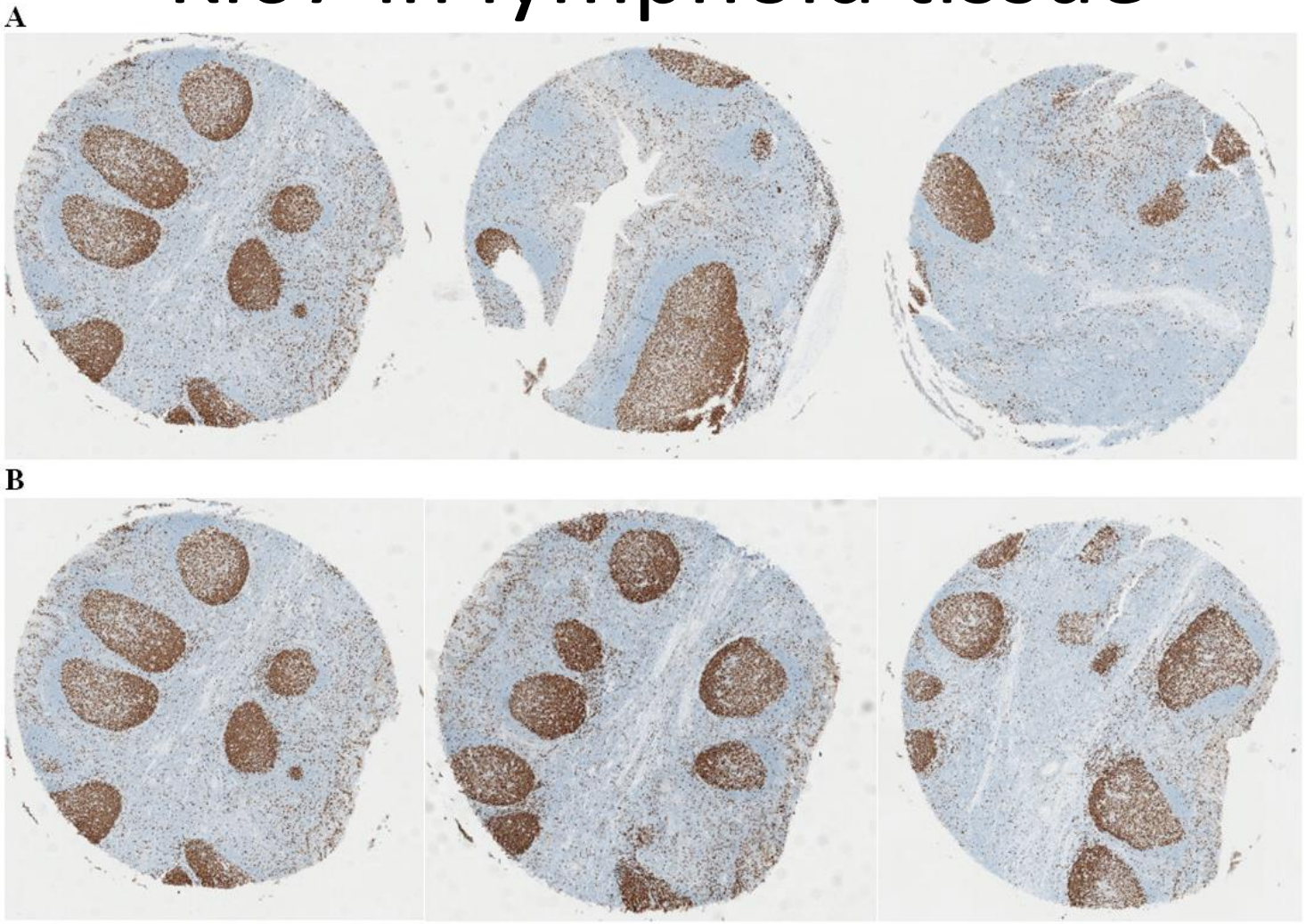


Figure 4.1: Tonsil contol tissue material. A shows three different tonsil control tissues. B shows the variance within the same tonsil control tissue through the tissue block.

Hansen, LS., Sørensen M., Nielsen S., Røge R., Vyberg M. 2015

DIA Control

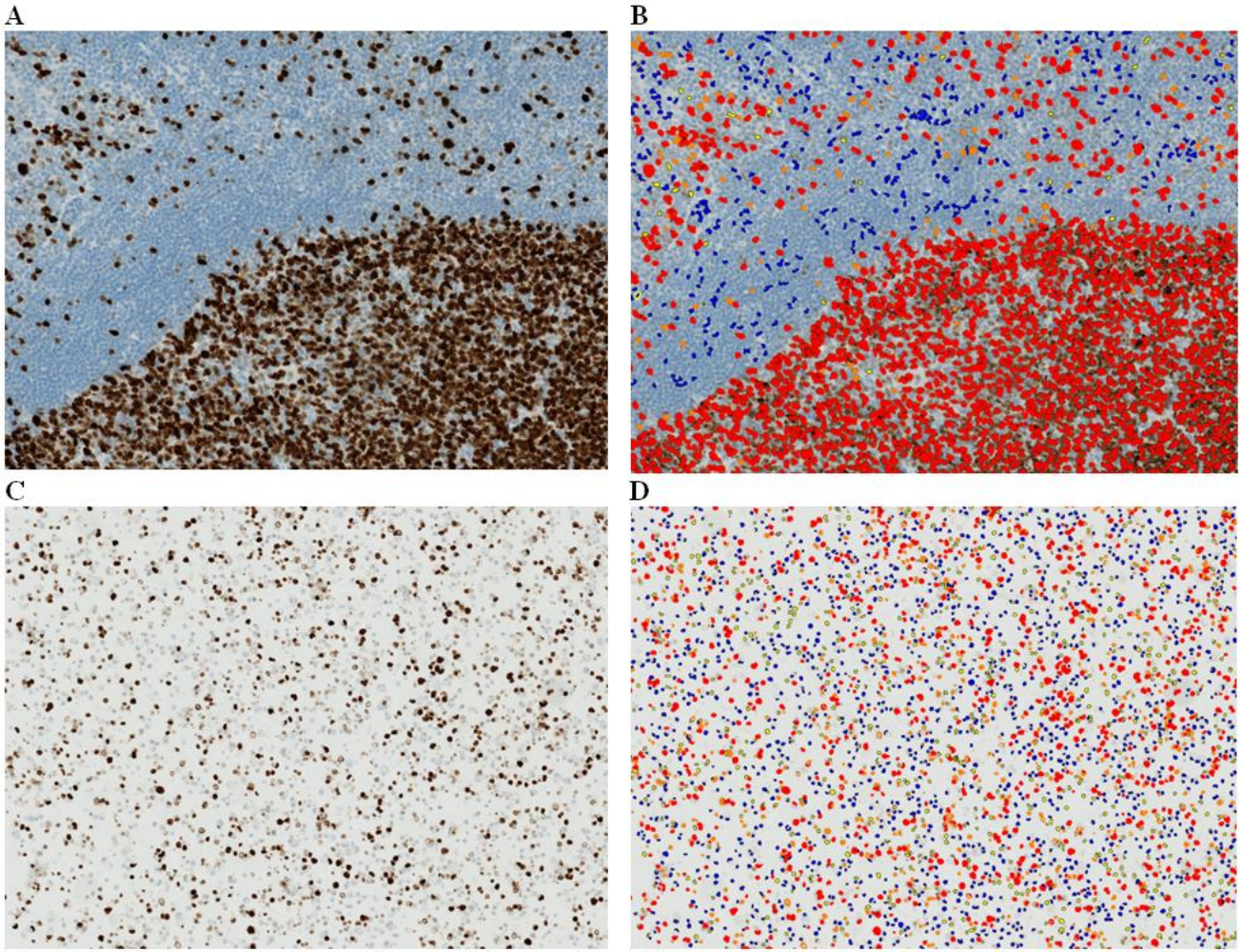
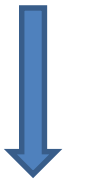


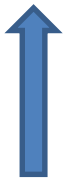
Figure 4.1: Ki67 staining of a tonsil control tissue and a cell line. A and C demonstrate stained tonsil control tissue and cell line. B and D demonstrate the same specimen with DIA performed. Red, orange and yellow colored elements indicate respectively strongly, moderately and weekly Ki67 stained nuclei. The blue elements are Ki67 negative cells.

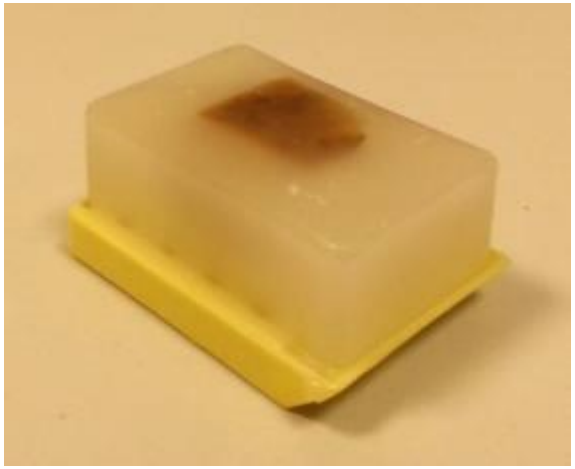
Hansen, LS., Sørensen M., Nielsen S., Røge R., Vyberg M. 2015

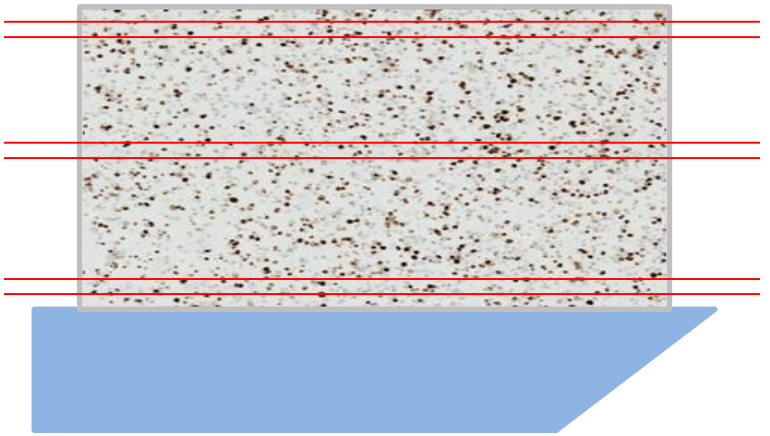
Paraffin block from cell cultures



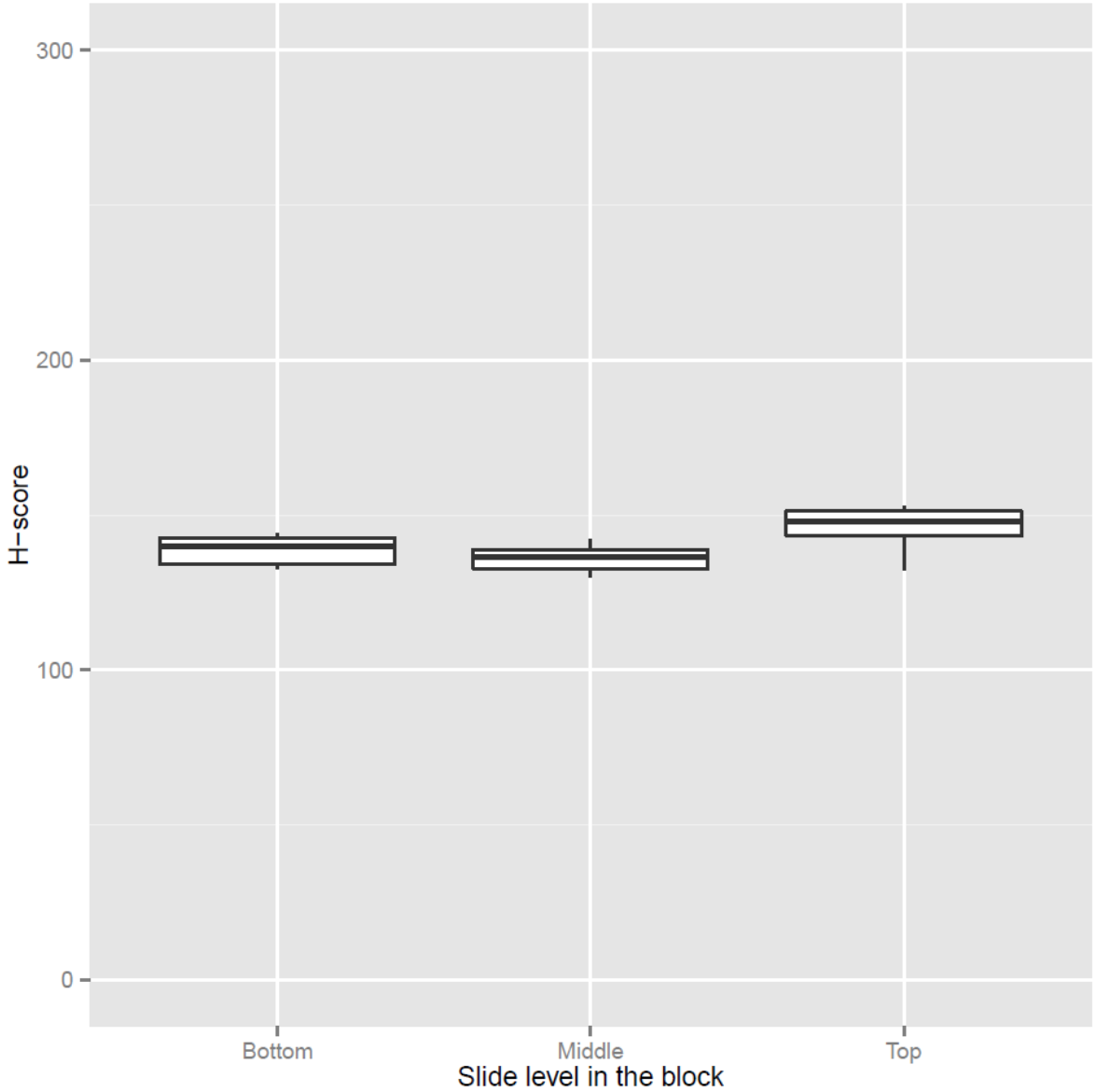
Stained for Ki67 (Mib1) in different antibody dilutions



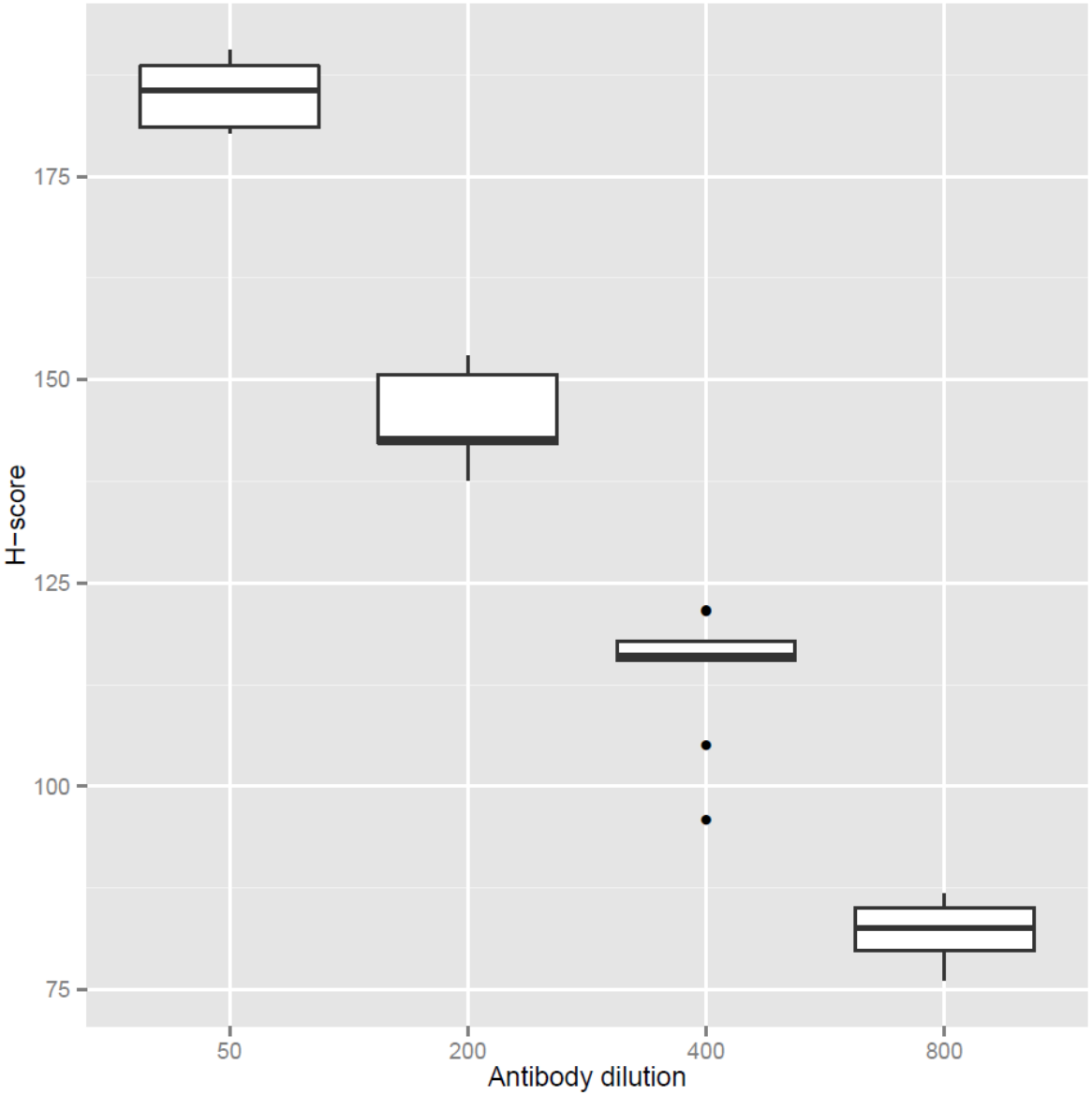




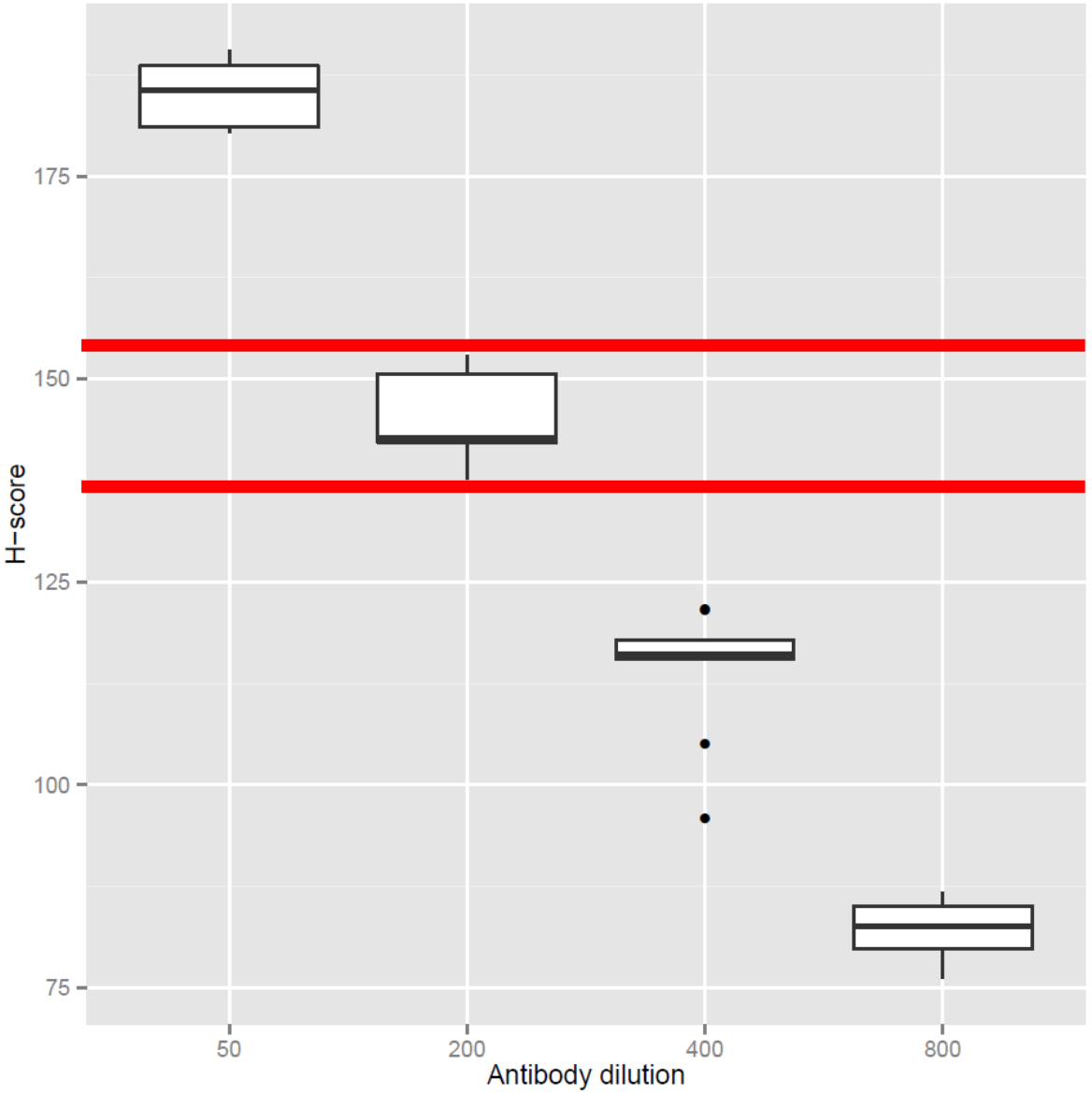
Ki67 H-score across the block



Ki67 H-score, different Ab conc



Ki67 H-score in cell cultures



Digital Image Analysis – Ki67

CLONES

Antibody clone comparison

Immunohistochemical assessment of Ki67 with antibodies SP6 and MIB1 in primary breast cancer: a comparison of prognostic value and reproducibility

Maria Ekholm,[1,2] Sanda Beglerbegovic,[3] Dorthe Grabau,[2,4] Kristina Lövgren,[2] Per Malmström,[2,5] Linda Hartman[2,6] & Mårten Fernö[2]

Conclusions: SP6 was not superior to MIB1, but the two antibodies were comparable in the assessment of Ki67. Both MIB1 and SP6 could therefore be considered for prognostic use in primary breast cancer.

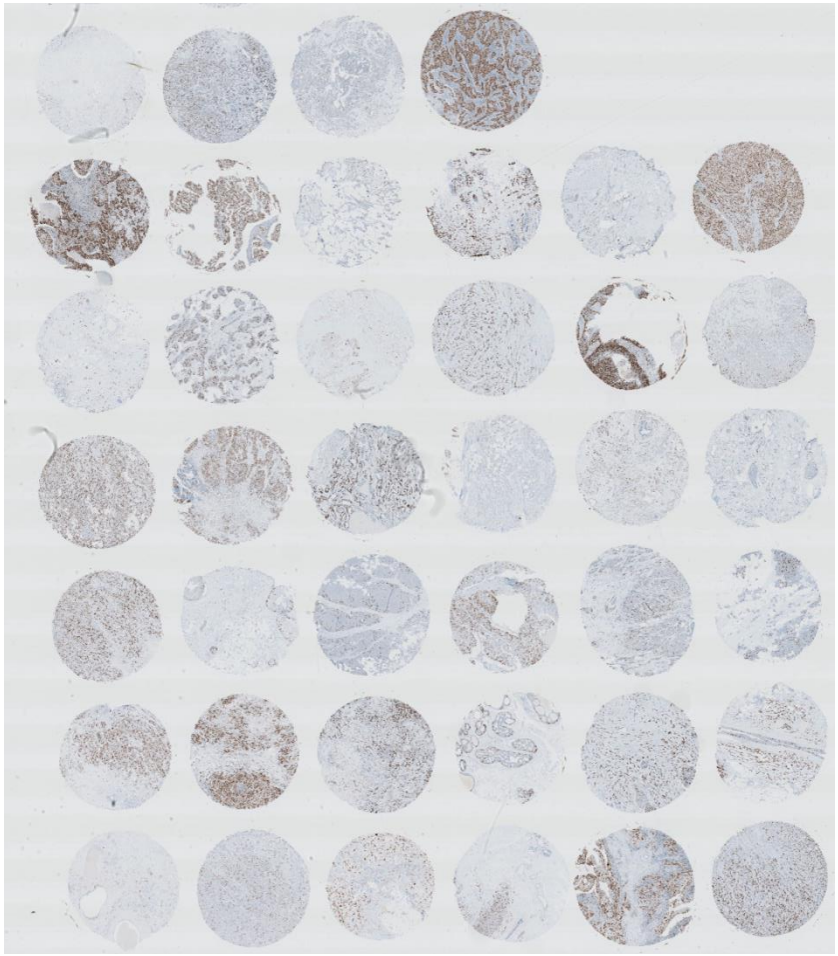
Comparative Validation of the SP6 and MIB1 Antibodies to Ki67 and Their Use in Tissue Microarray (TMA) and Image Analysis for Breast Cancer.

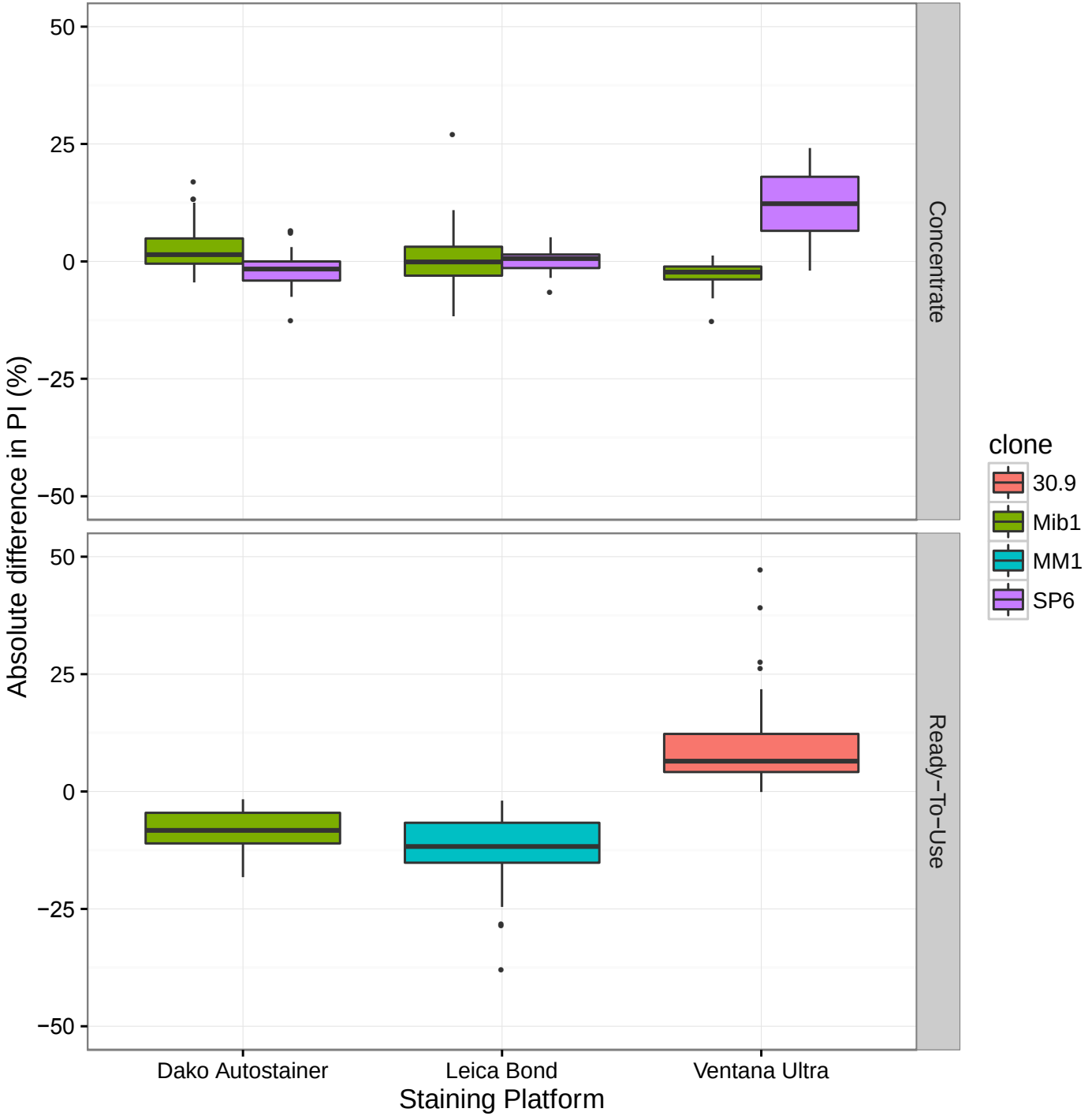
L. Zabaglo[1], L. Zabaglo[2], J. Salter[1], J. Salter[2], H. Anderson[1], H. Anderson[2], M. Hills[1], R. A'Hern[3], M. Dowsett[1], and M. Dowsett[2]

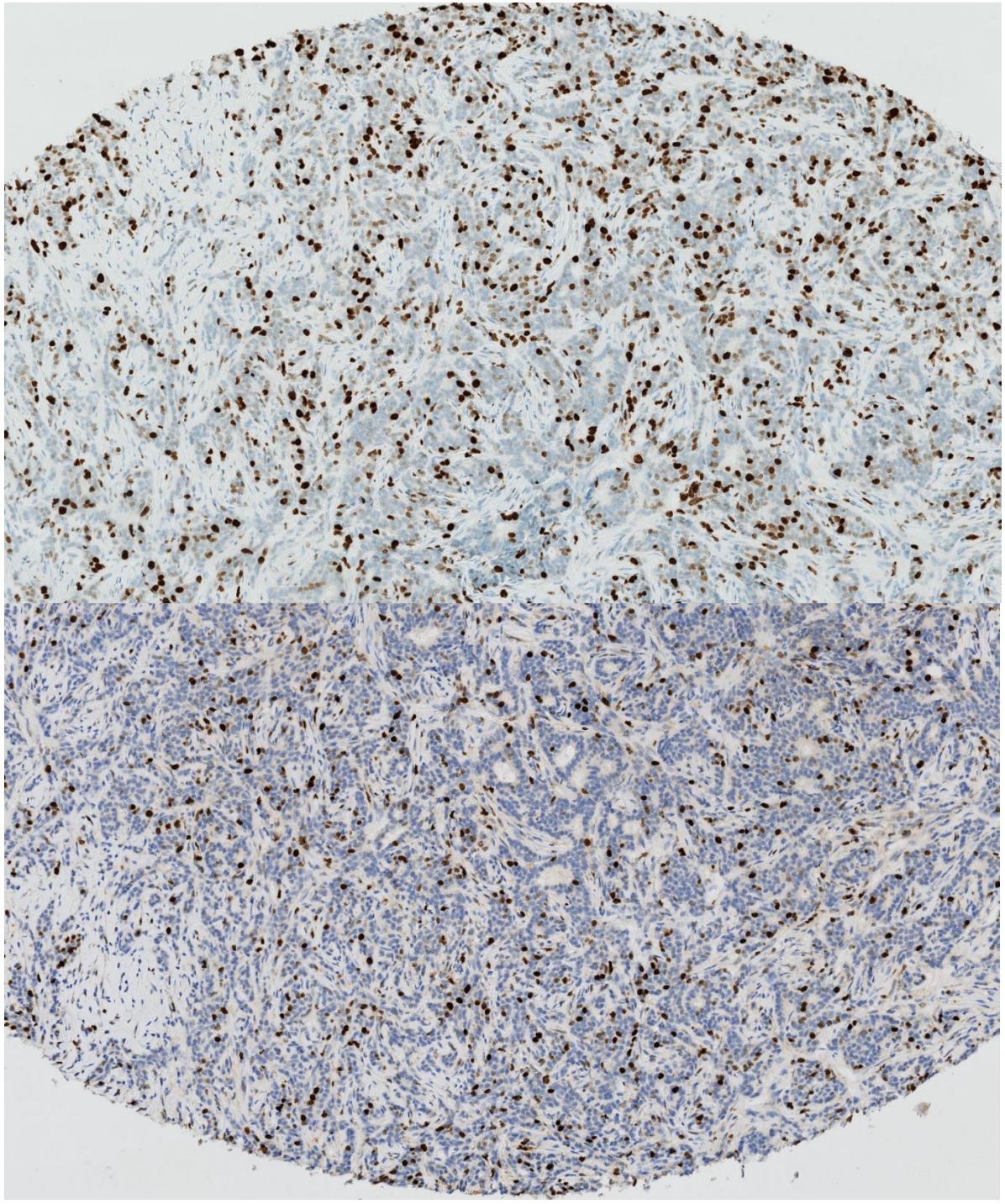
Conclusions: SP6 and MIB1 provide highly comparable measures of Ki67 that predict progression of advanced disease similarly. SP6 is substantially better suited than MIB1 to image analysis, and is now our preferred antibody for future studies.

Experimental setup

- TMA with 40 breast cancers
- Stained using most commonly used mAb: Mib1, SP6, 30.9, MM1
- Stained using both (if available) Ready-To-Use format and concentrated format (In-House optimized protocol)
- Stained on all major staining platforms
- Parallel slide stained for PCK
- Proliferation Index calculated using Virtual Double Staining




Absolute difference in PI (%)
50
25
0
−25
−50
Concentrate
Ready−To−Use
Dako Autostainer
Leica Bond
Ventana Ultra
Staining Platform
clone
30.9
Mib1
MM1
SP6



SP6 concentrate,
Ventana platform

Proliferation Index:
38 %

MM1 RTU,
Leica platform

Proliferation Index:
12 %

Digital Image Analysis – Ki67

IMAGE ANALYSIS IN NORDIQC ASSESMENTS

Image analysis in EQA?

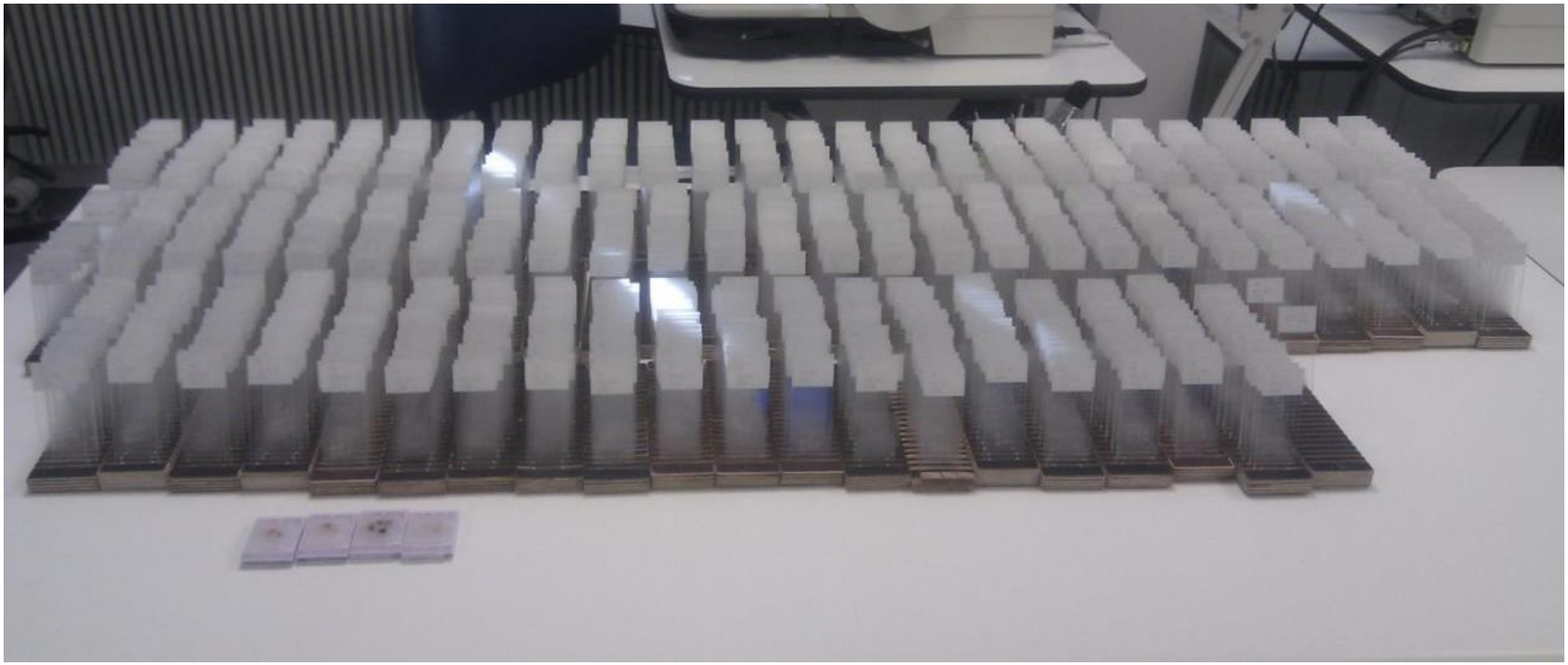
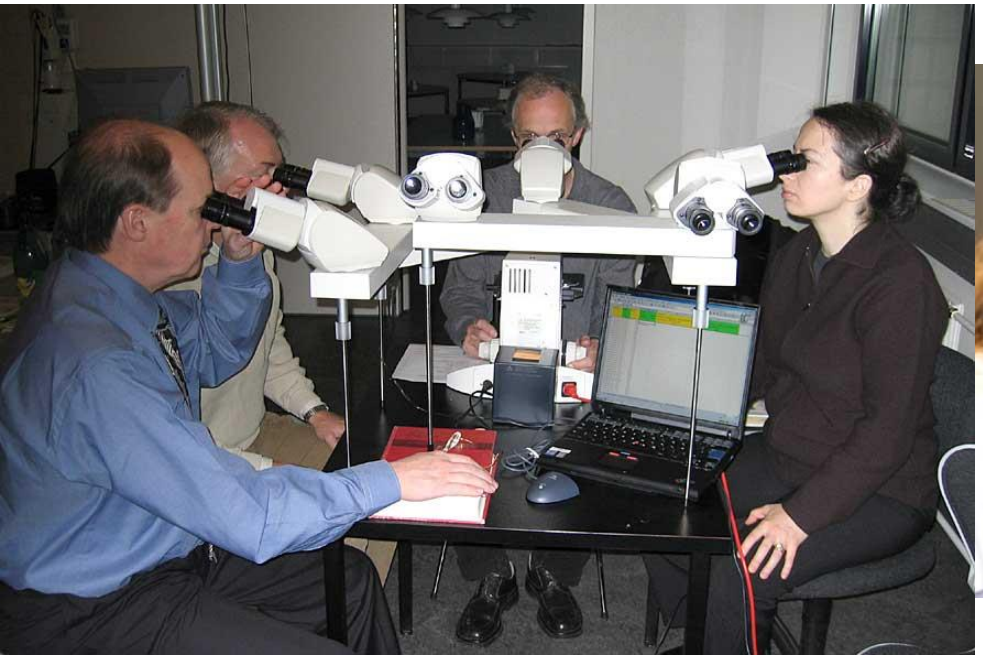


Image analysis in EQA?



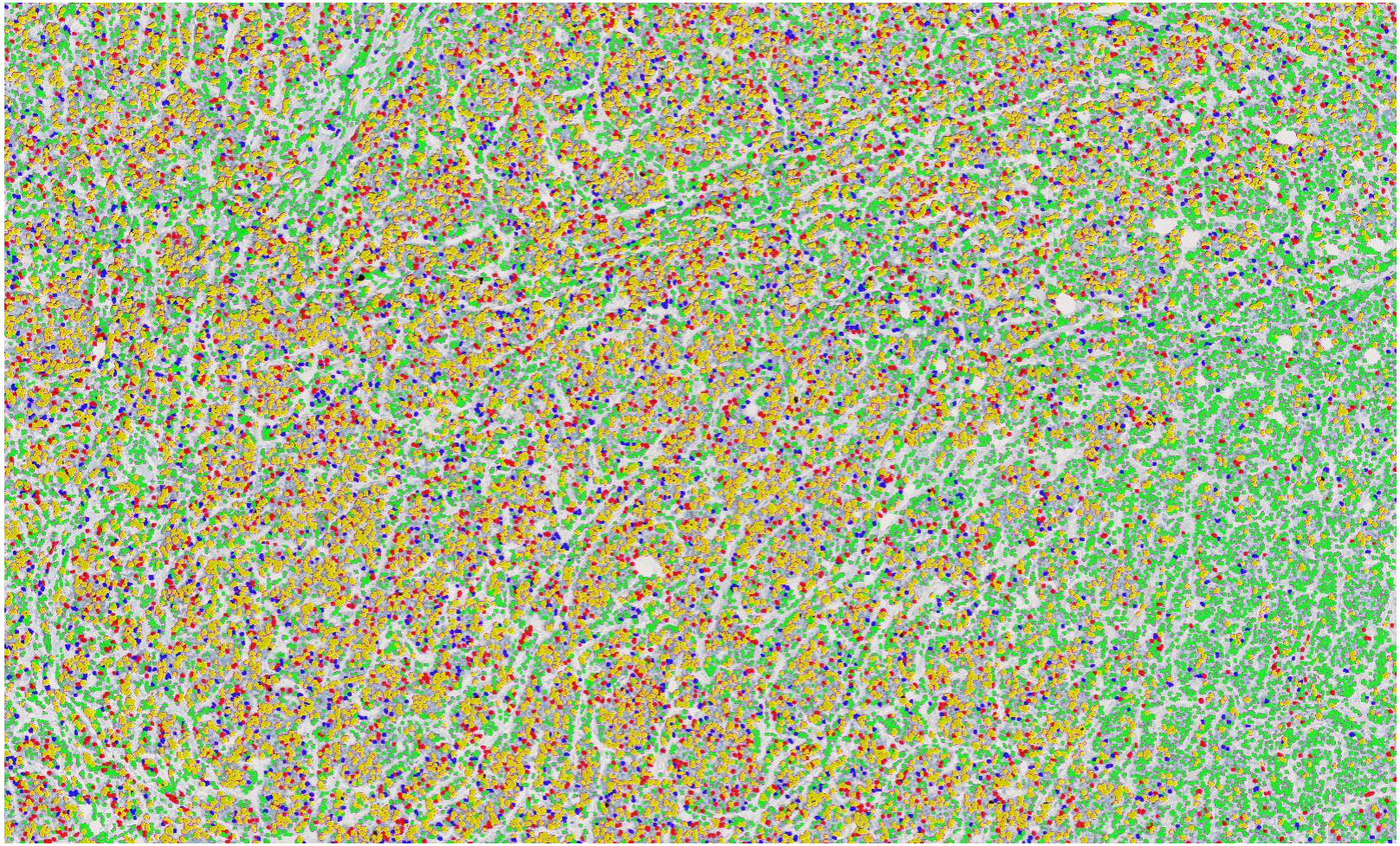
Pilot experiment

- One run (B12) of NordiQC assessment for Ki67
- 229 participants

	Optimal	Good	Borderline	Poor
Total	166	39	18	6
Proportion	72%	**17%**	**8%**	**3%**

- All slides were scanned
- Slides contained 1 core of breast carcinoma
- All cells in this core were categorised as negative or positive (3 grades)
- H-score (based on intensity and extension)

Segmentation of cells



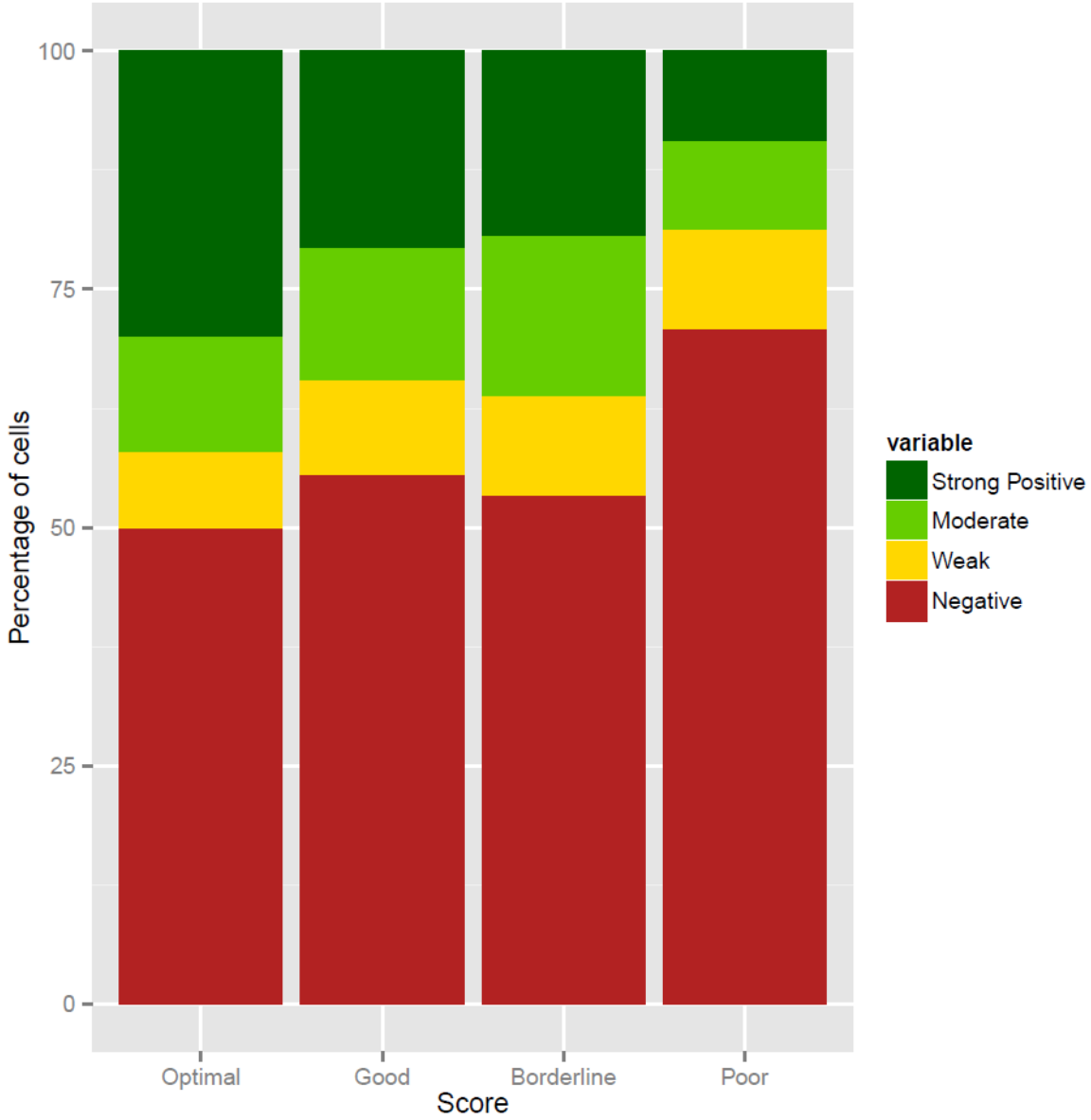
Segmented nuclei

H-score:

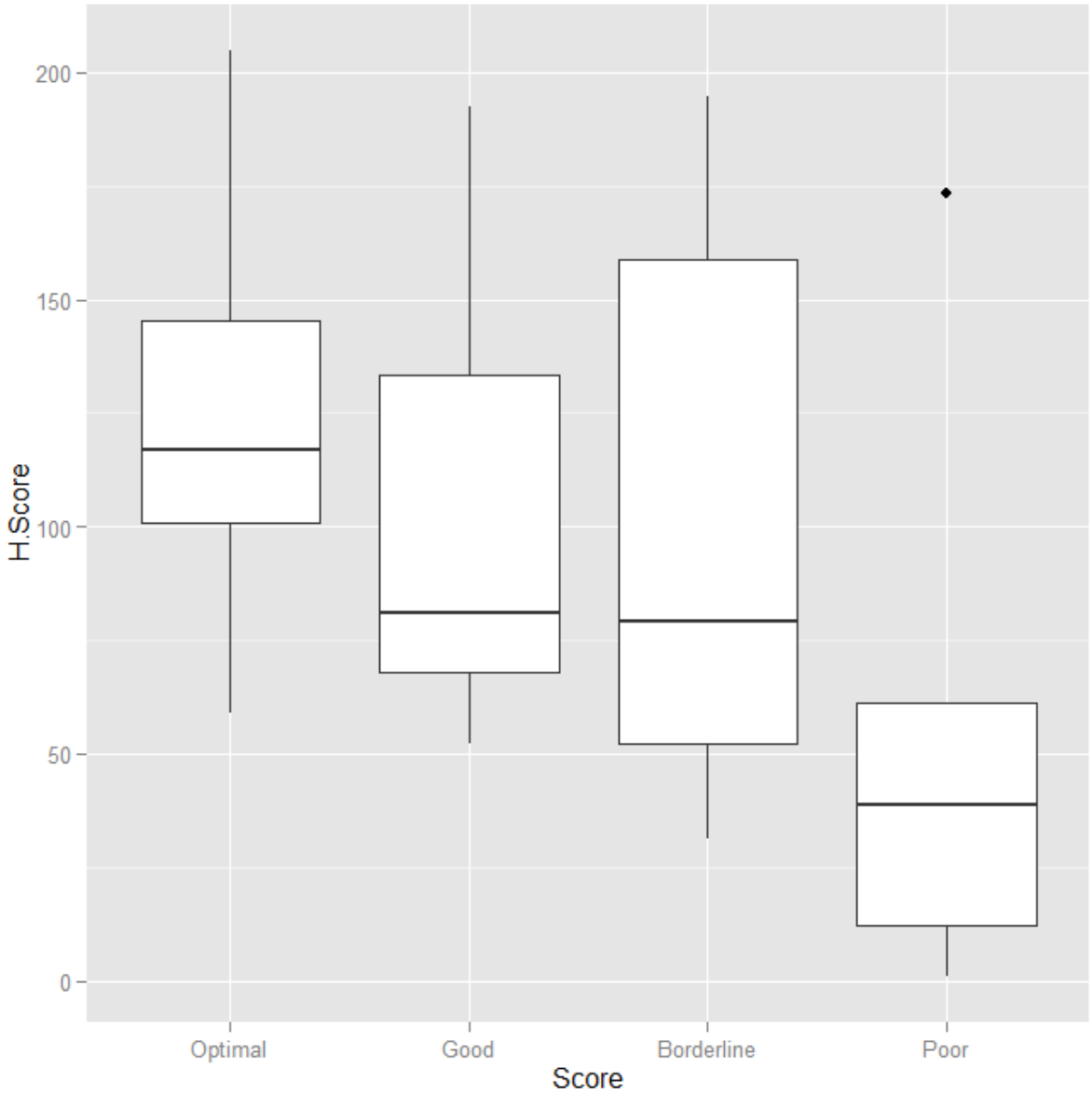
1 x (% Weakly) +

2 x (% Moderate) +

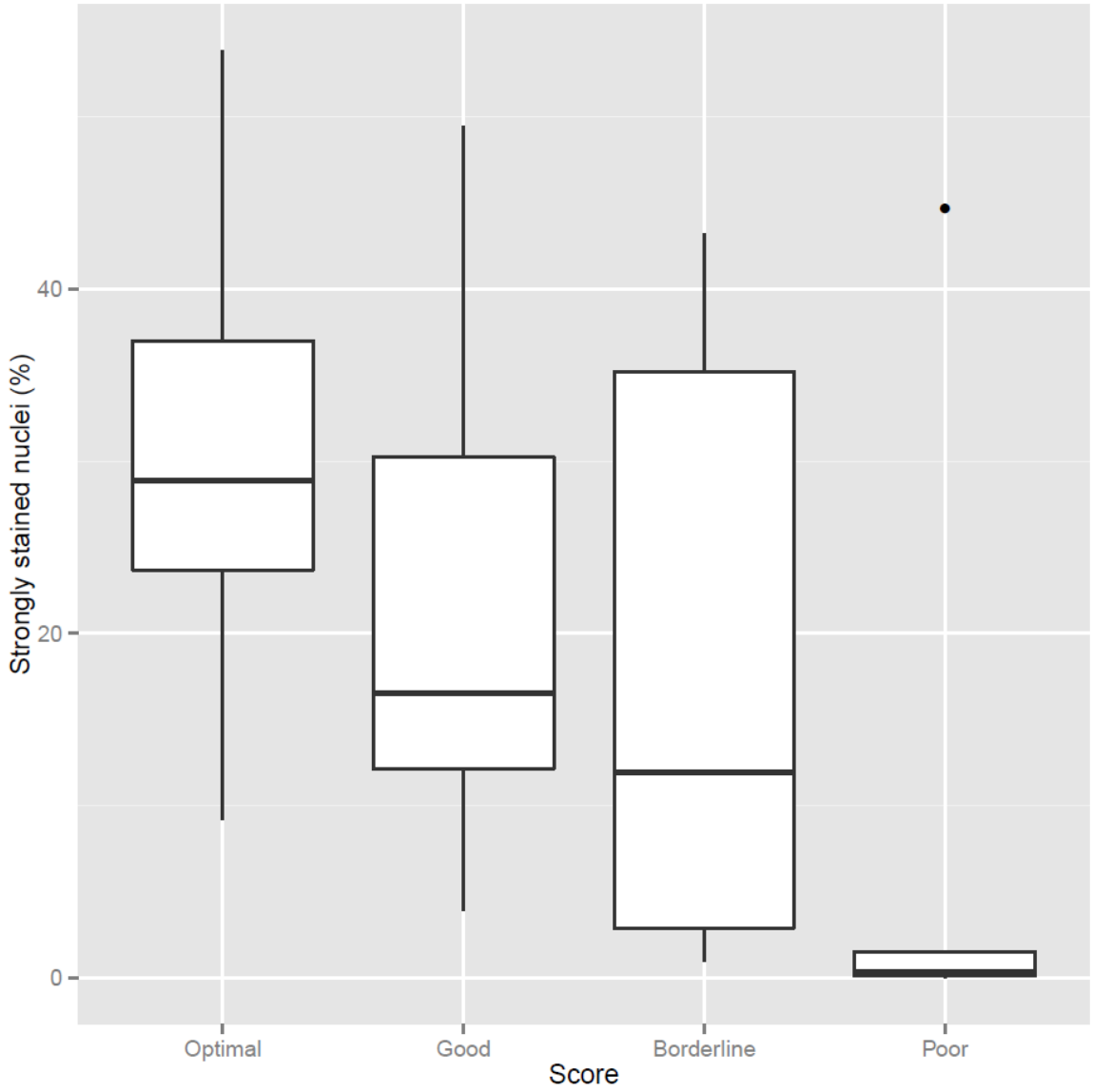
3 x (% Strong)



H-score



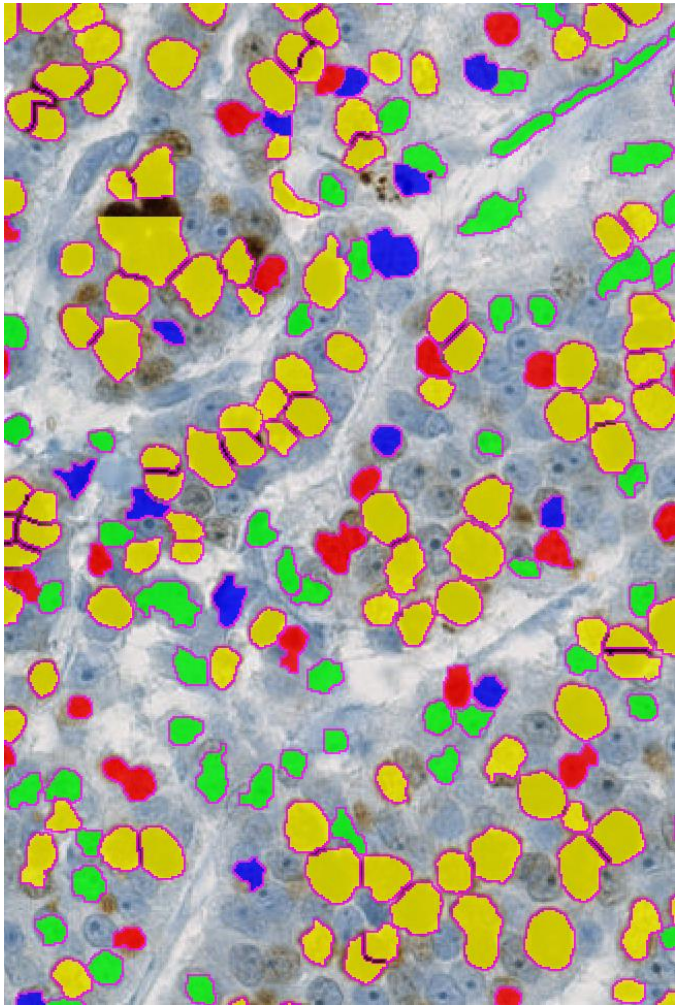
Strongly stained nuclei



Discussion

- Still experimental, algoritm not yet optimized for variance in staining protocols/platforms

- Challenged when nuclei overlap or cell borders are blurry

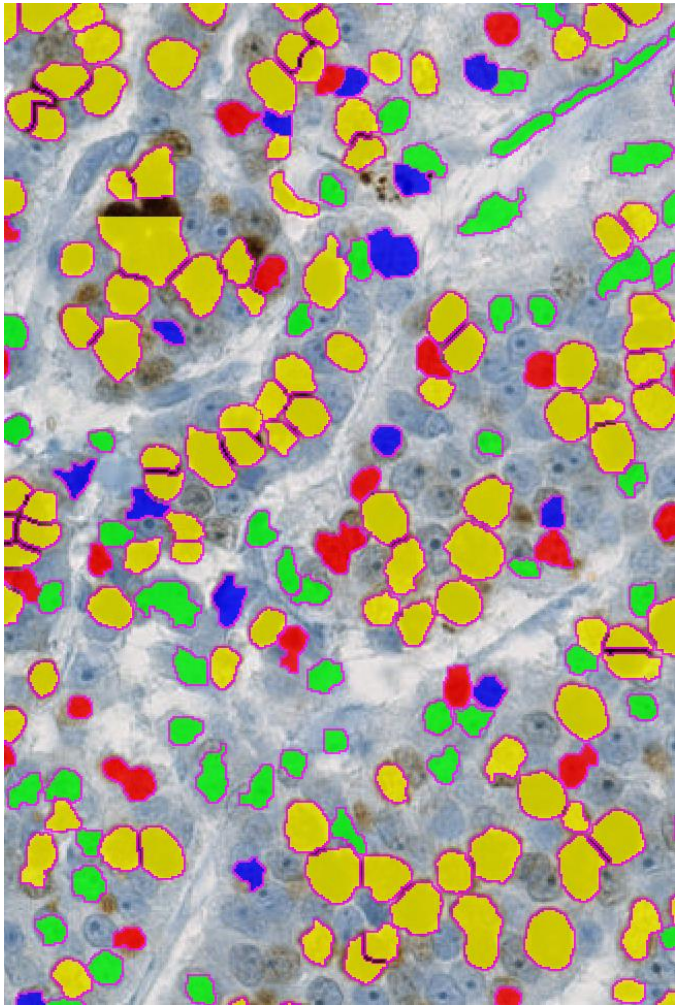


Digital Image Analysis – Ki67

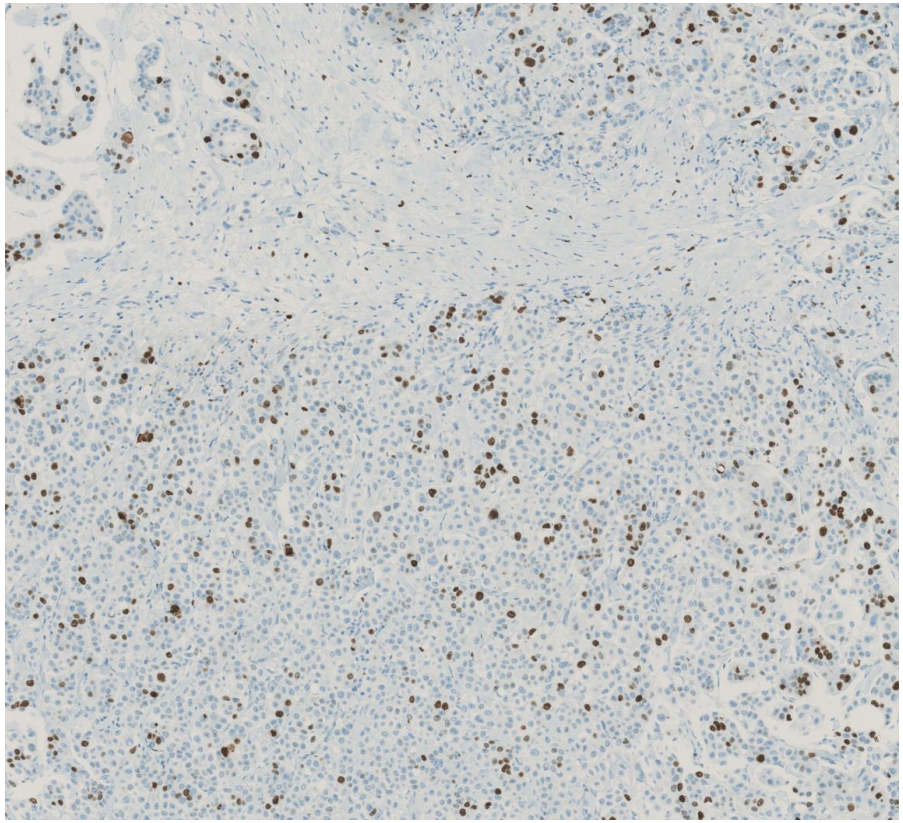
DISCUSSION & FUTURE PERSPECTIVES

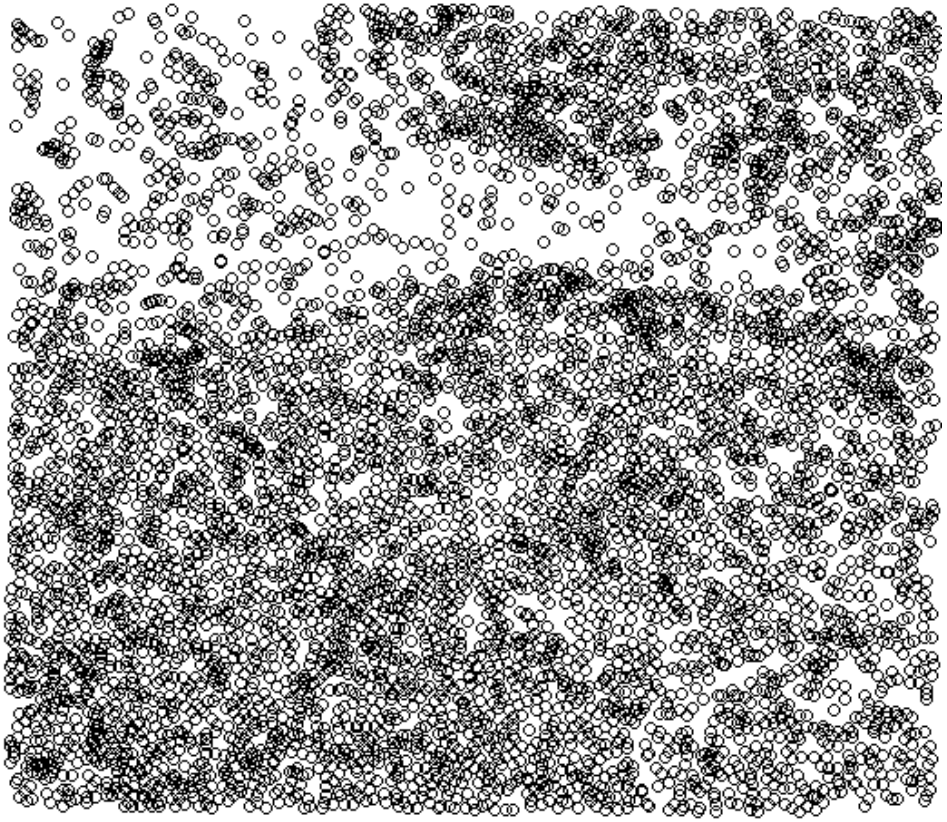
Discussion

- Still experimental, algoritm not yet optimised for variance in staining protocols/platforms
- Challenged when nuclei overlap or cell borders are blurry

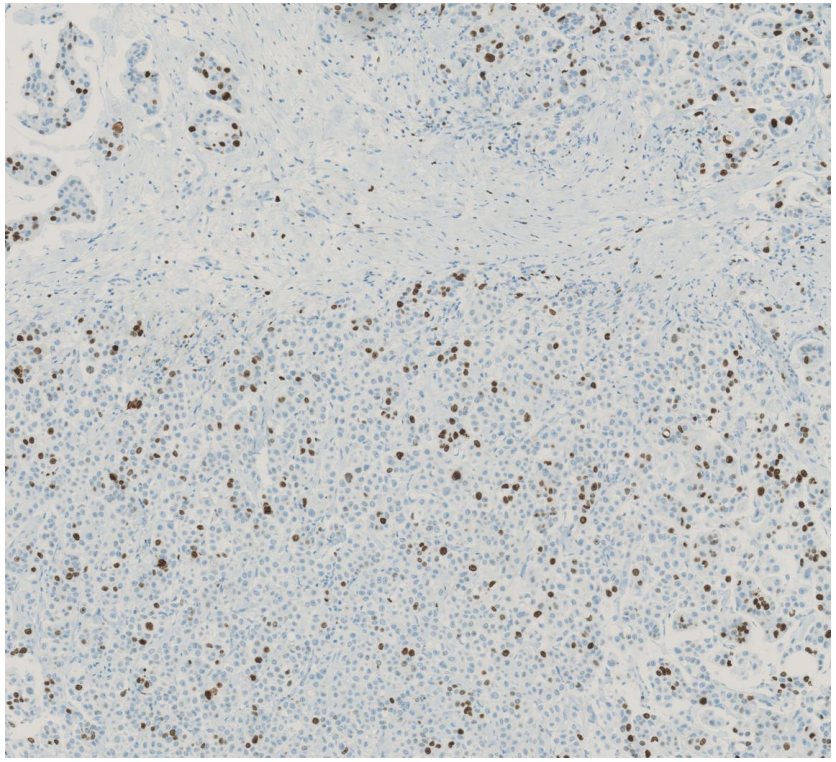


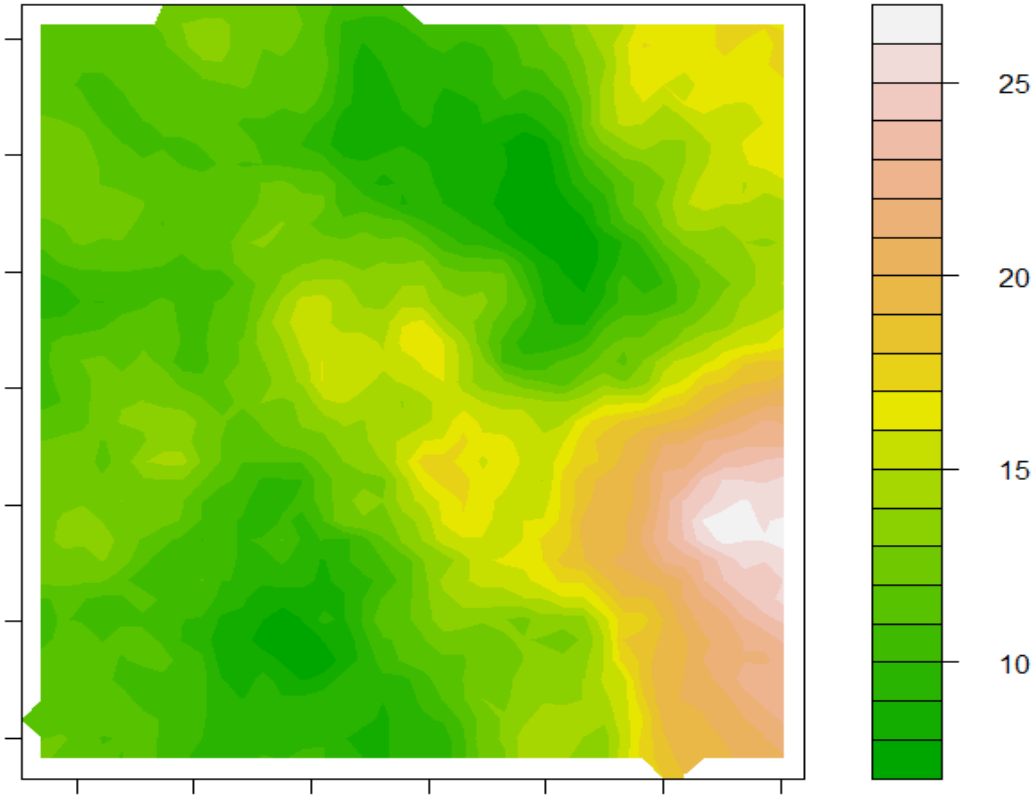
Future perspectives





Future perspectives





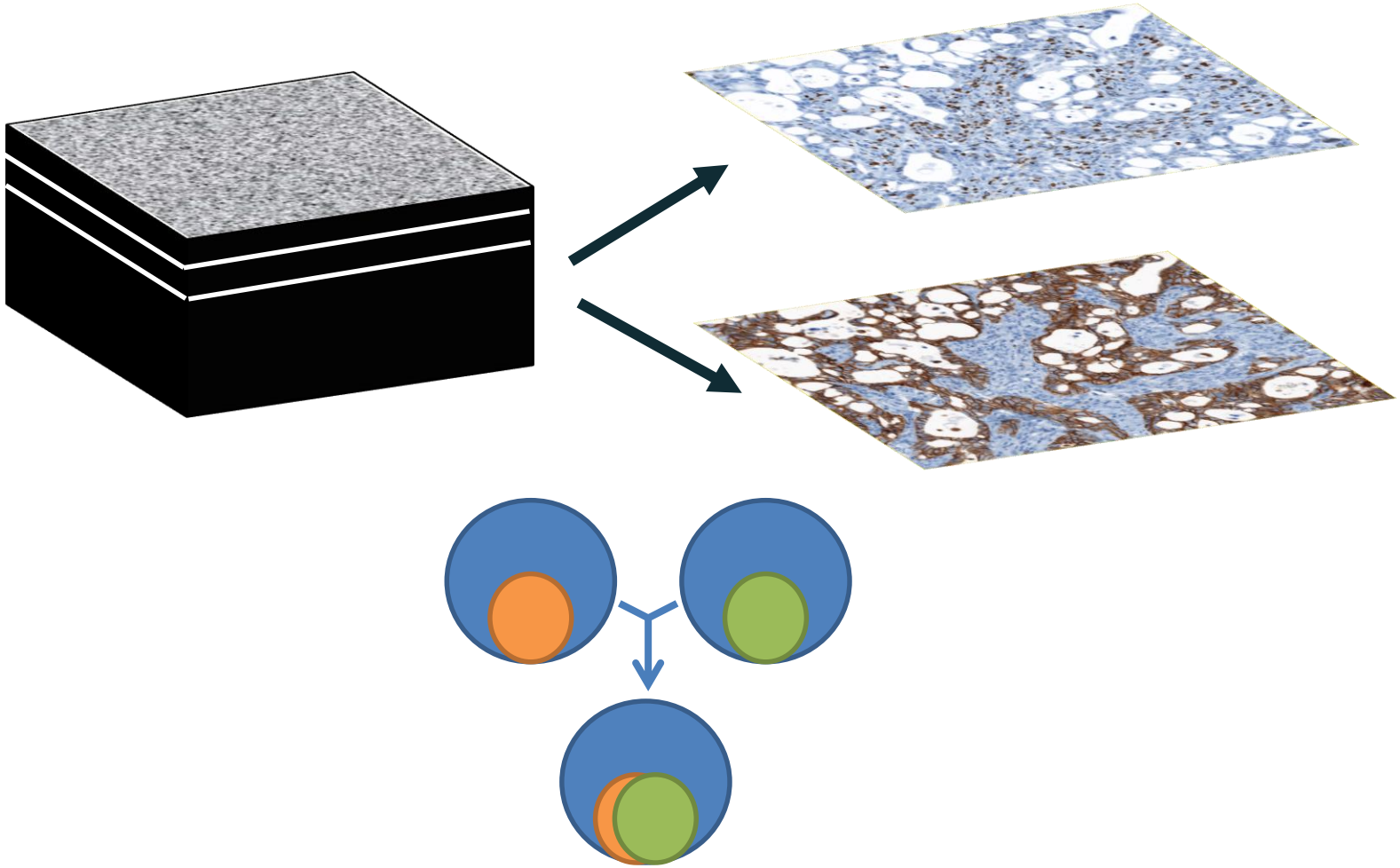
Ki67 proliferation index (%) - Heat map

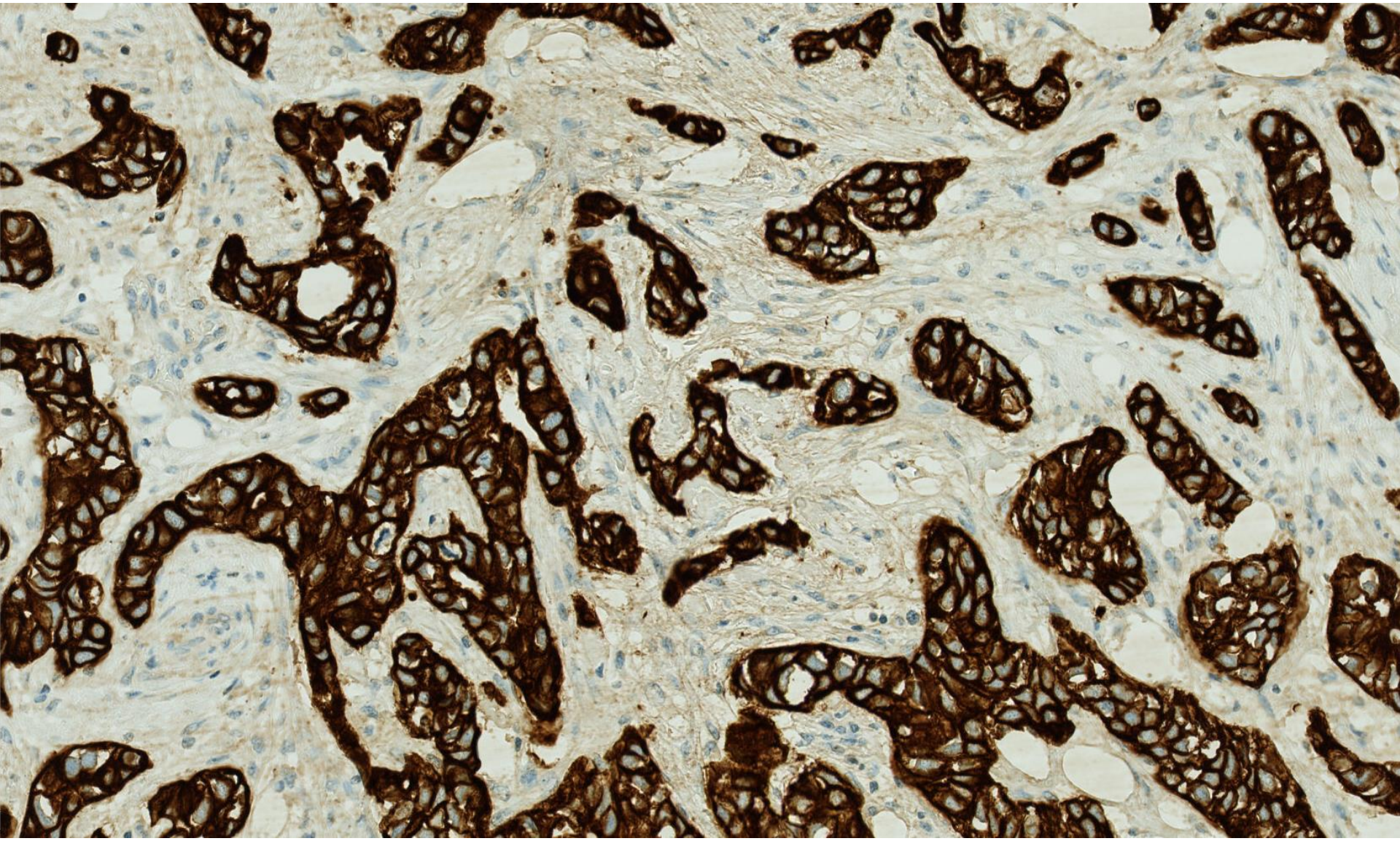
Thank you for your attention!

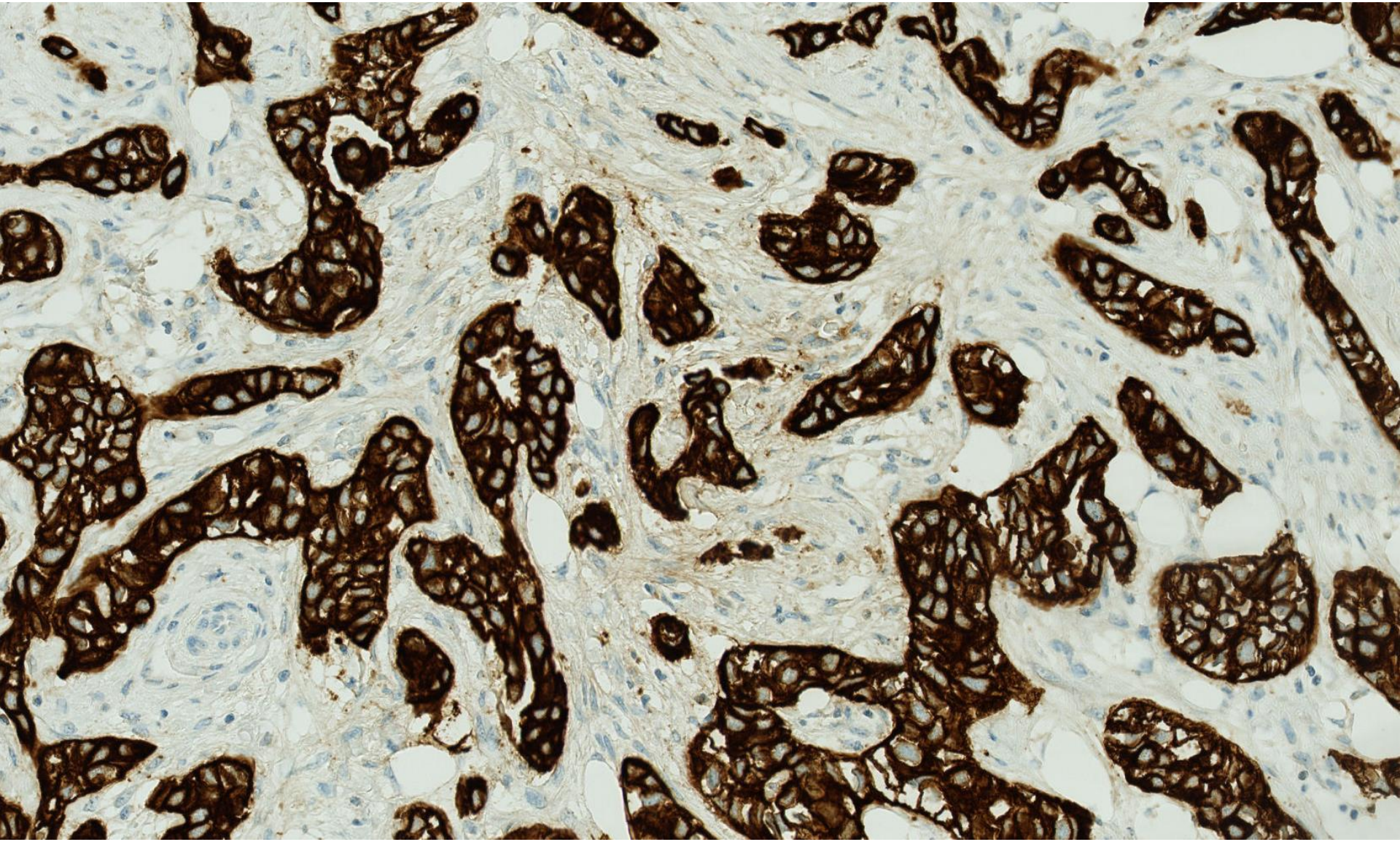
Collaborators

Søren Nielsen
Rikke Riber-Hansen
Line Sloth Hansen
Marina Sørensen
Mogens Vyberg

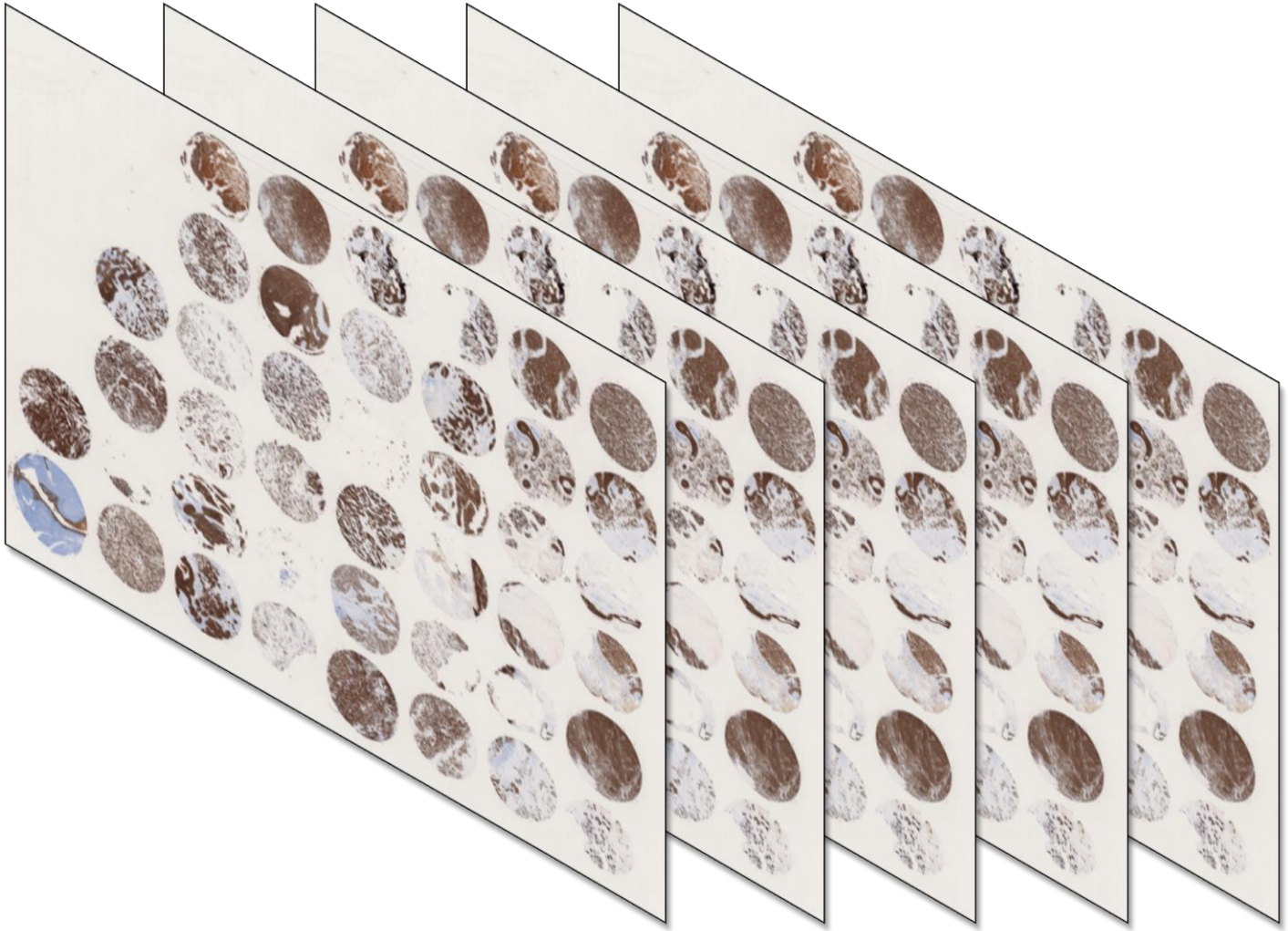
Validation of alignment







Five parallel slides of PCK



PCK-Alignment

- 5 parallel slides from TMA containing 40 breast cancers
- All stained for PCK TMA
- Only 26 (of 40) cores were usable
- Exclusion were due to
  - Missing cores in one or more slides
  - Damaged cores

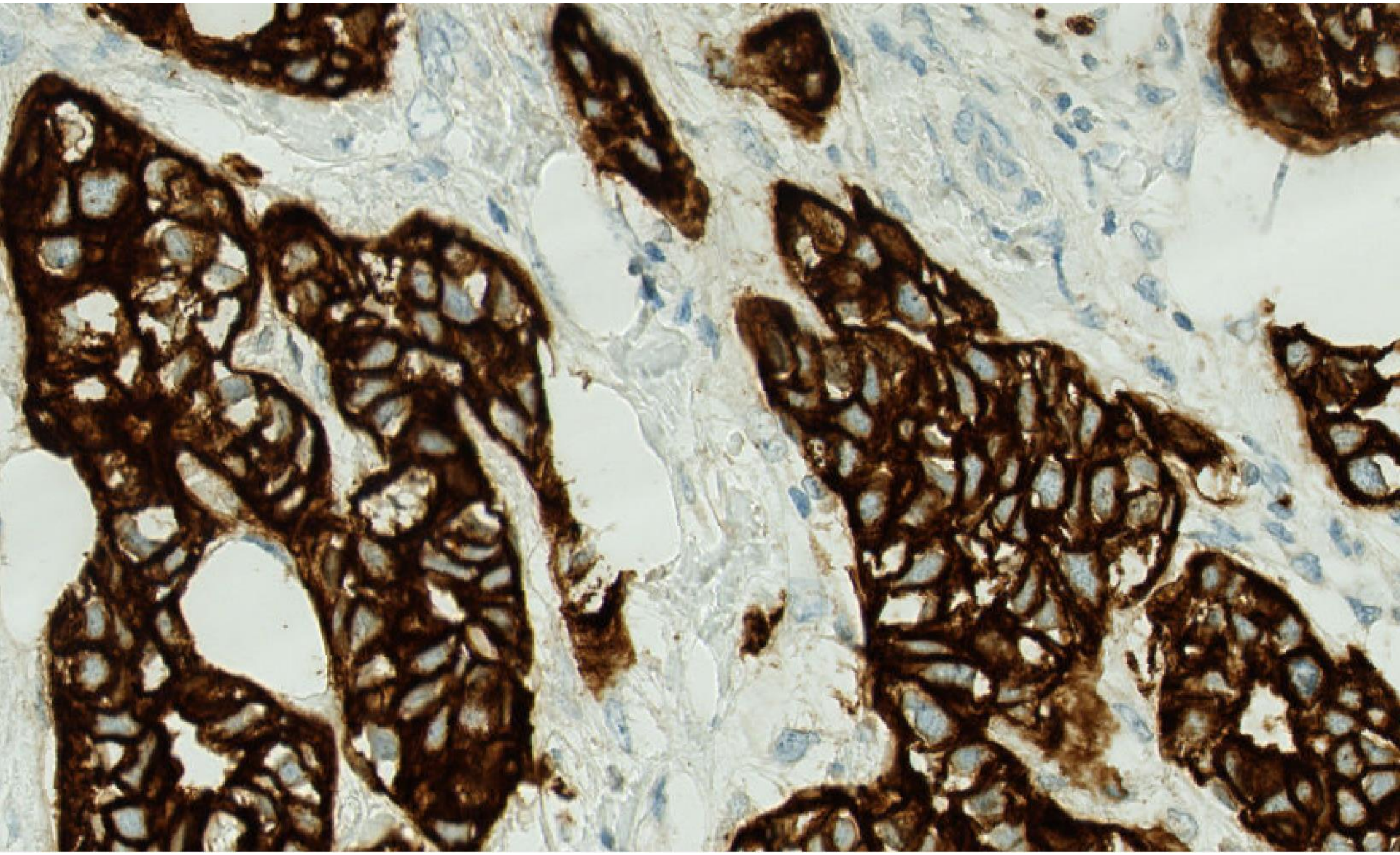
PCK-Alignment

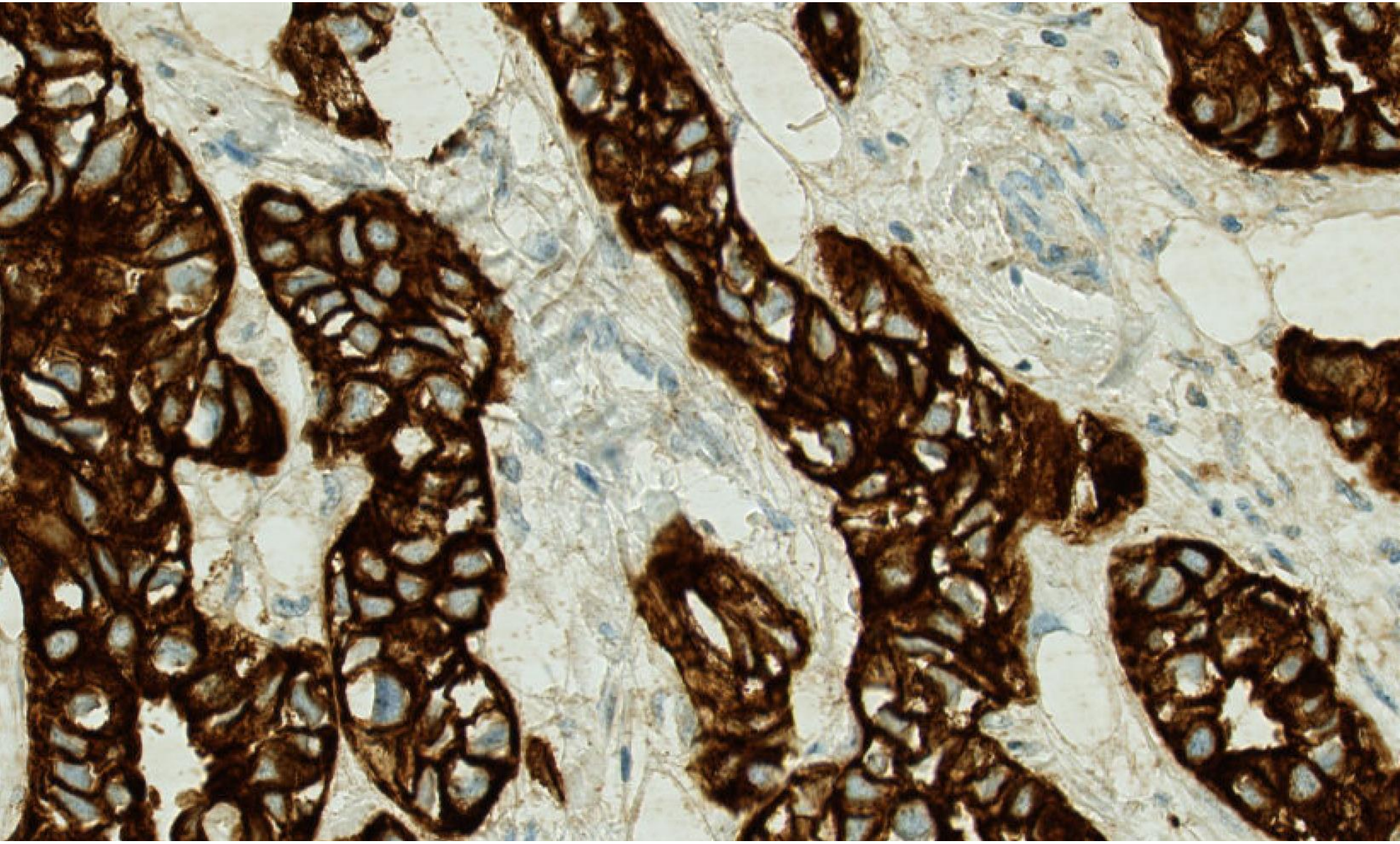
- Algorithm was developed that segmented 2 slides based on PCK expression
- Four categories based on PCK status in slide 1 and slide 2:

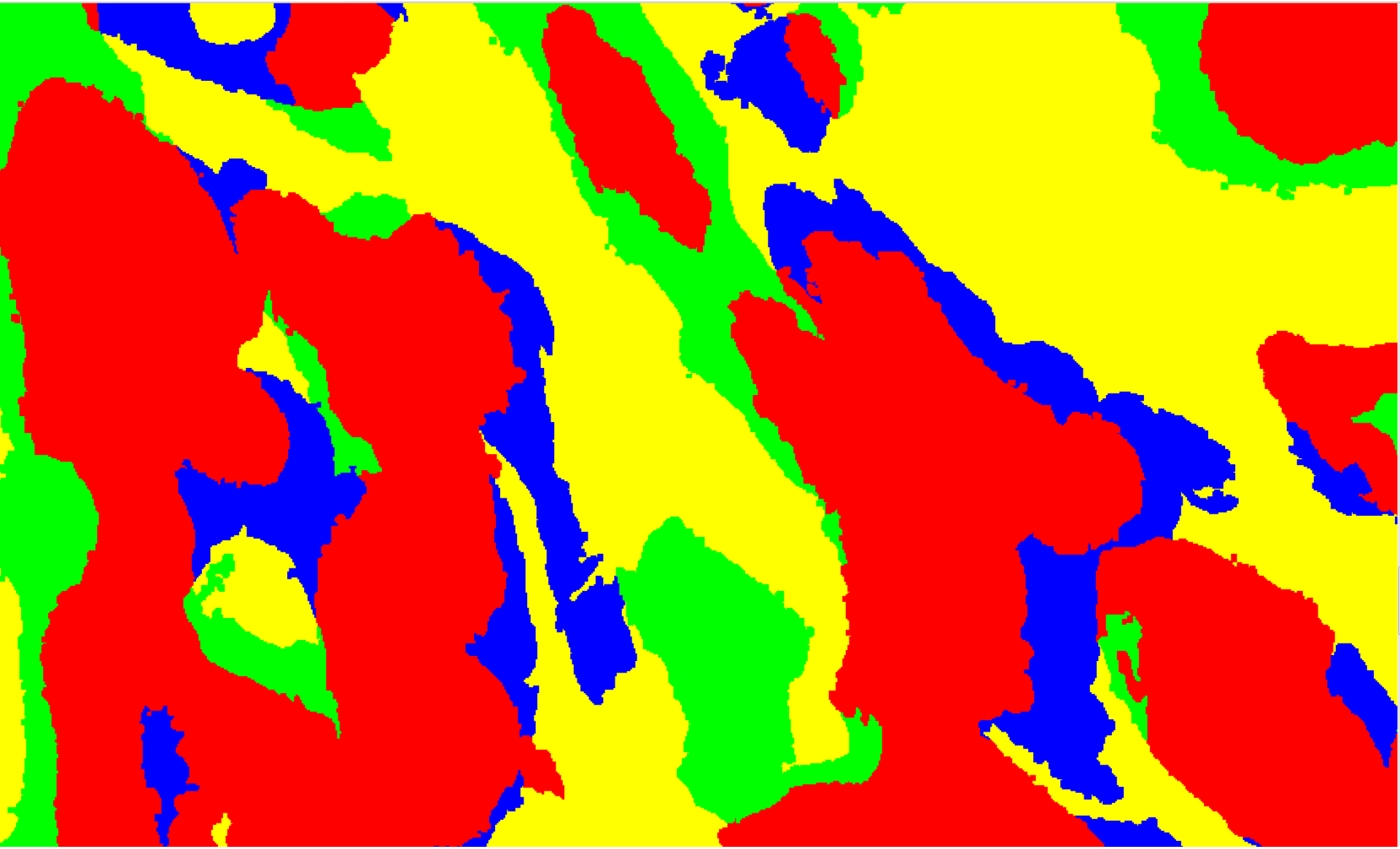
  + / + : PCK positive in both slides

  - / - : PCK negative in both slides

  + / - or - / +: PCK positive in only one slide







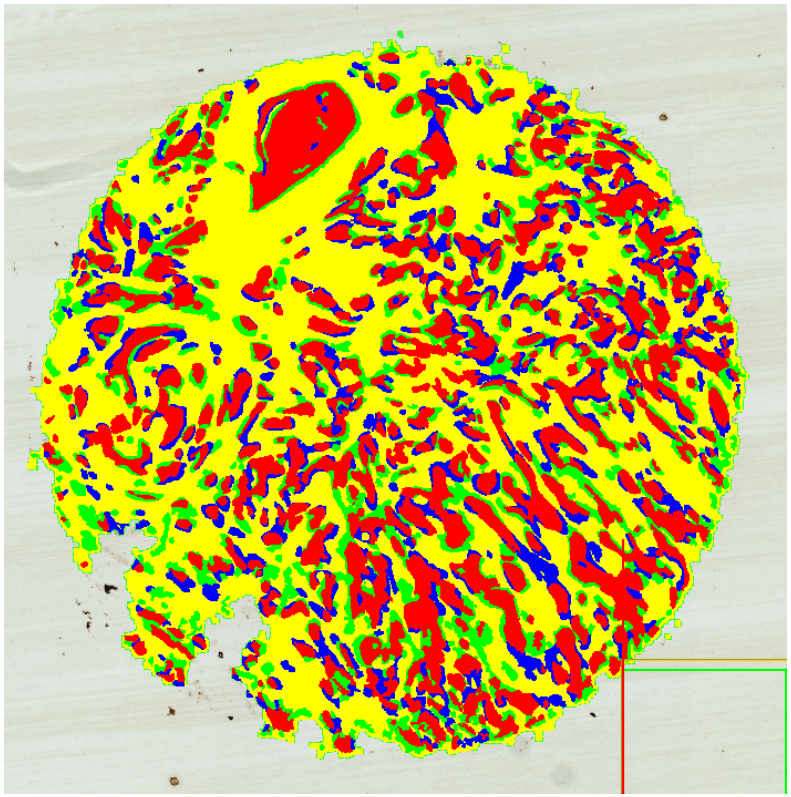
Overlap/agreement (%)

- Calculated as:

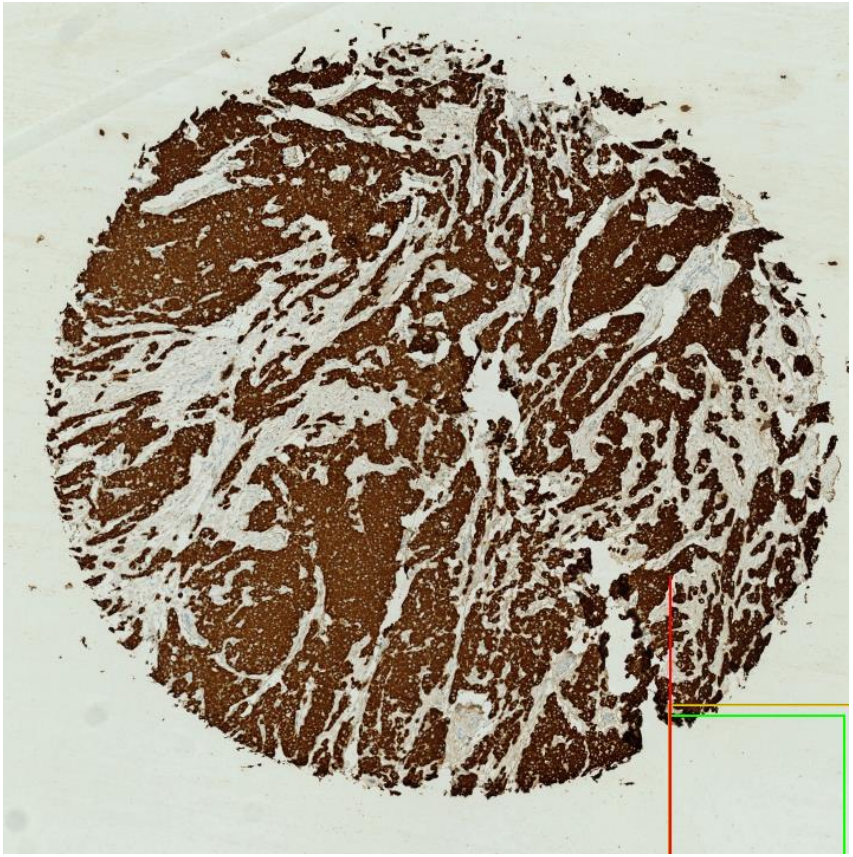
PCK positive area in both slides + 

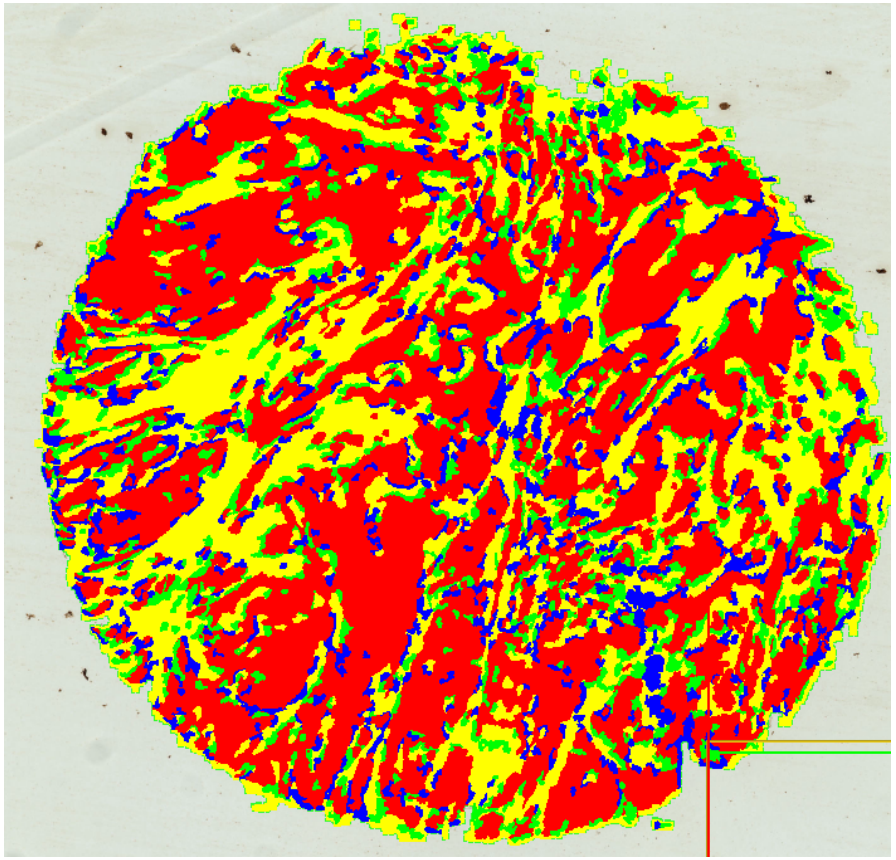
PCK negative area in both slides 

Divided by total area

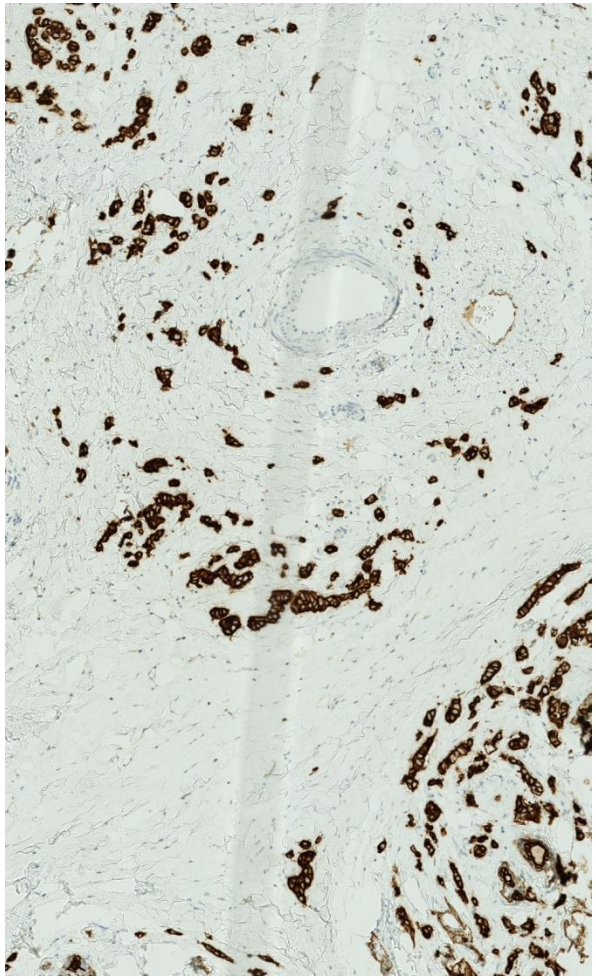


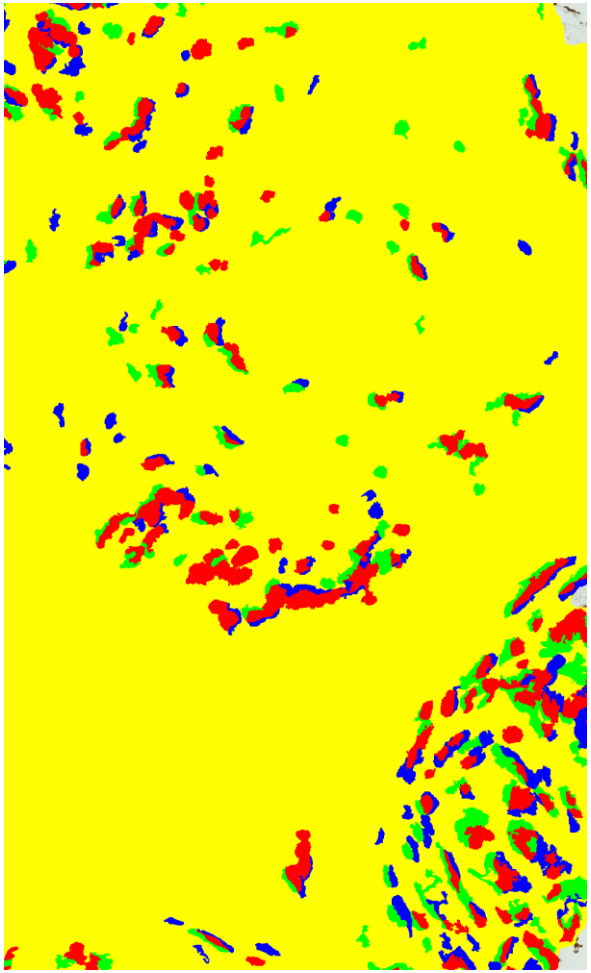
Good agreement (>90 %)

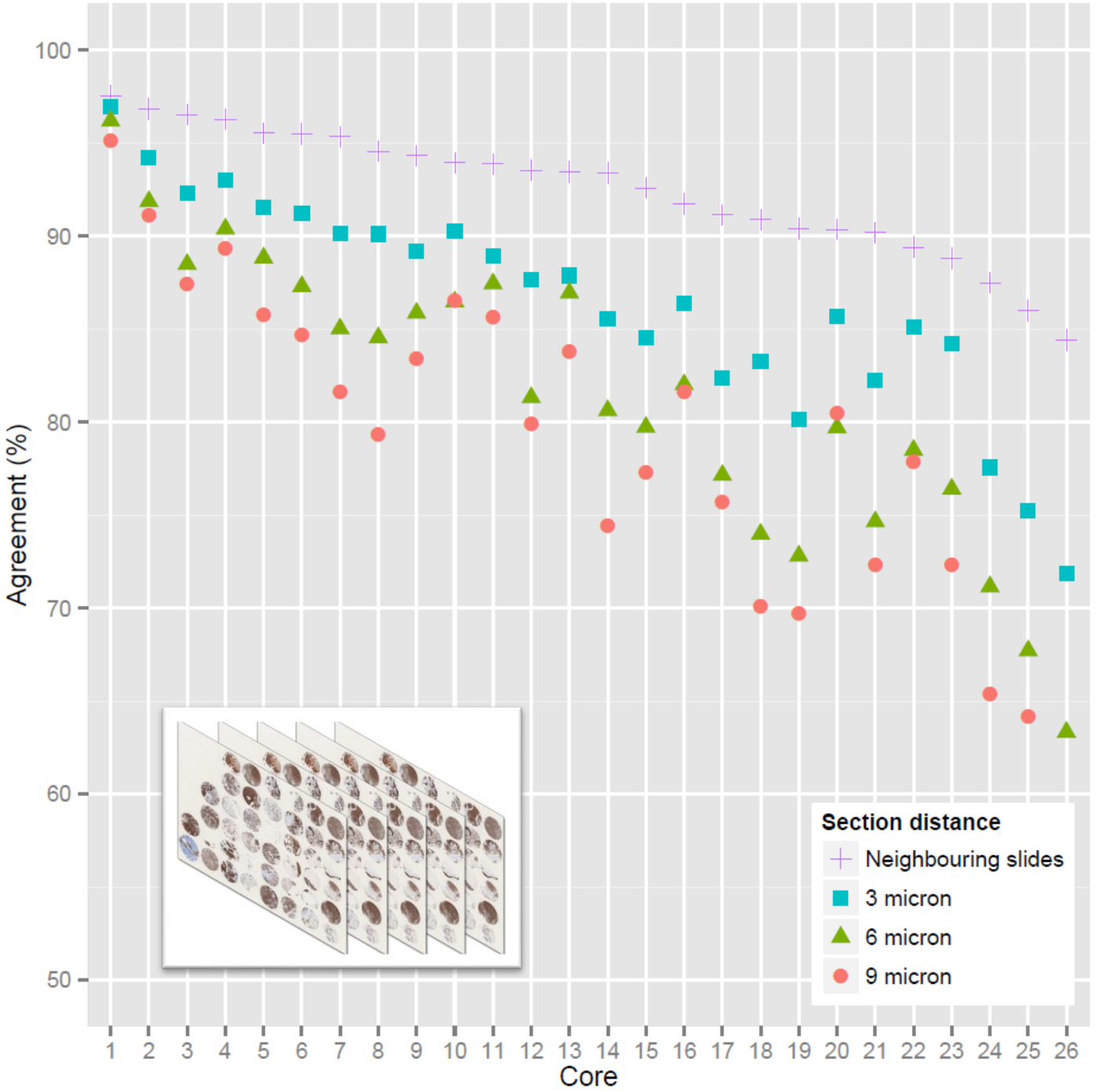




Less good agreement






100
90
80
70
60
50
Agreement (%)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
Core
Section distance
Neighbouring slides
3 micron
6 micron
9 micron