Image analysis in IHC - overview, considerations and applications

Workshop in Diagnostic Immunohistochemistry Oud St. Jan/ Old St. John – Brugge (Bruges), Belgium June 13th – 15nd 2018

Rasmus Røge, MD, NordiQC scheme organizer

When?

- Time consuming repeatable tasks
- Standardizable
- Output are simple or quantifiable parameter:
  - Count
  - Length
  - Area
  - Volume
  - Regions of Interest with specific characteristics
  - Categorical

When not?

- “We will just solve that by some image analysis…”
- “Ready by Friday…?”
- Very complex setups that requires (human) interpretation
- Jobs that could easily be solved in another way

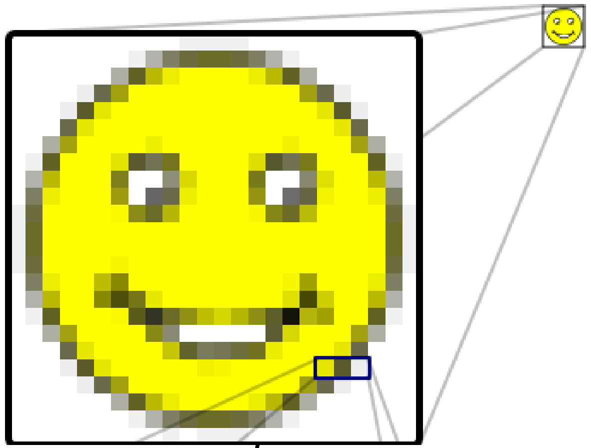
Theory

Image analysis in IHC - overview, considerations and applications

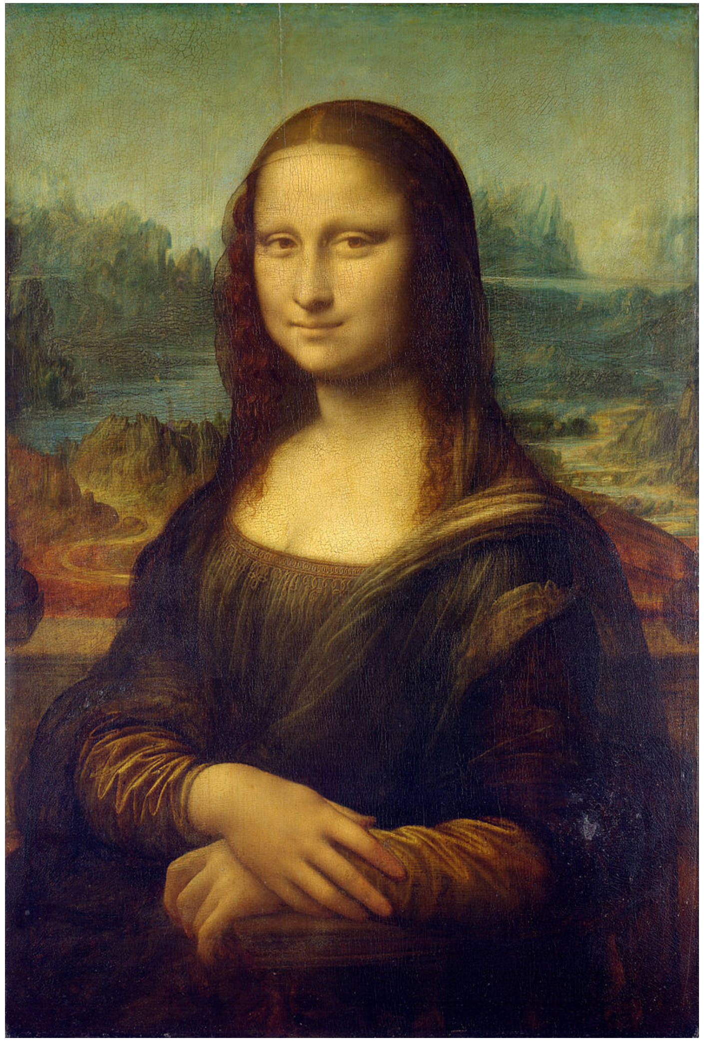
NordiQC

Theory

- Digital Image – numeric representation of two-dimensional image
- Either
  - Raster type: coordinate system of pixels, resolution-fixed (bmp, jpg, gif)
  - Vector type: build from primitive geometrical shapes, not-resolution-fixed (pdf, ps, fonts)

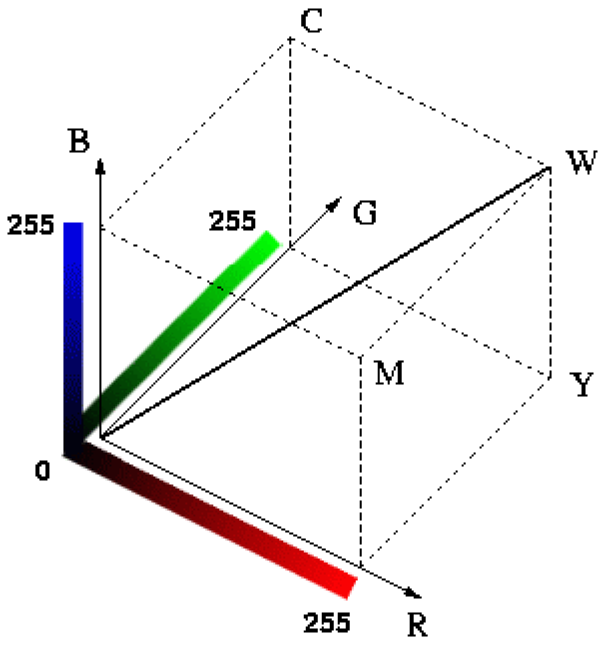


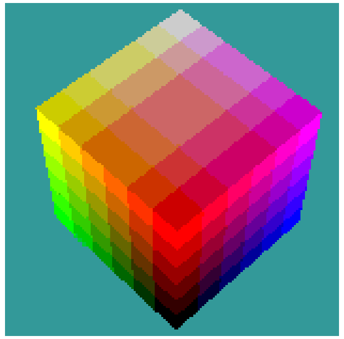
Pixels



RGB colour model

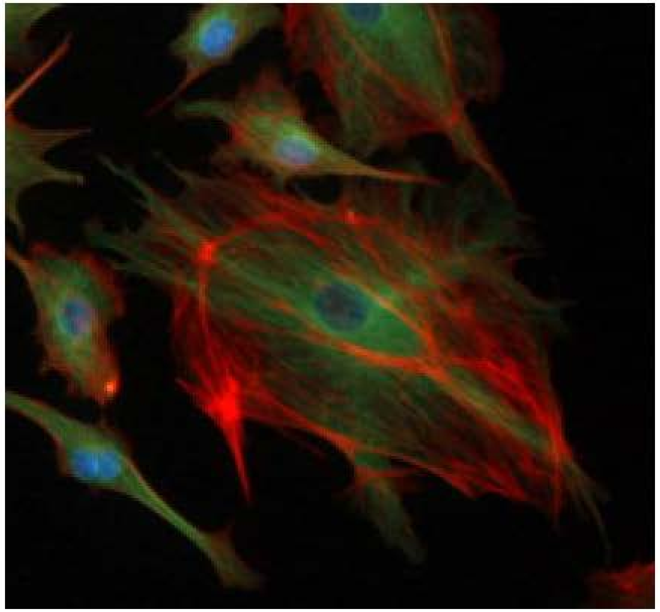
- Additive colour model
- Red, green and blue light
- System to encode representation of colour



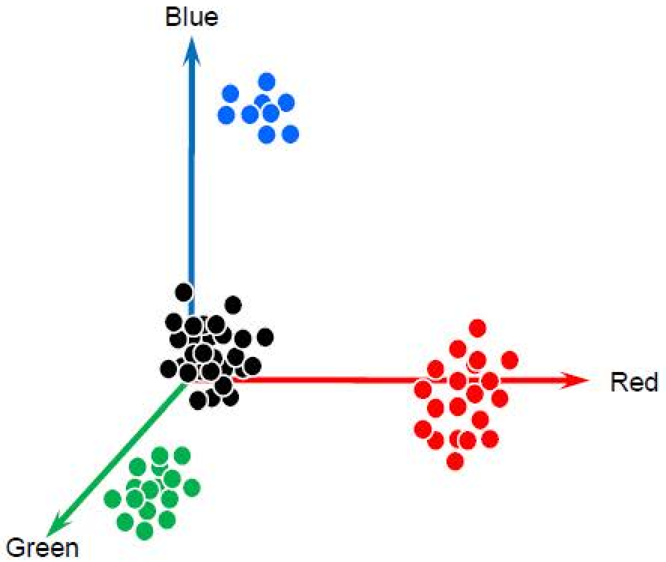


A FEW WORDS ON PIXELS

Grouping in color space



$$\overline{P}(x,y) = \begin{pmatrix} R(x,y) \\ G(x,y) \\ B(x,y) \end{pmatrix}$$



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

Color Models

IHS/HSV (Intensity, Hue, Saturation / Hue, Saturation, Value)

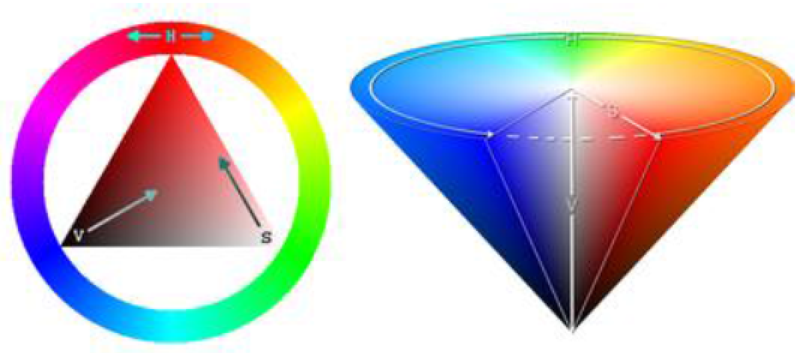
$$I = \frac{1}{3}(R+G+B)$$

$$H = \begin{cases} \theta & \text{if } B \le G \\ 360-\theta & \text{if } B > G \end{cases}$$

with

$$\theta = \cos^{-1}\left(\frac{\frac{1}{2}[(R-G)+(R-B)]}{\sqrt{(R-G)^2+(R-B)(G-B)}} \right)$$

$$S = 1 - \frac{3}{(R+G+B)}\min(\mathrm{R,G,B})$$



From Wikimedia Commons

Color Models

Color chromaticities

- Normalize out intensity, relative amount of each RGB color component

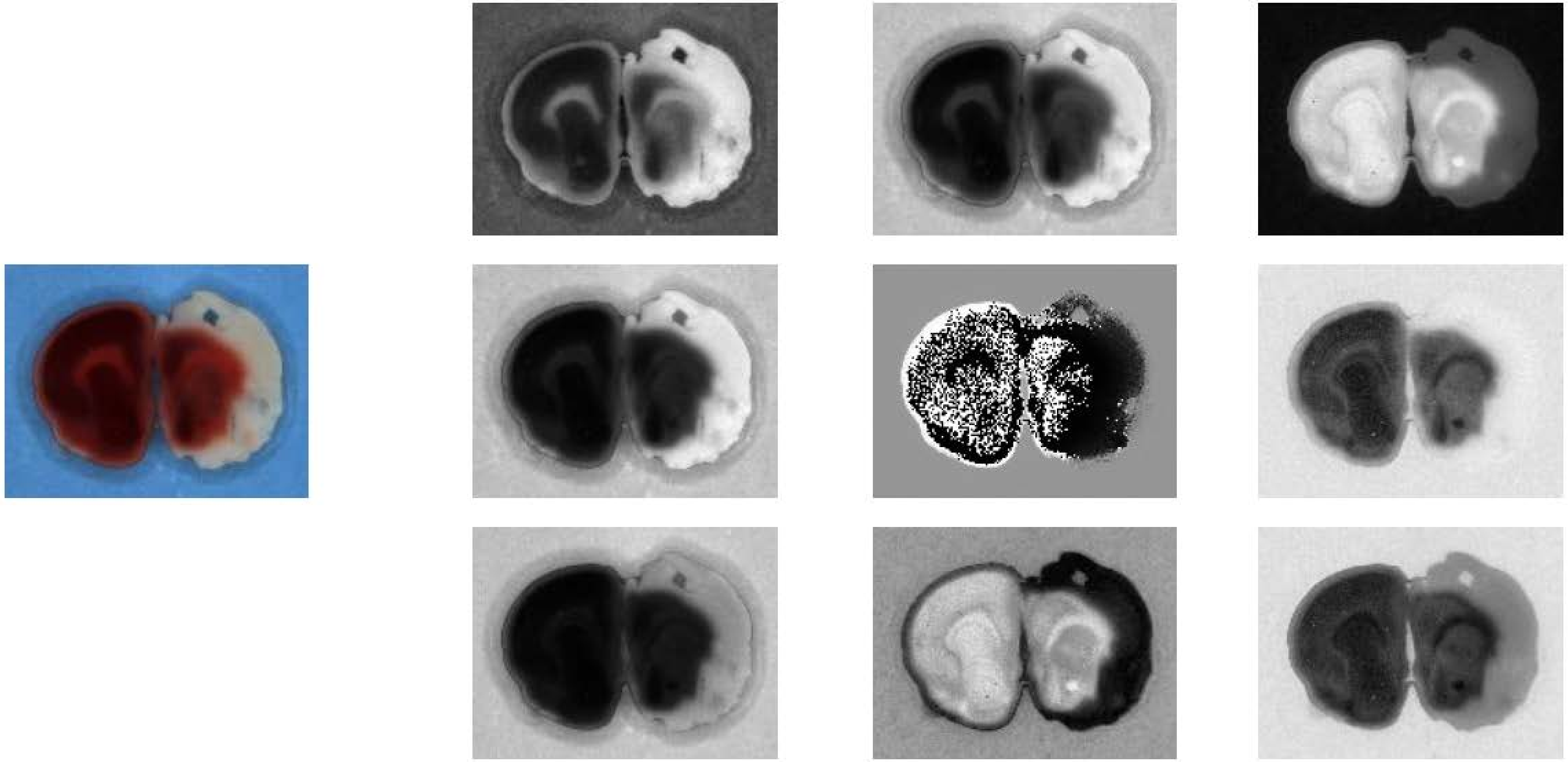
$$r = \frac{R}{R+G+B}$$

$$g = \frac{G}{R+G+B}$$

$$b = \frac{B}{R+G+B}$$

Color Models

R,G,B   I,H,S   r,g,b



Digitization – microscope / scanners

Camera mounted on microscope

- Pro
  - Area of interest
  - Quick
- Con
  - Time consuming
  - Not standardizable
  - Area of interest only

Slide scanner

- Pro
  - Standardizable
  - Quality
- Con
  - Price
  - Time
  - File size

Slide scanner

- Single or multi-slide scanner

- Whole experiment on same scanner!

- Whole experiment after calibration

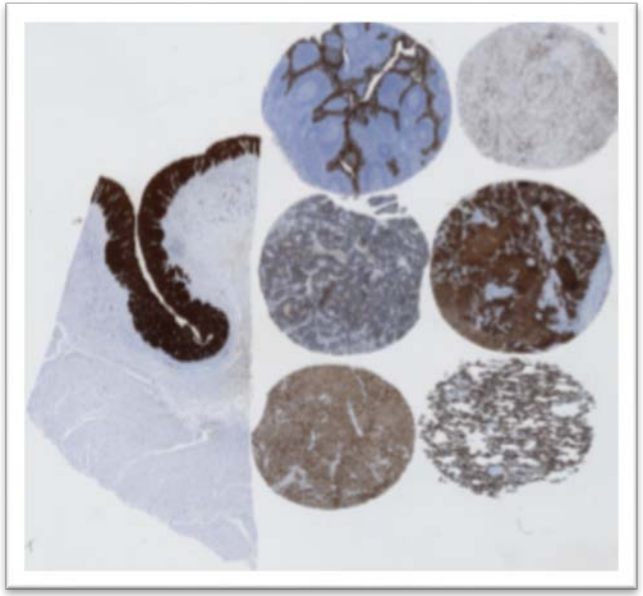
NordiQC

Image analysis

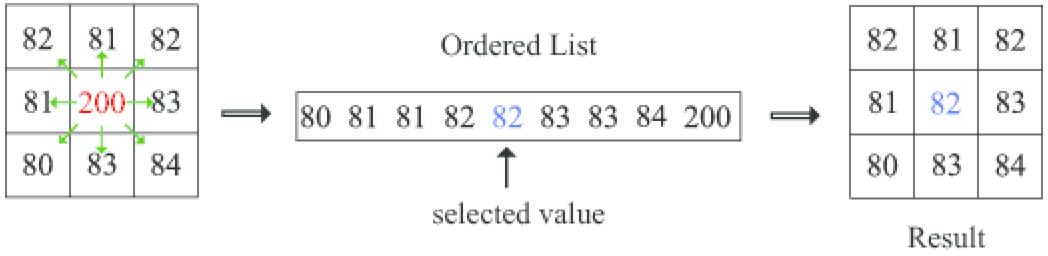
- Selection of filters
- Preprocessing – optimization of image to classification
  - Noise filtering, enhancement
- Classification / Segmentation
- Post processing
- Report of quantitative results

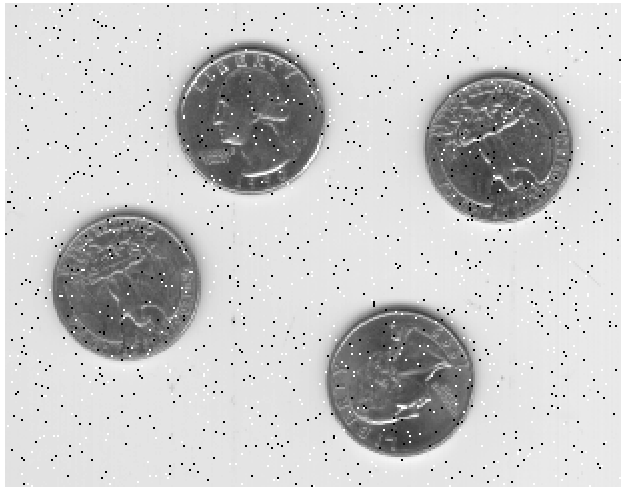
Selection of relevant tissue

- TMA will often contain several irrelevant or less interesting areas
- Algorithm will analyse whole image or ROI (Region of interest)
- Manually or automatic detection of ROI?



Noise filtering





Edge Enhancement

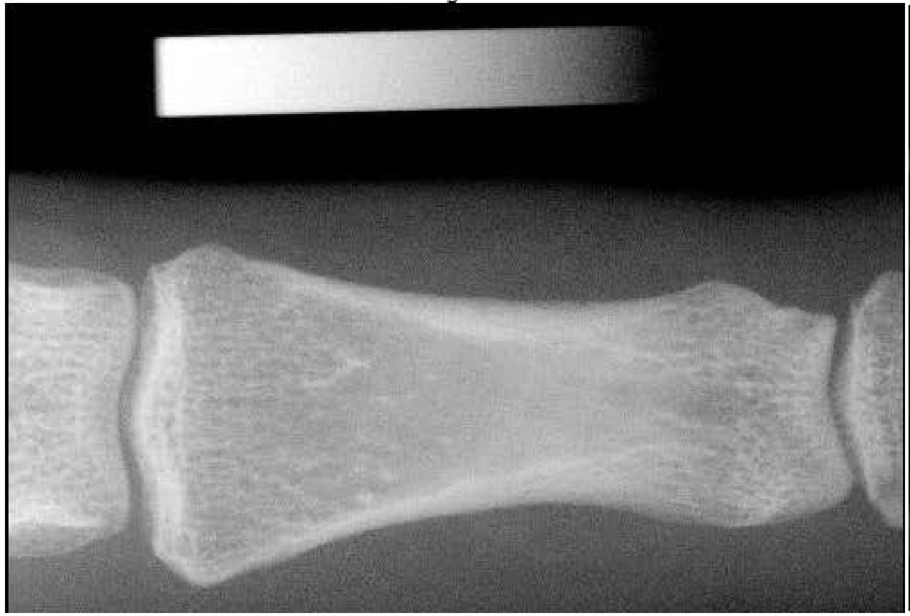
Standard deviation filter

Edge Enhancement

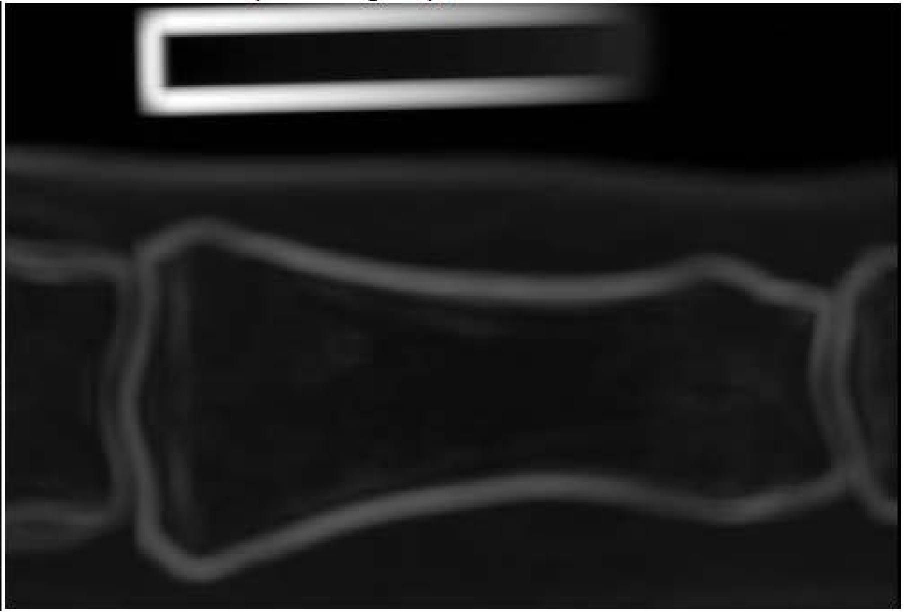
Standard deviation filter

$$\begin{bmatrix}100 & 100 & 100\\ 100 & 100 & 100\\ 100 & 100 & 100\end{bmatrix} \rightarrow 0 \qquad \begin{bmatrix}200 & 198 & 7\\ 255 & 132 & 3\\ 150 & 150 & 2\end{bmatrix} \rightarrow 96$$

Original



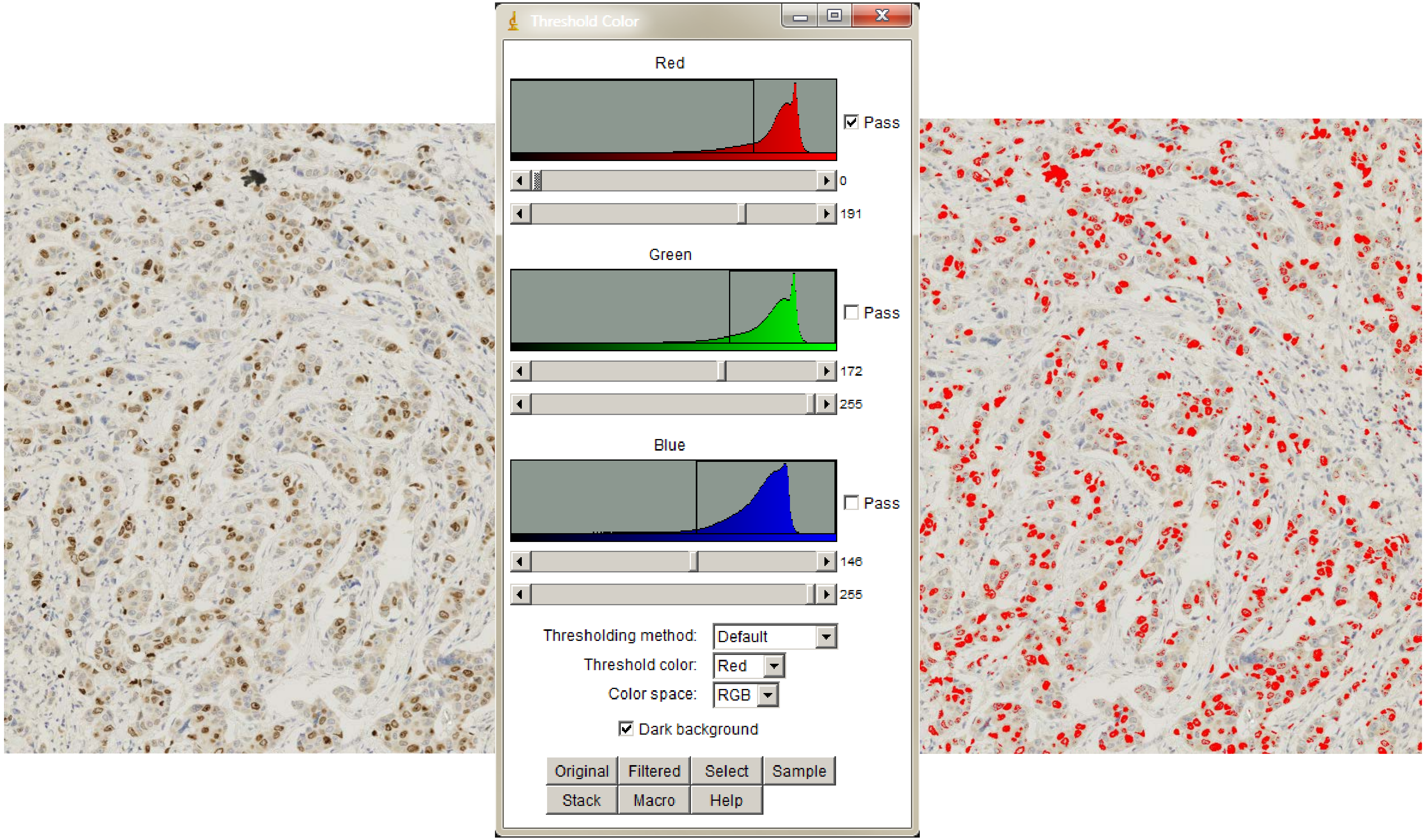
After the processing step, with a filter size of 35x35.



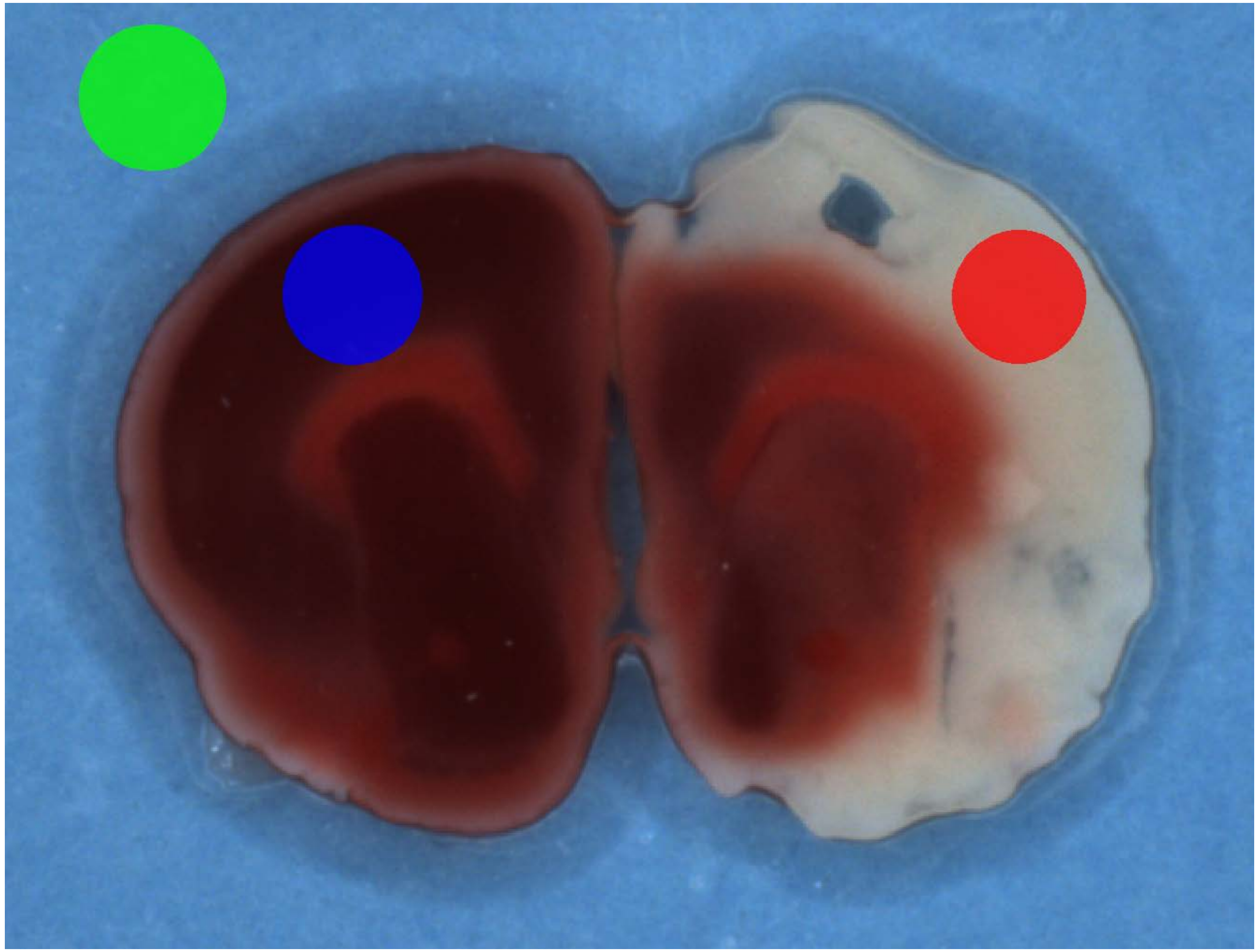
Classification / segmentation

- 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

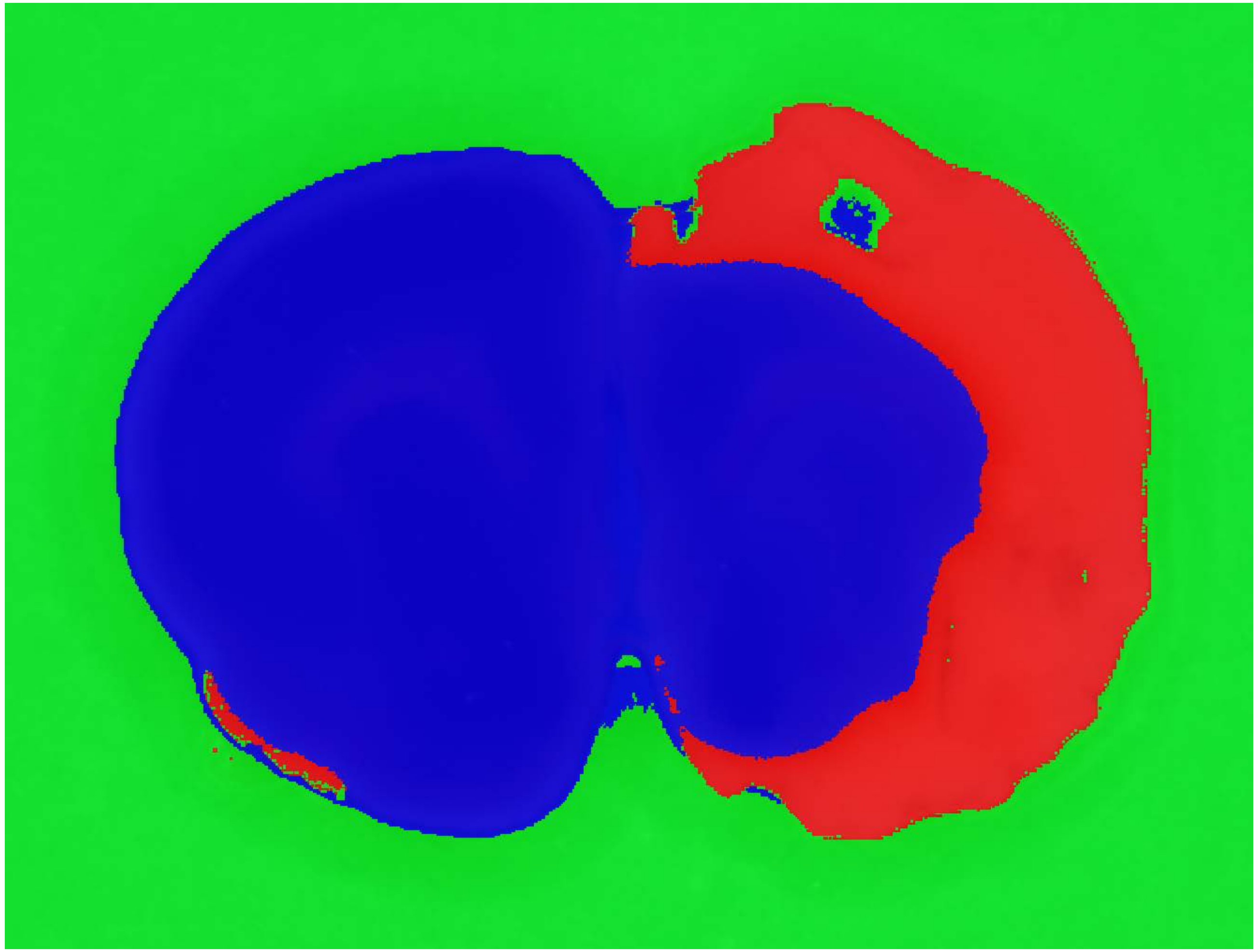
Threshold


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

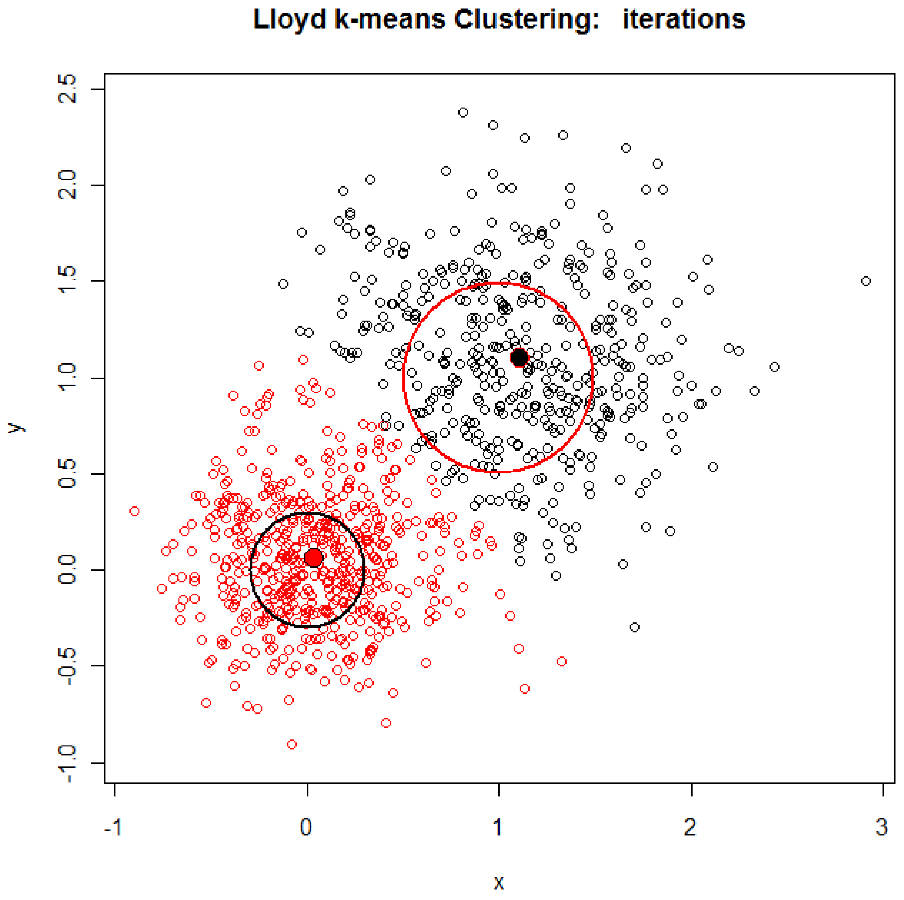


Bayesian

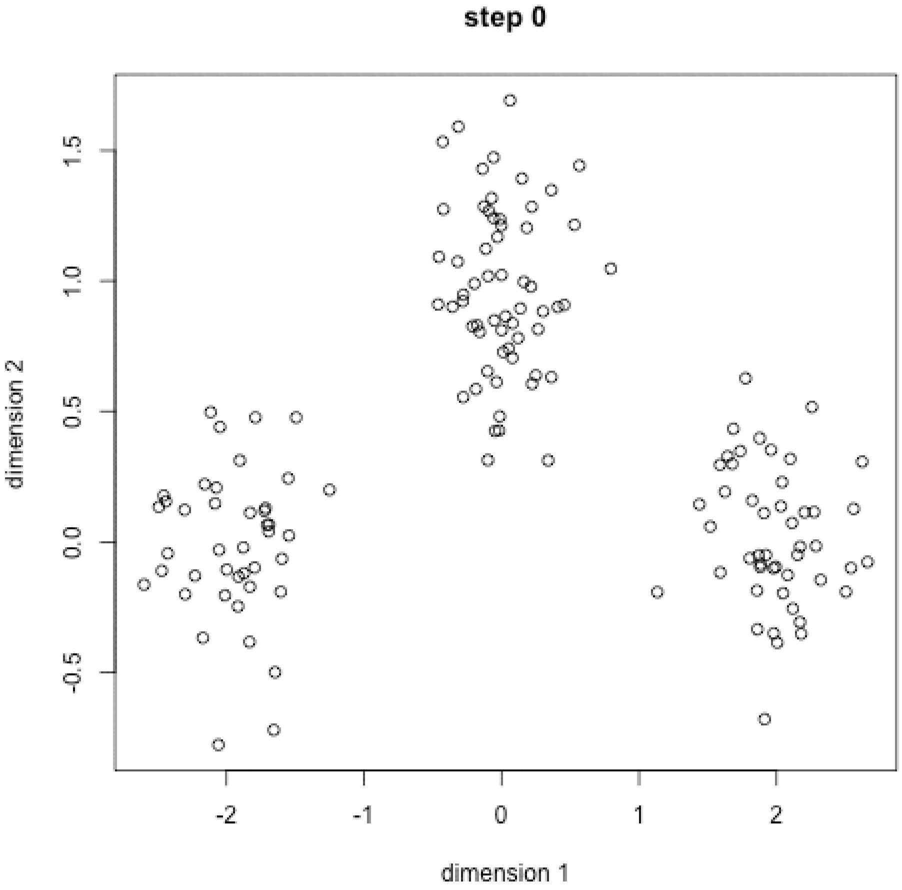


K-means

- 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

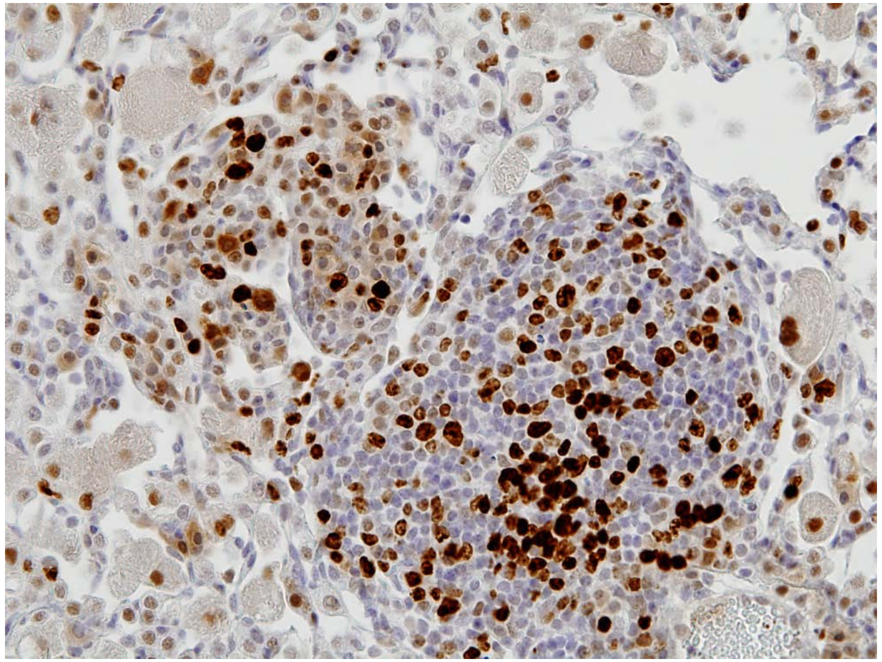


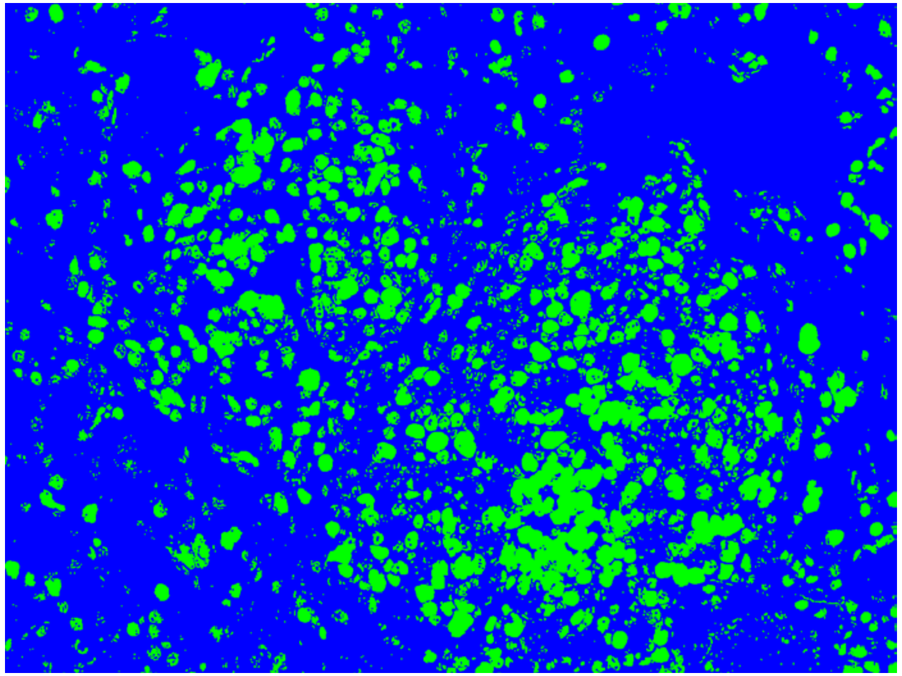
K-means



K-means

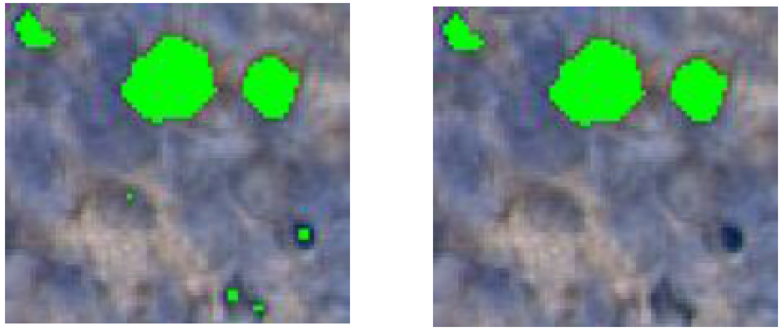
K-means



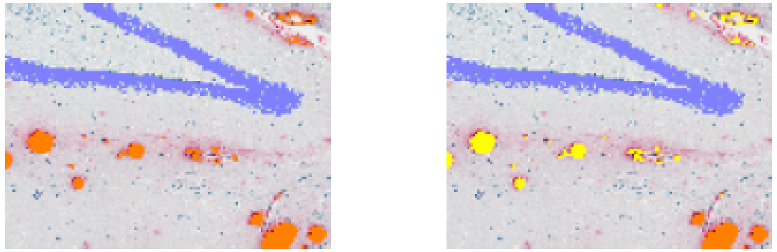


Post processing

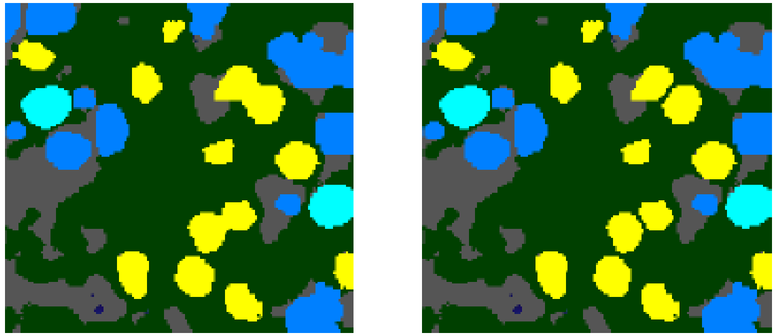
Clean-up / Noise removal: elimination of small or large objects



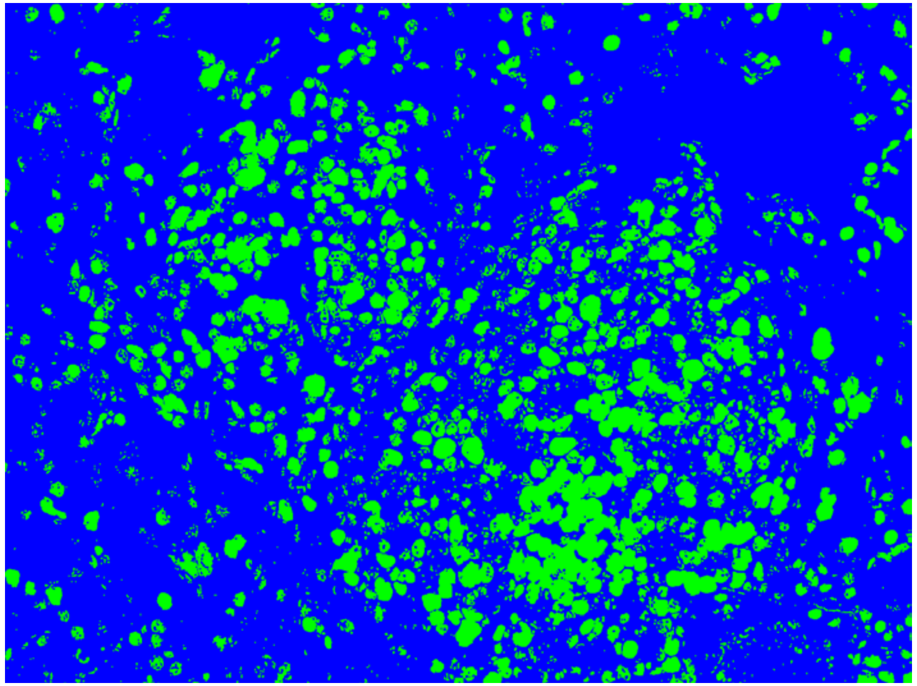
Discriminate objects based on distance to other objects

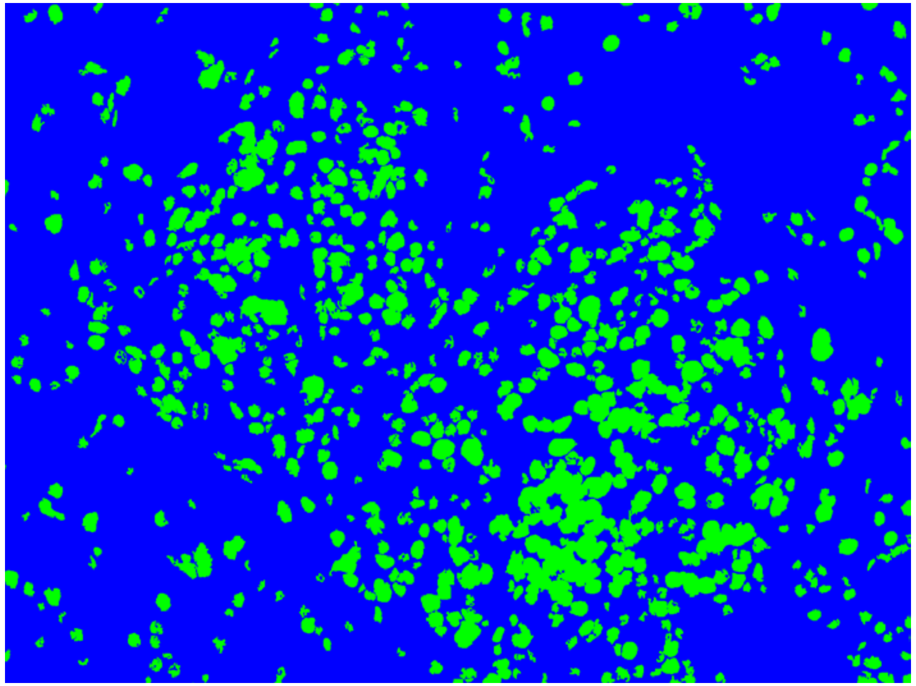


Separate objects, change based on shape or surroundings, erode, dilate, open, close, skeletonize, mark maxima, ...



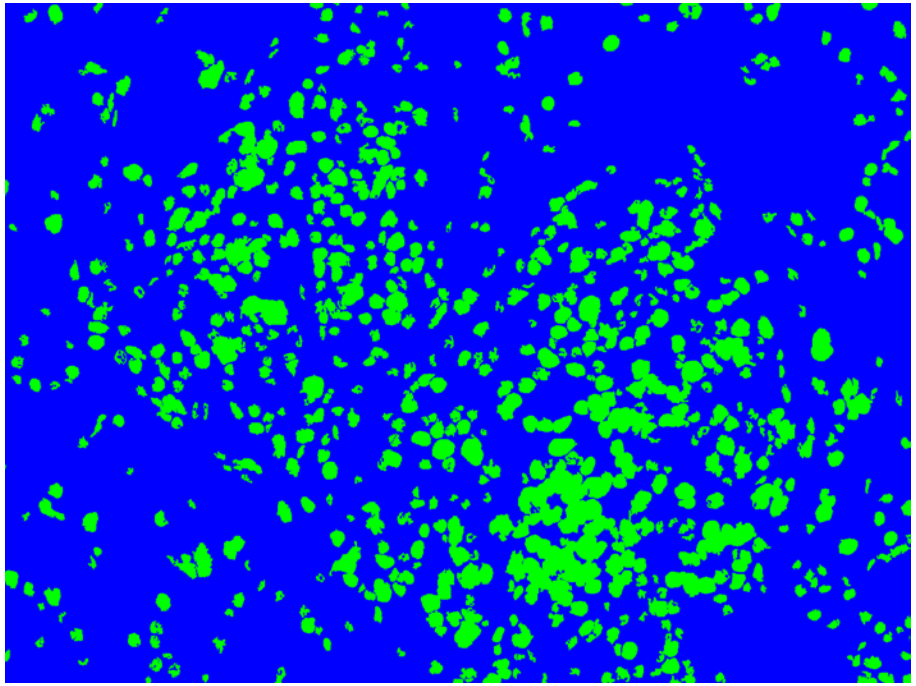
Post processing

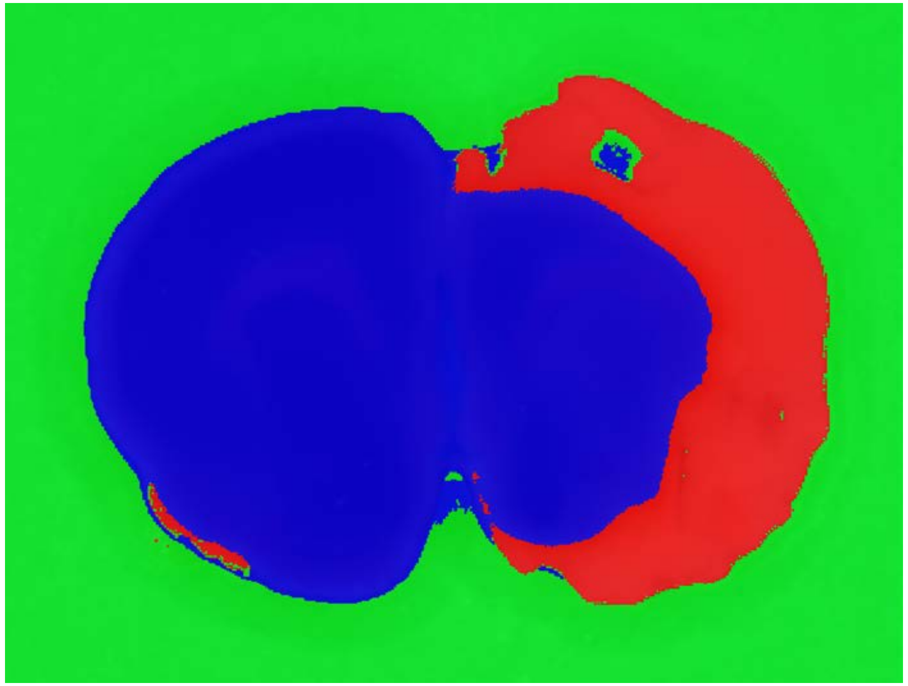




Post-processing:
Small green area, replaced by blue
Small blue area, replaced by green

Report of quantitative results





COUNT:
Typical number or fraction of objects

AREA:
Area of each category

Image analysis – example 1

Image analysis in IHC - overview, considerations and applications

Ki67 & Virtual Double Staining

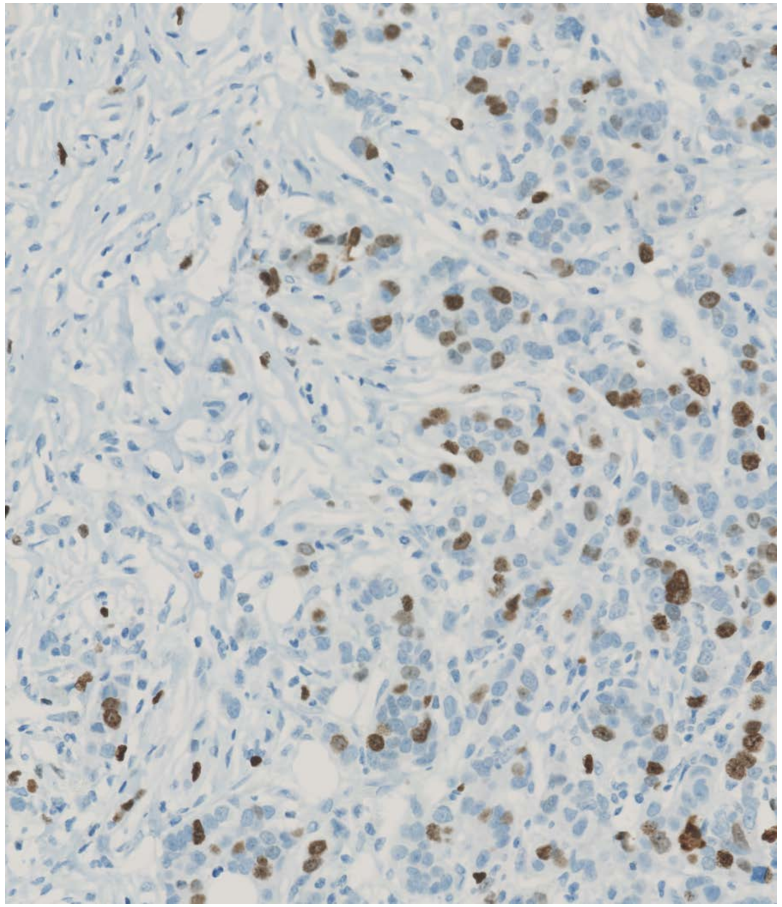
Ki67 – why is it important?

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

Digital Image Analysis

Criteria

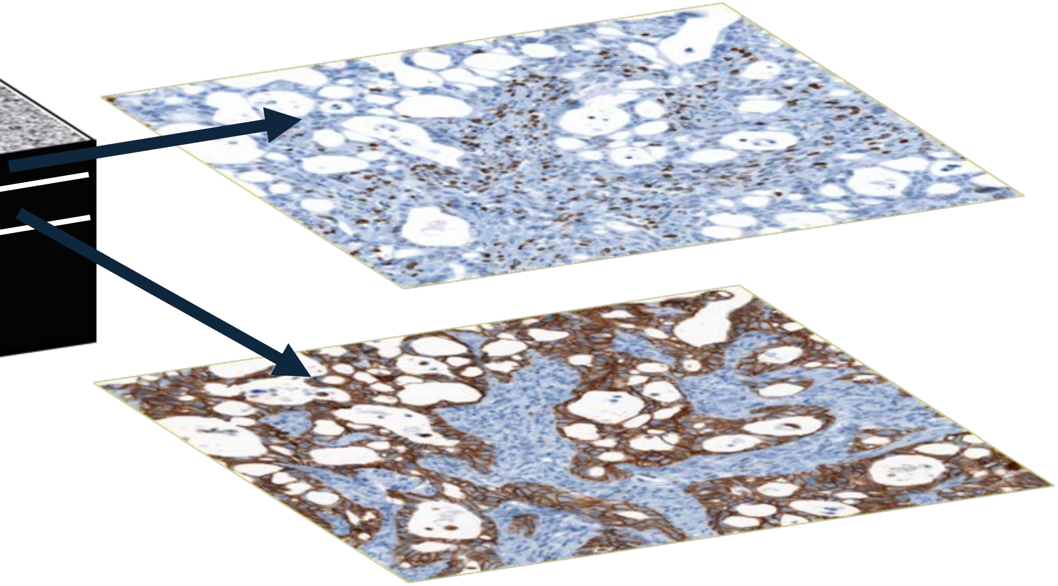
- Identify nuclei
- Distinguish Ki67 positive and negative nuclei
- Exclude non-tumour cells from analysis



Virtuel Double Staining: concept

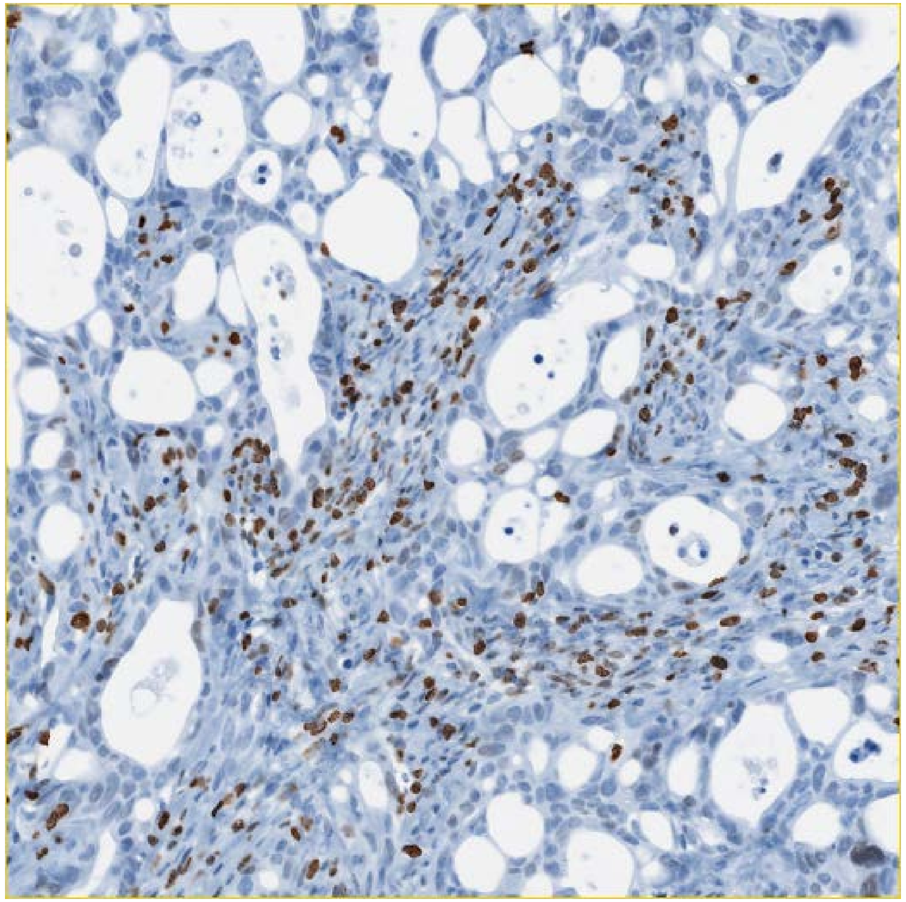
Cut serial sections (3µm):

- Slide stained for Ki67

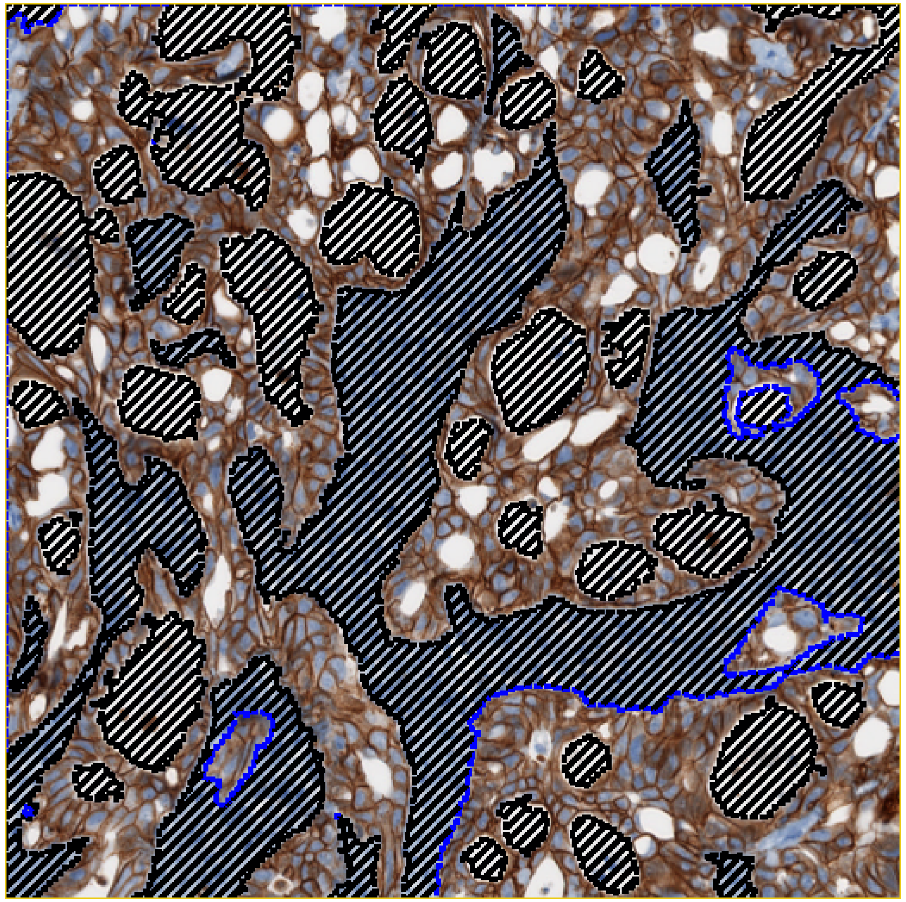


- Neighboring slide stained for pancytokeratin

Image analysis for identification of tumor

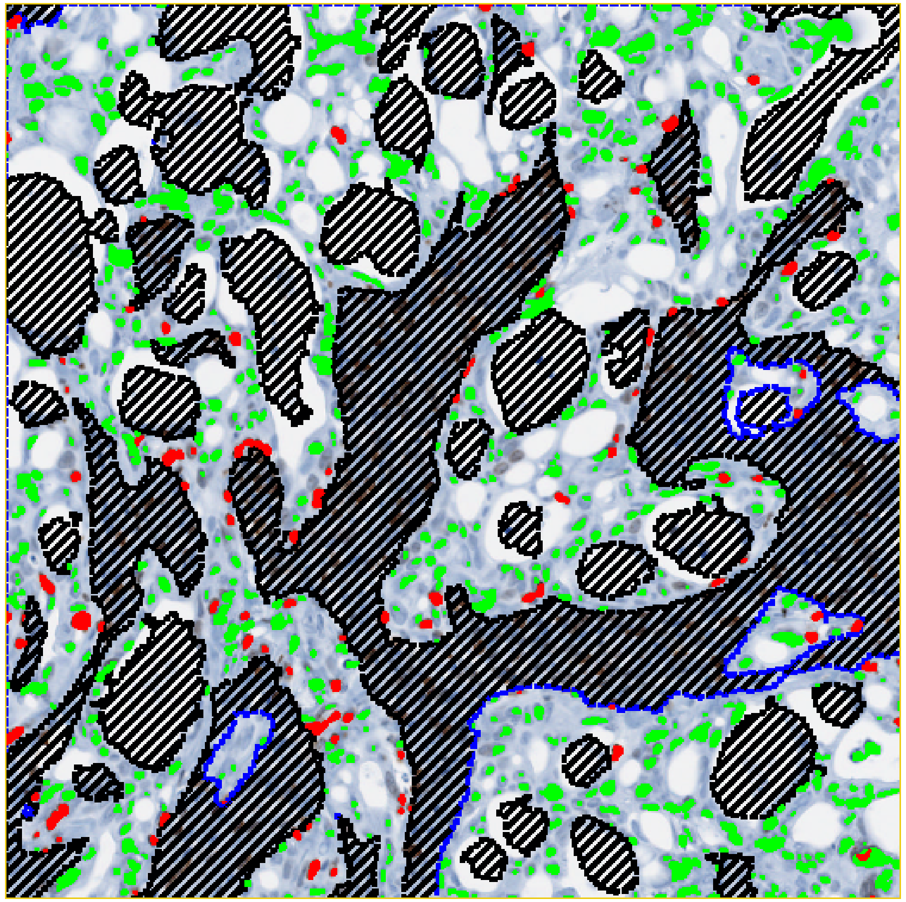


Ki67

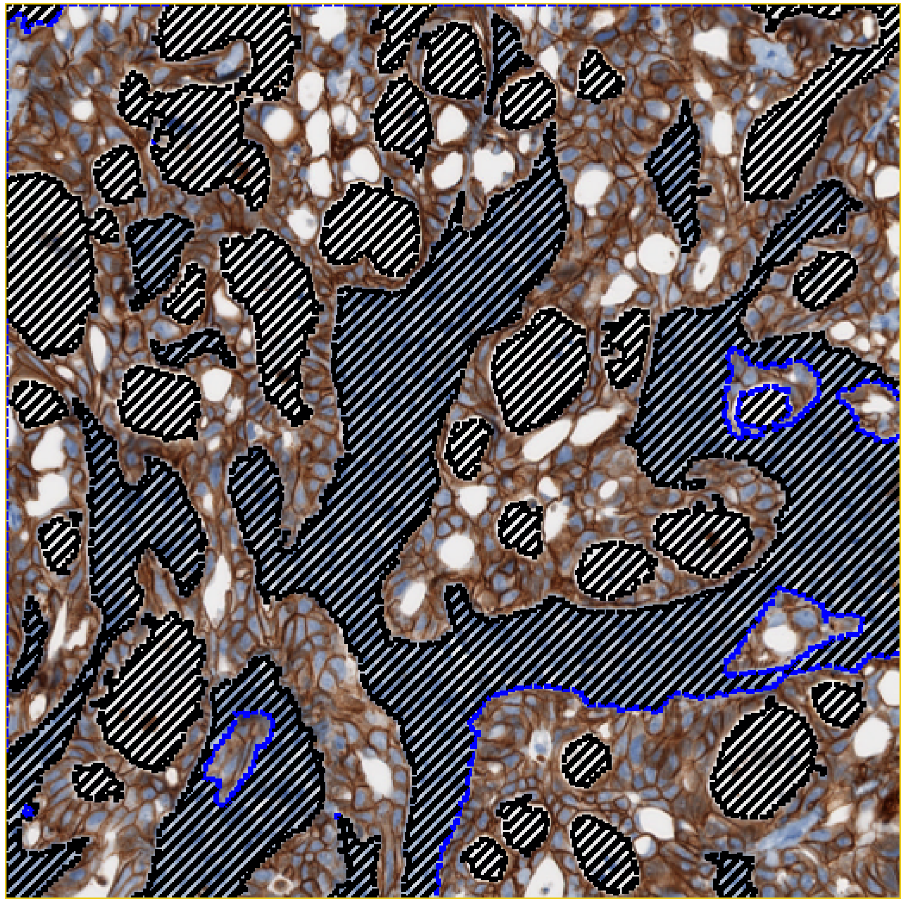


Pancytokeratin

Image analysis for identification of biomarker (Ki67)



Ki67



Pancytokeratin

Validation of VDS + Ki67 counting

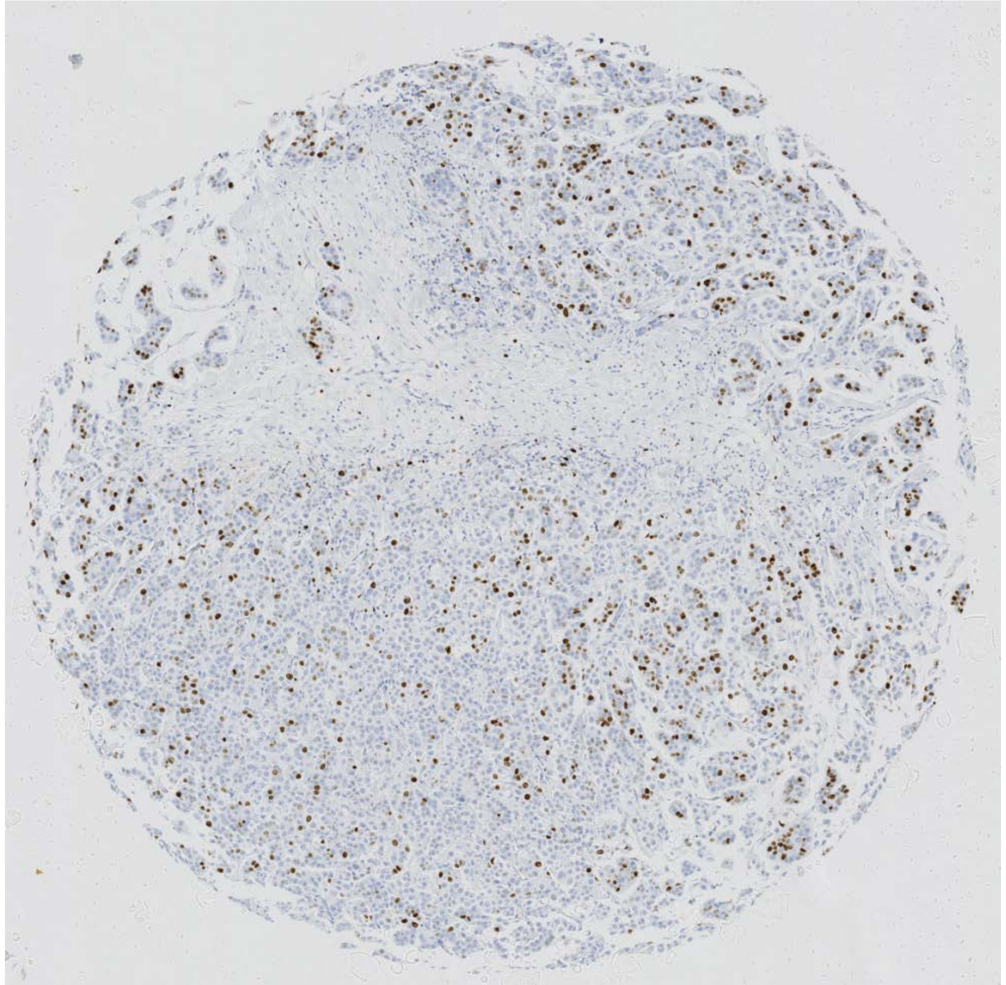
- Validation of the Nuclear detection and segmentation (number of positive and negative nuclei)

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

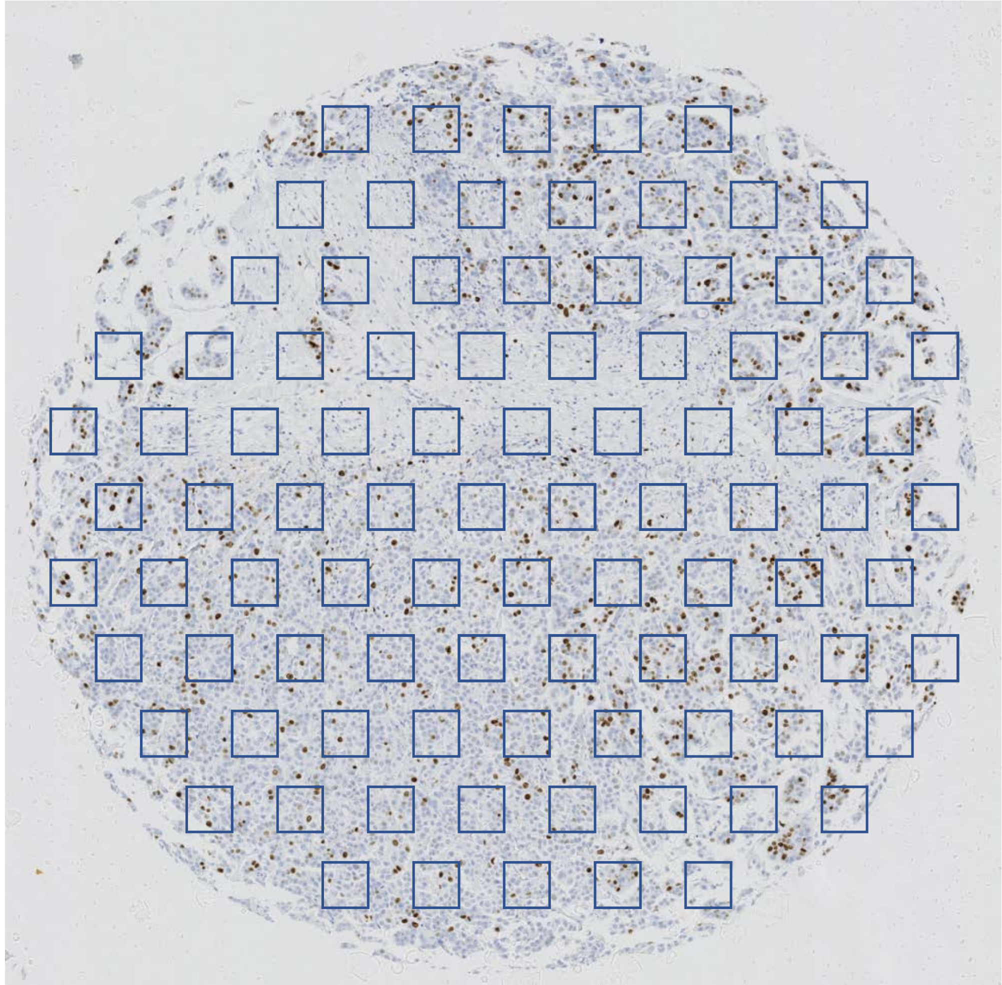
Method

- 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

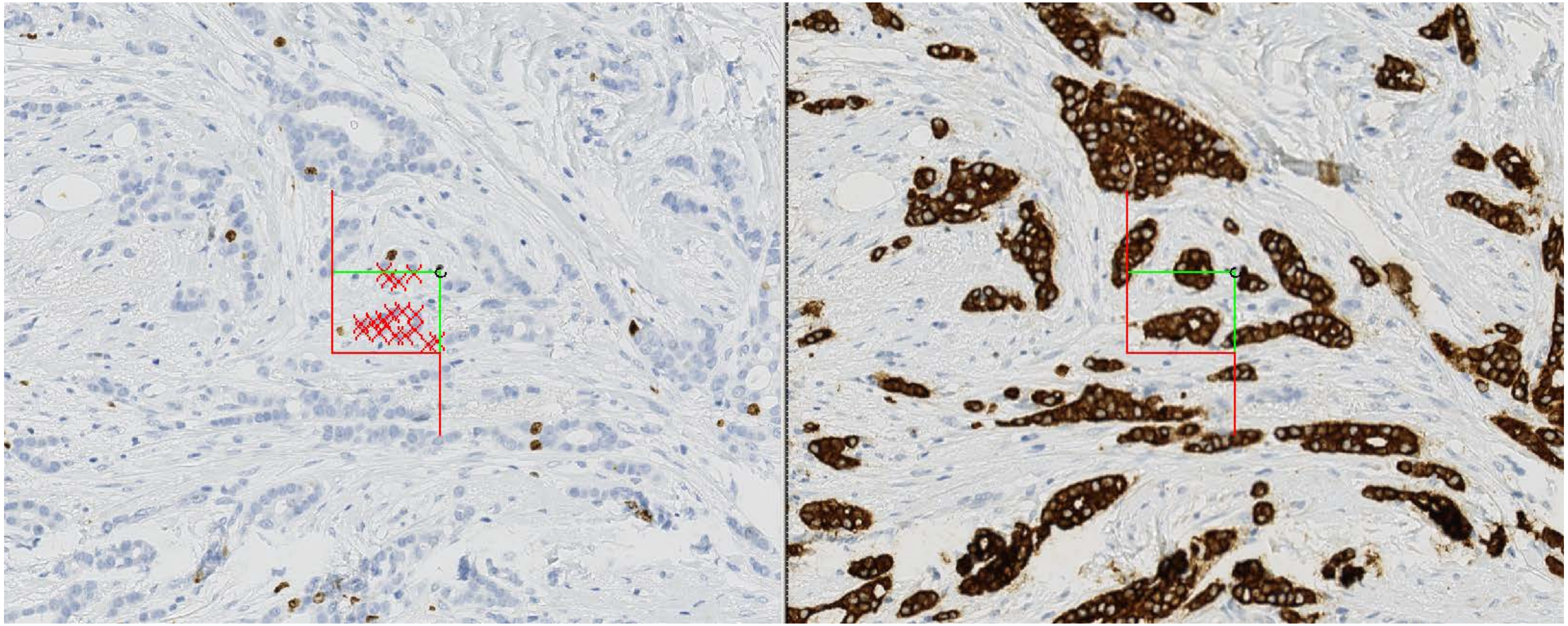


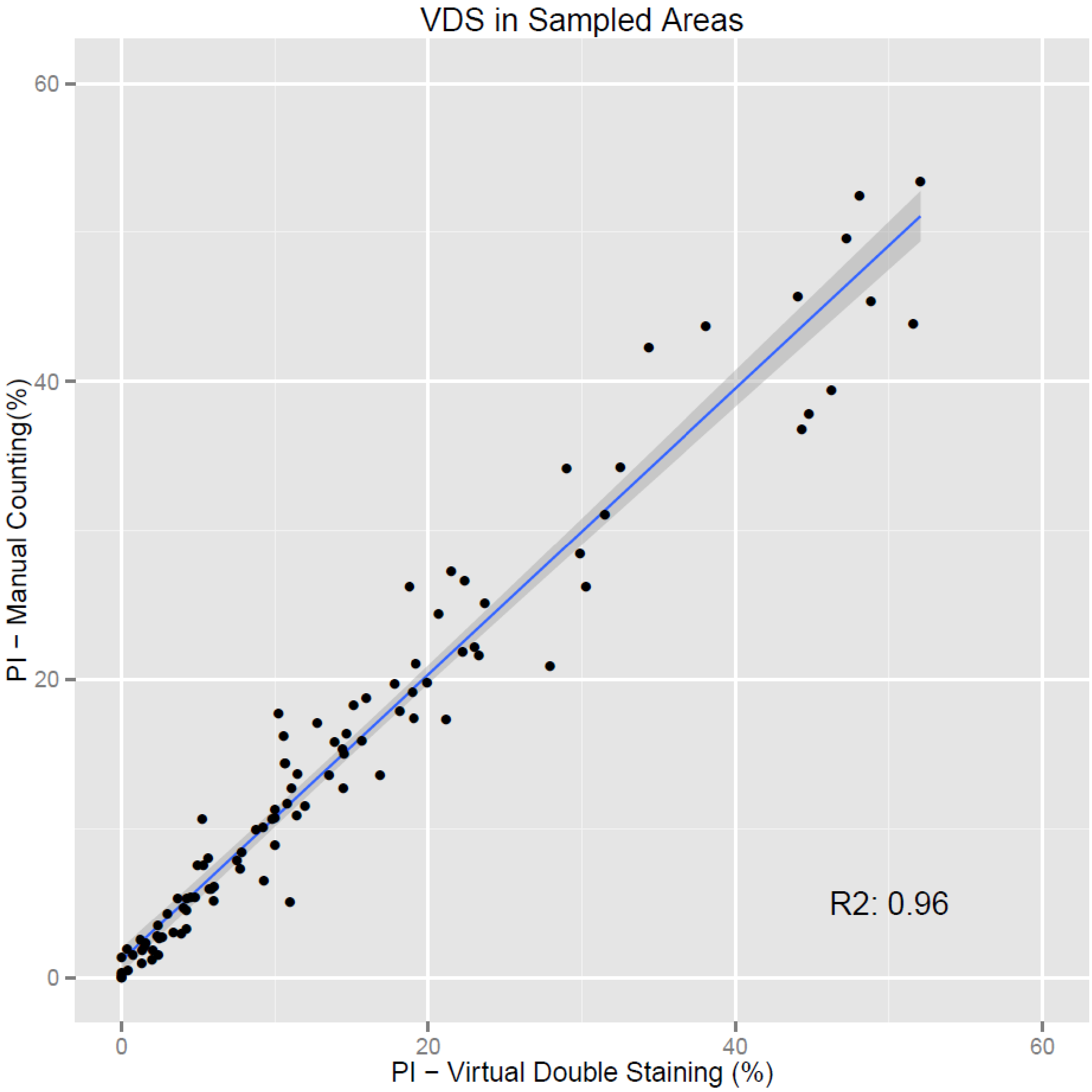
Systematic Random Sampling

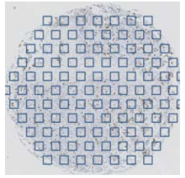


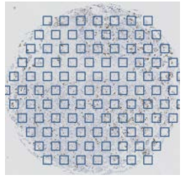
- Grid of frames randomly placed on core
- Positive and negative tumour cells counted manually in each frame
- Each frame extracted as an image for Virtual Double Staining

Stereological counting




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





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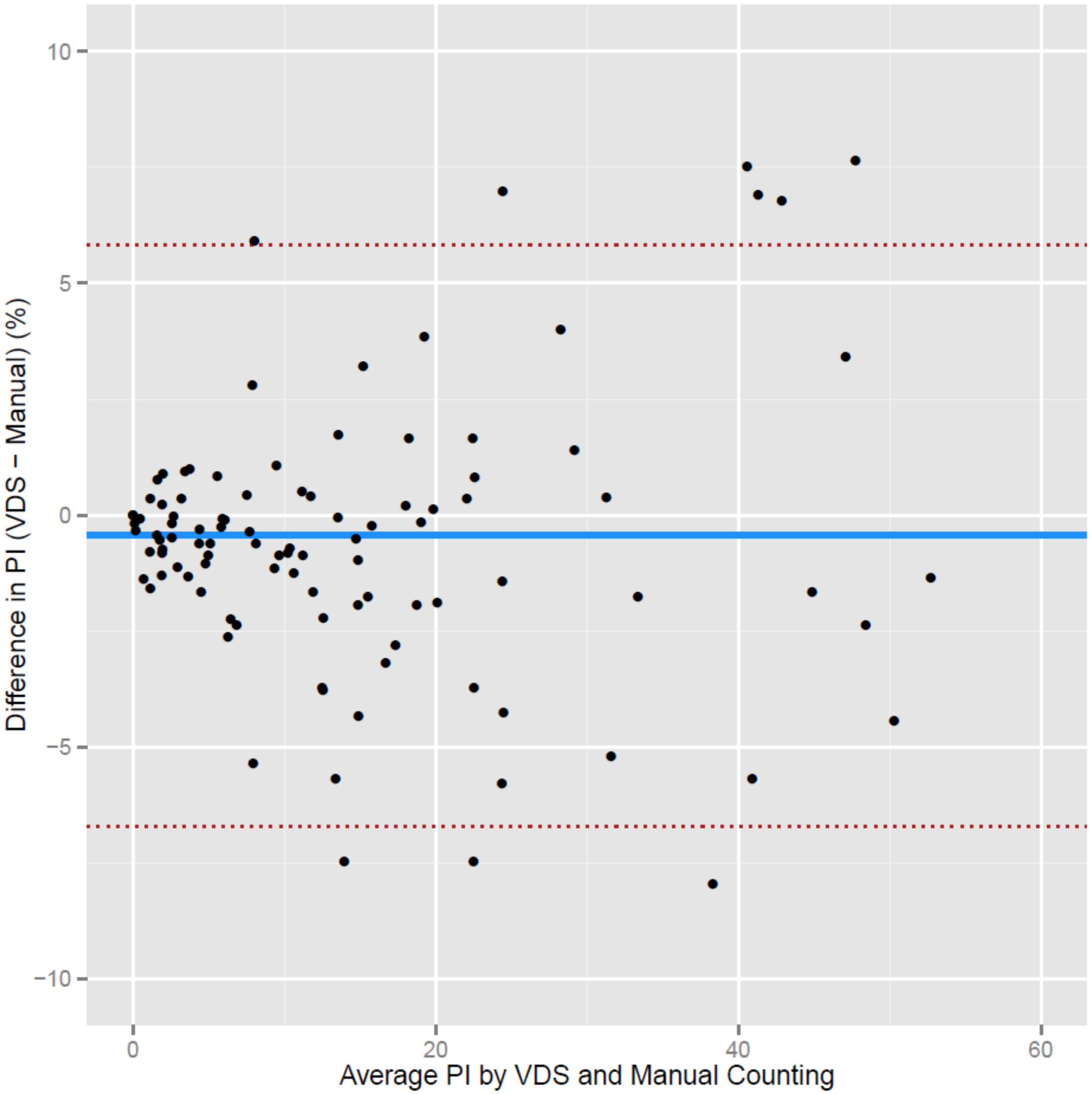
Bland-Altman



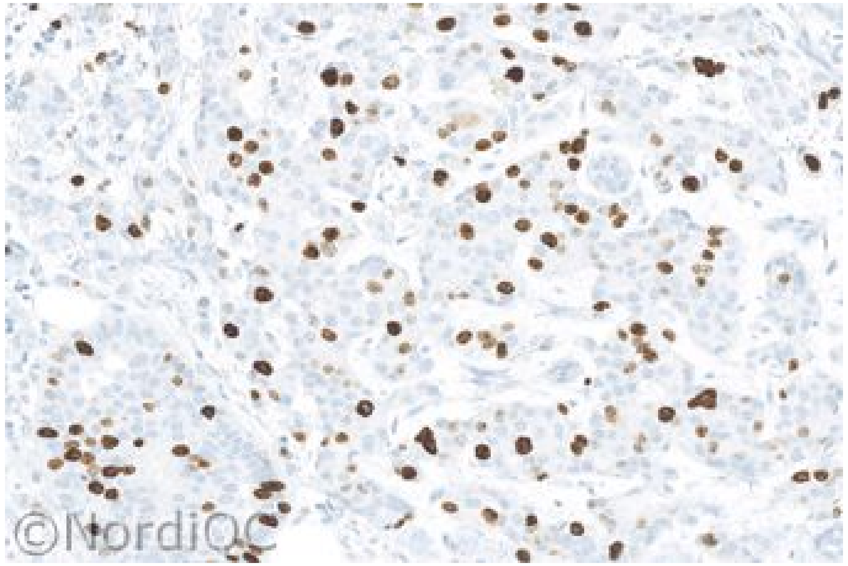
NordiQC

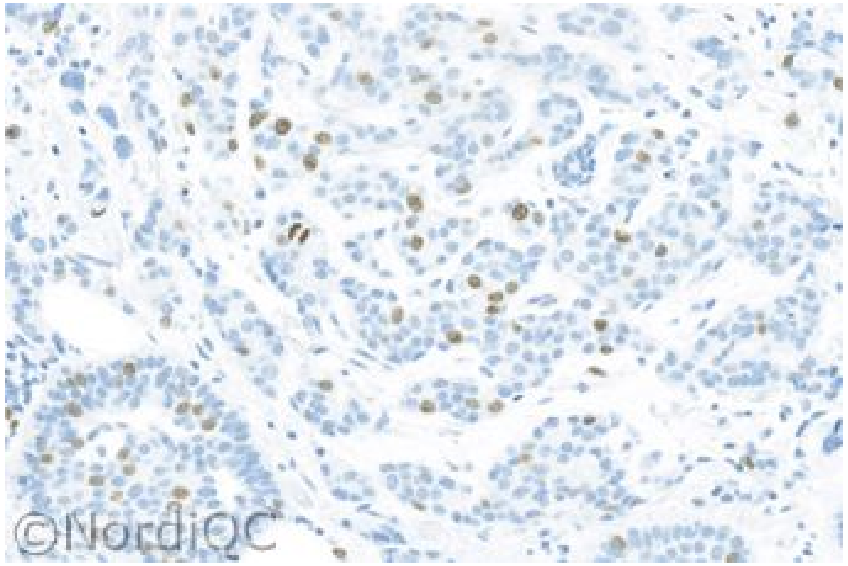
Image analysis – example 2

Image analysis in IHC - overview, considerations and applications

Ki67 clone comparison

Ki67 – why staining quality is important





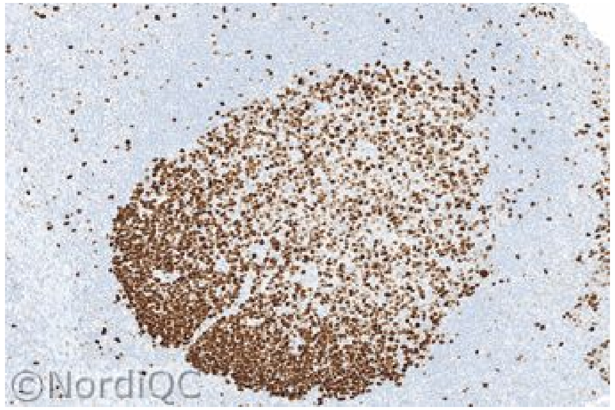
Ki67 - NordiQC

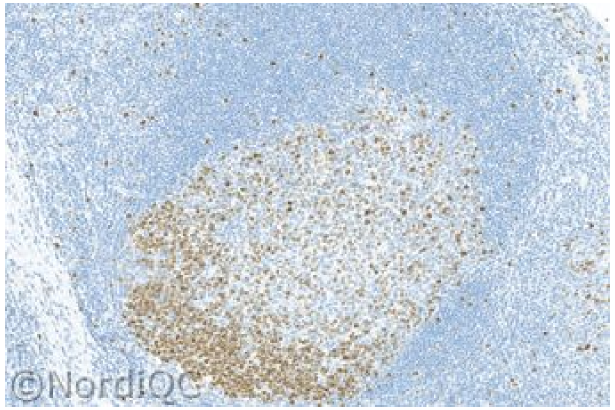
Performance in 4 NordiQC runs

	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%**





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.

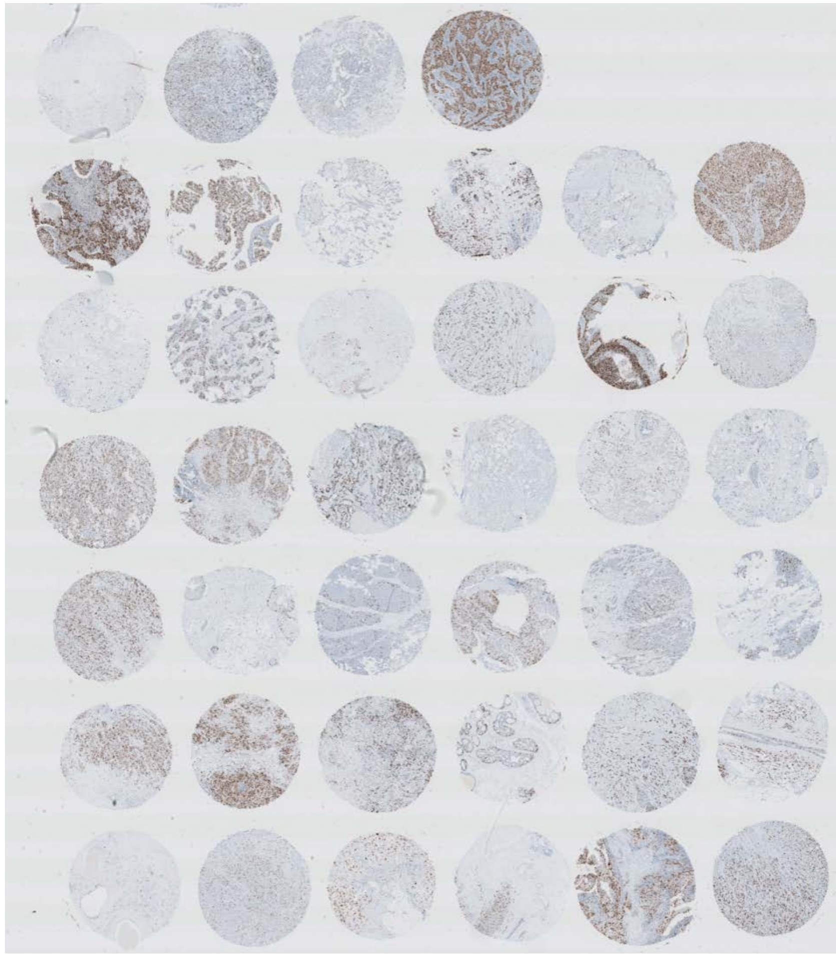
Comparative Validation of the SP6 and MIB1 Antibodies to Ki67 and Their Use in Tissue Microarray (TMA) and Image Analysis for Breast Cancer.

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



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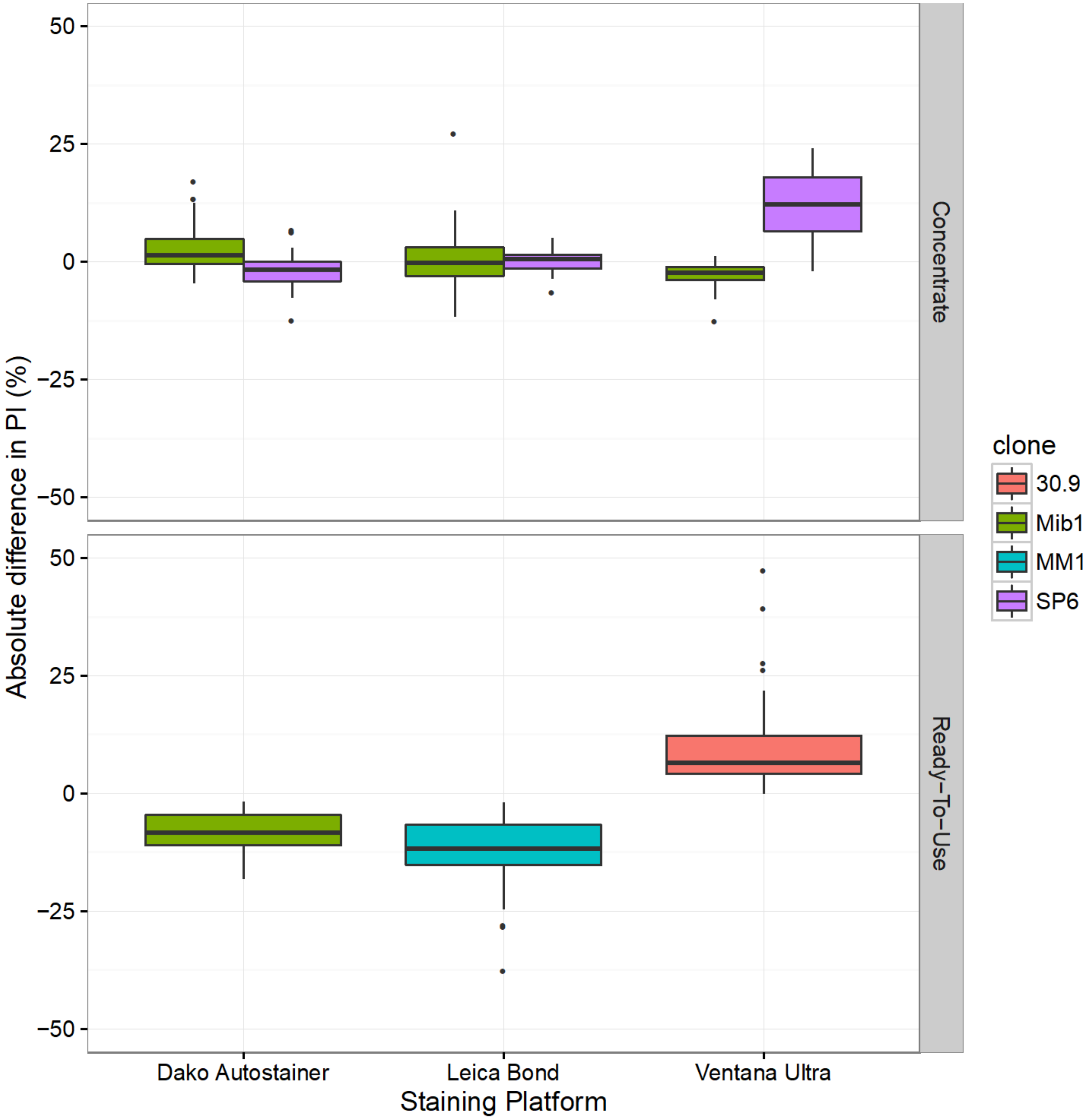
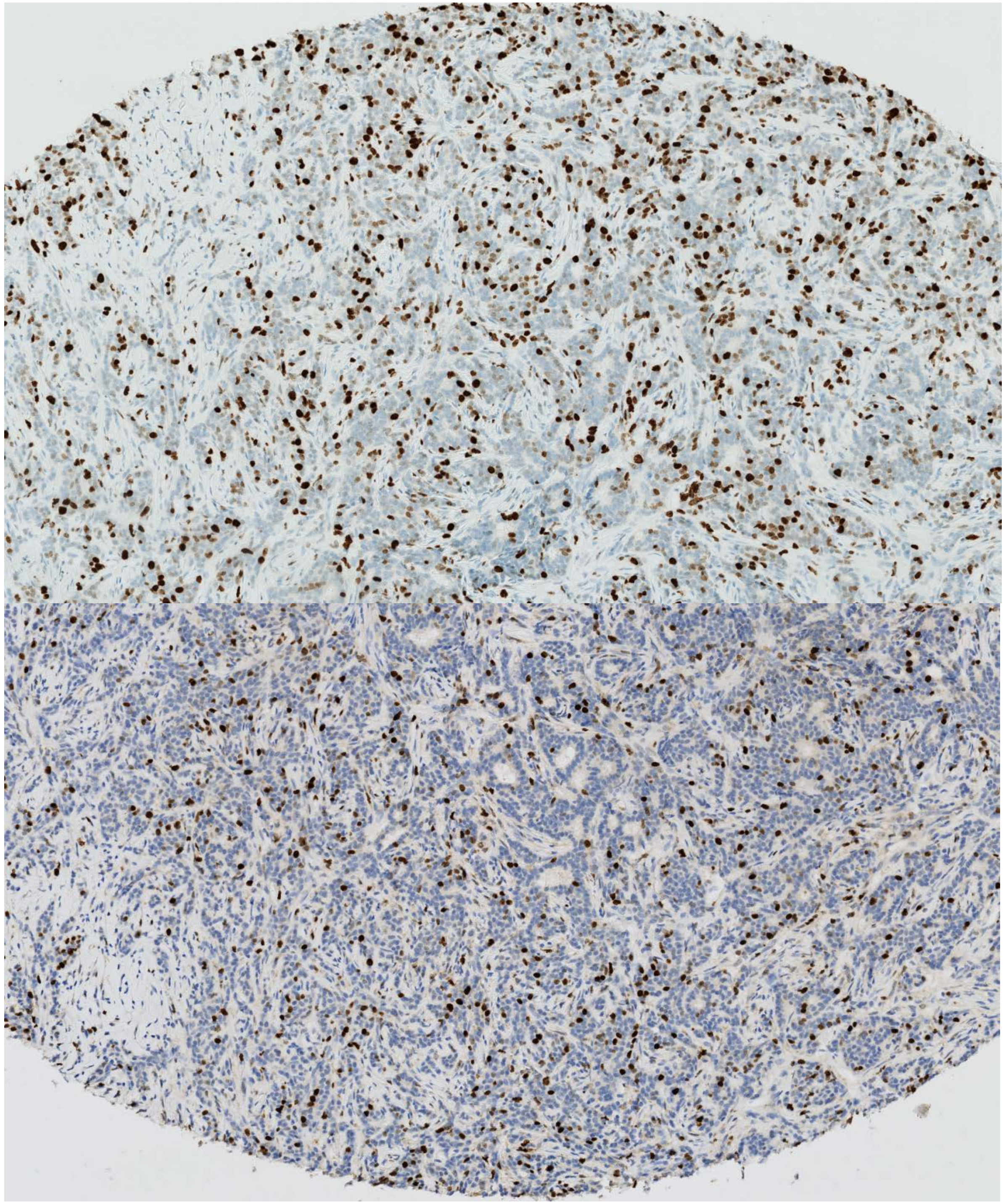
Results



50
25
0
−25
−50
50
25
0
−25
−50
Absolute difference in PI (%)
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 %

Image analysis – example 3

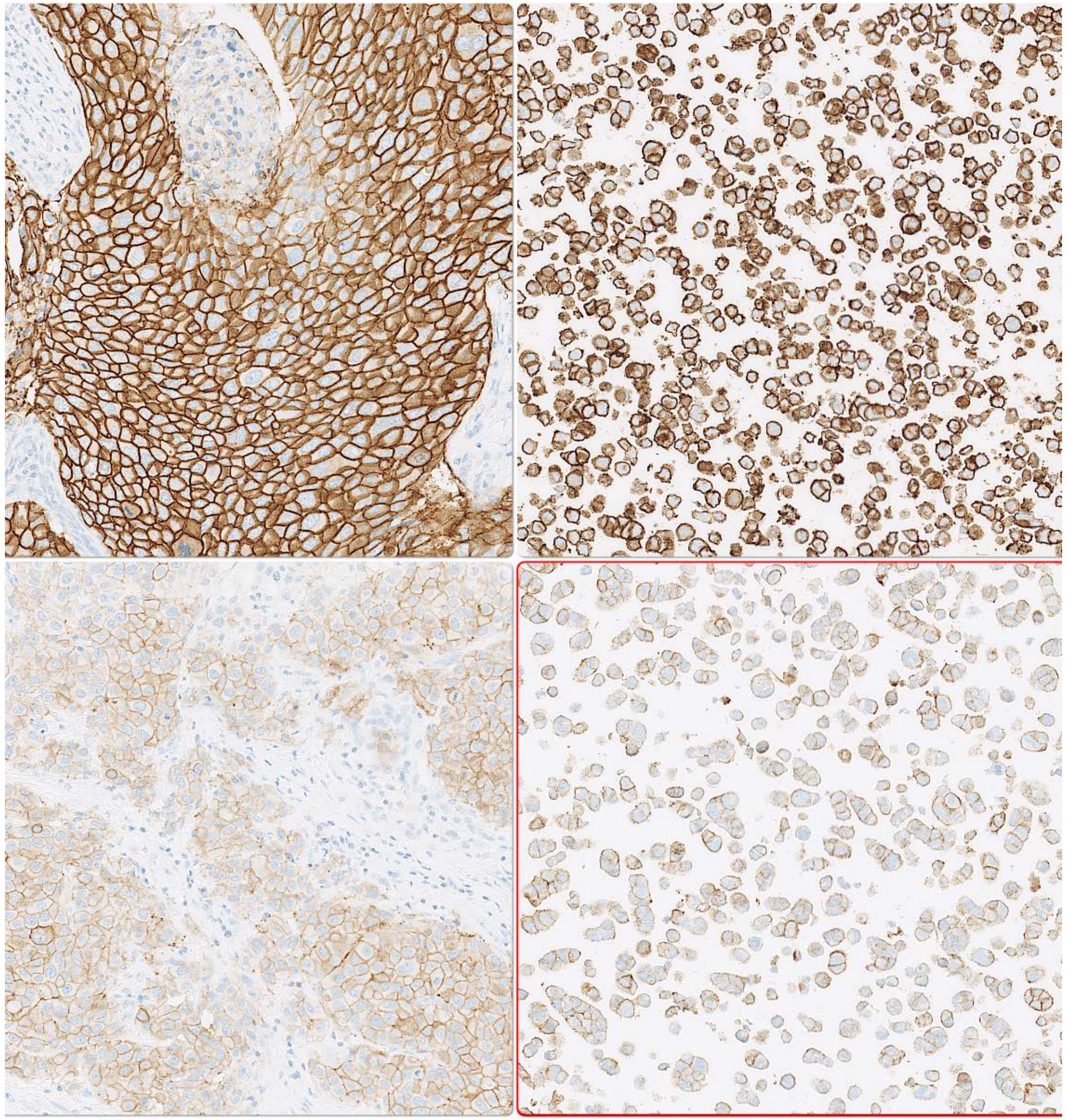
Image analysis in IHC - overview, considerations and applications

HER2 connectivity and cell lines

Control material for HER2 IHC: performace control / consistency

Histology:
3+ tumour

<u>2+ tumour</u>



Cell lines:
3+

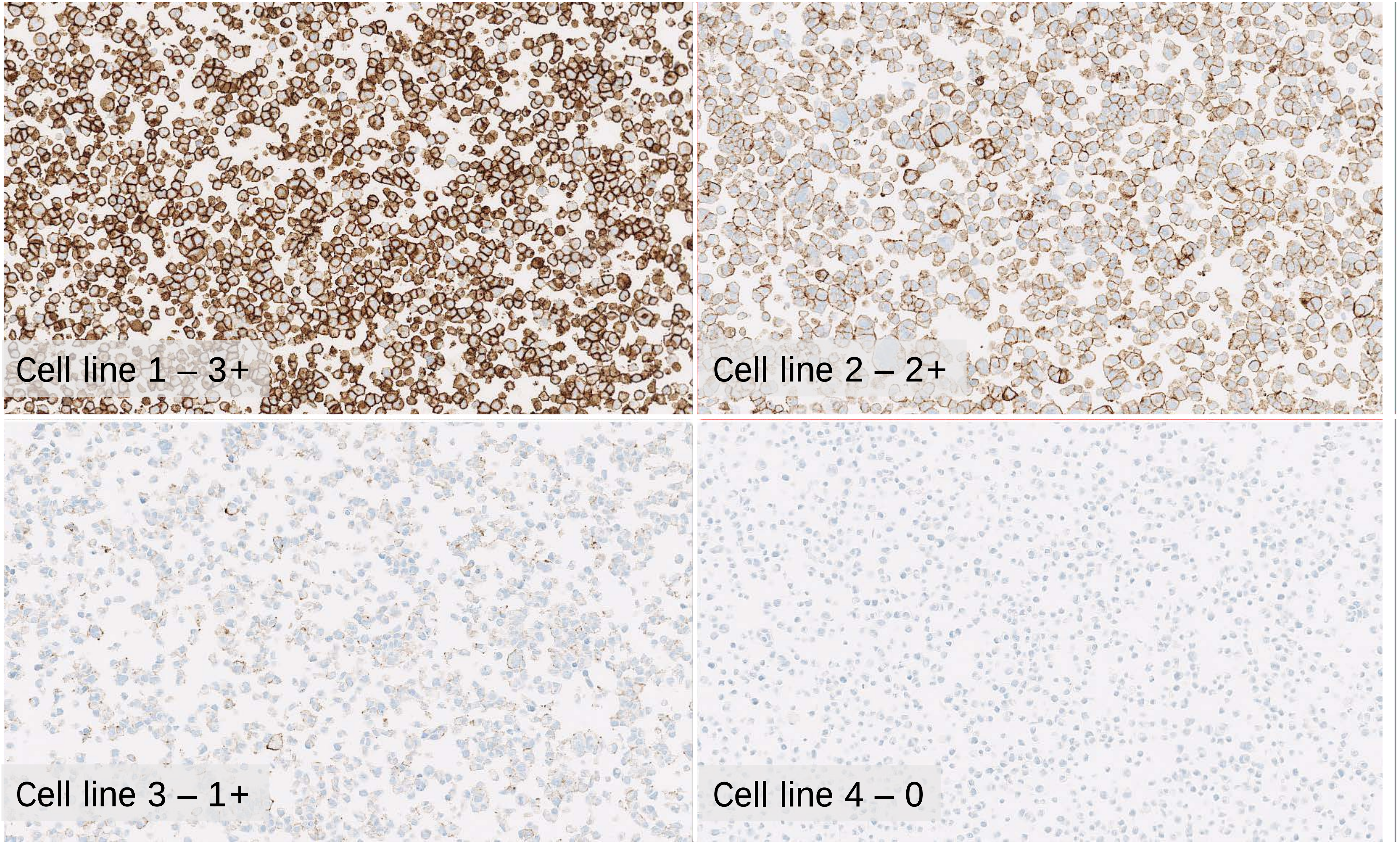
<u>2+</u>

Applicable for DIA & ref data comparing run-to-run

Courtesy of S. Nielsen

Control material for HER2 IHC: performace control / consistency

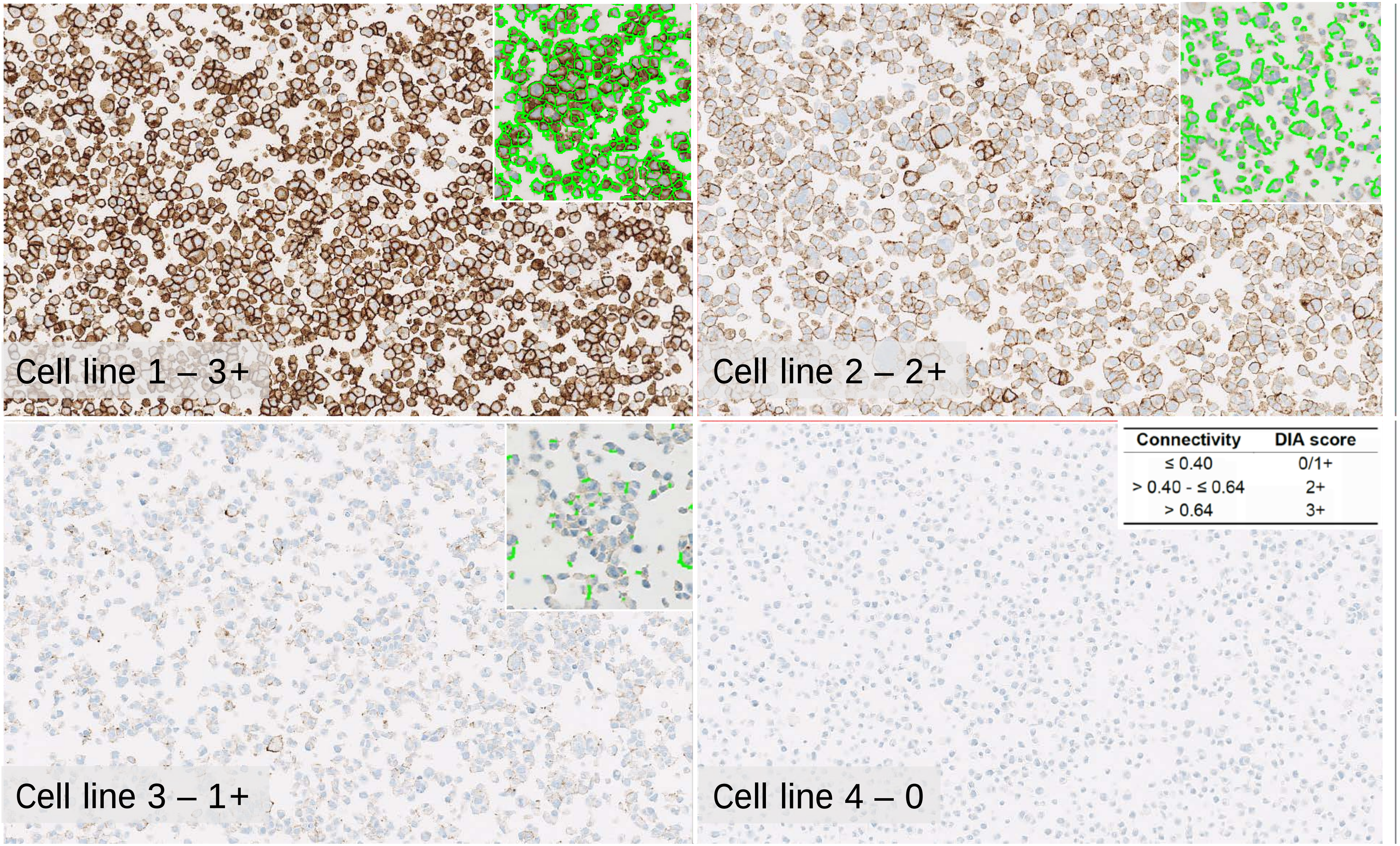
Histocyte cell lines HER2: PATHWAY IHC



Courtesy of S. Nielsen

Control material for HER2 IHC: performace control / consistency

Histocyte cell lines HER2: PATHWAY IHC



Courtesy of S. Nielsen

NordiQC

Software

Image analysis in IHC - overview, considerations and applications

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
- INCA
- Aperio (Leica)
- PathXL / Philips
- Matlab

Thank you for your attention!

Collaborators

Søren Nielsen

Rikke Riber-Hansen

Alex Skovsbo Jørgensen

Lasse Riis Østergaard

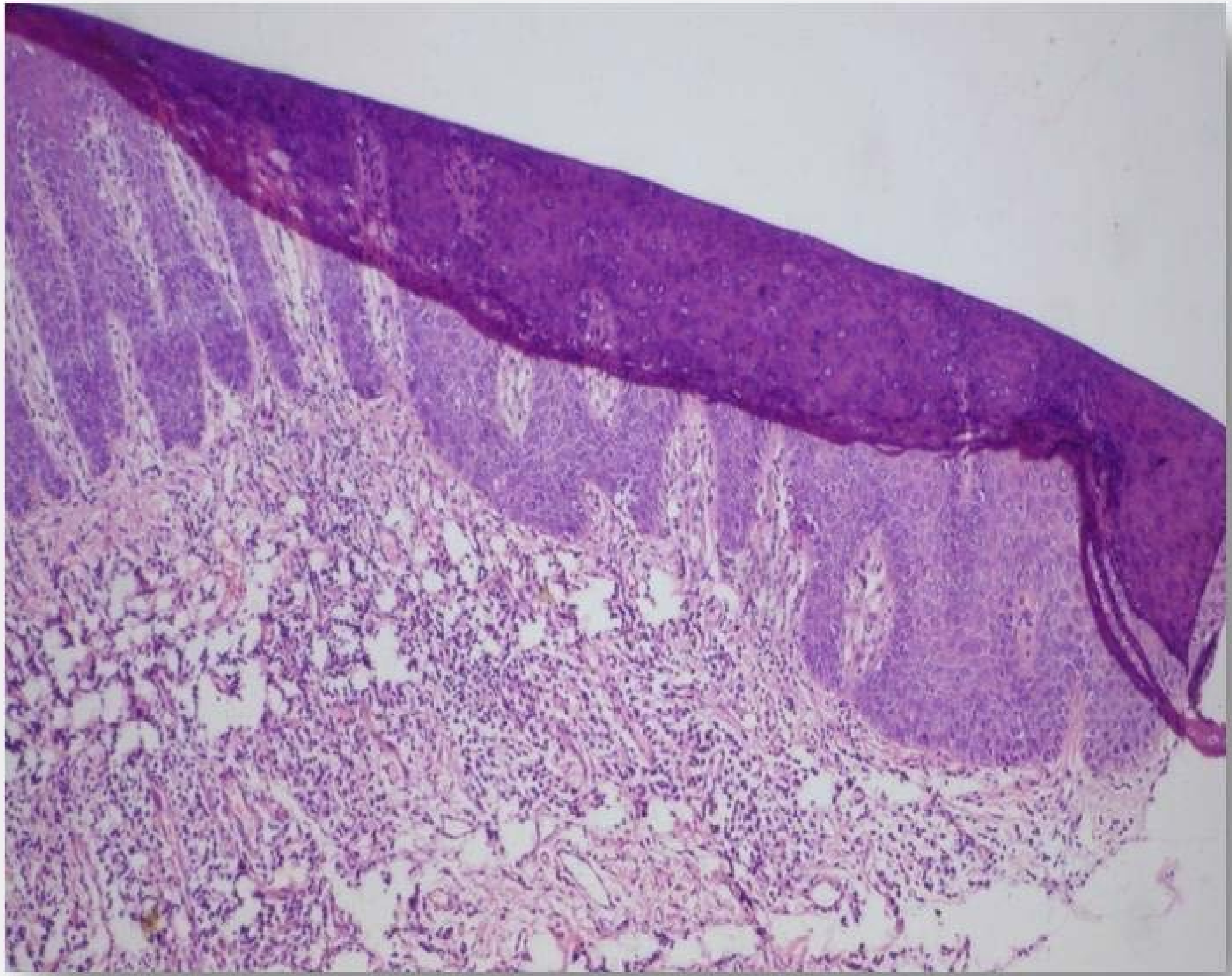
Mogens Vyberg

NordiQC

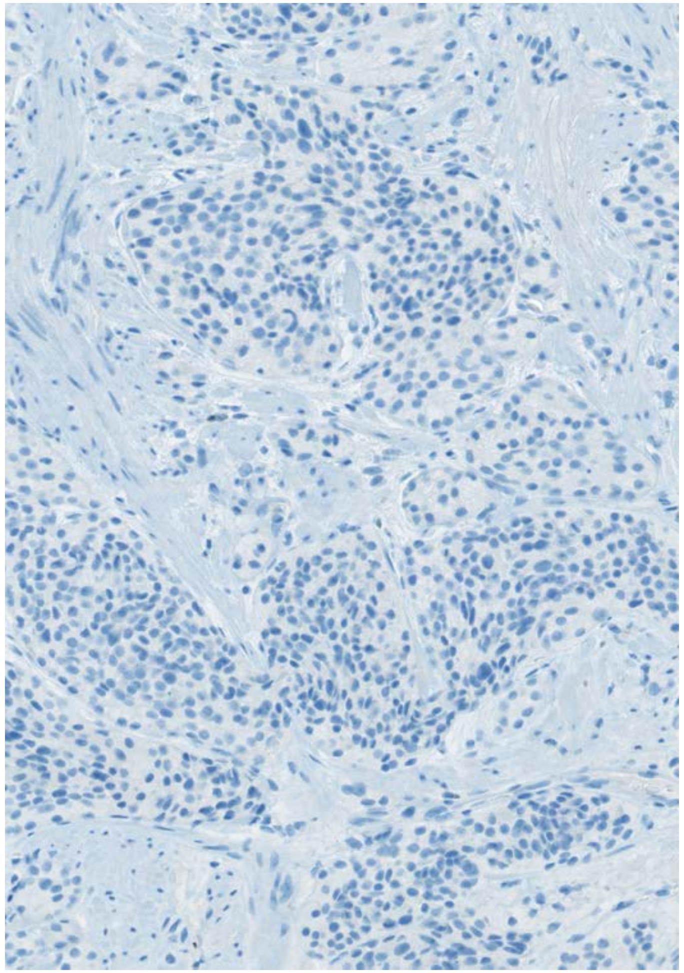
Pitfalls

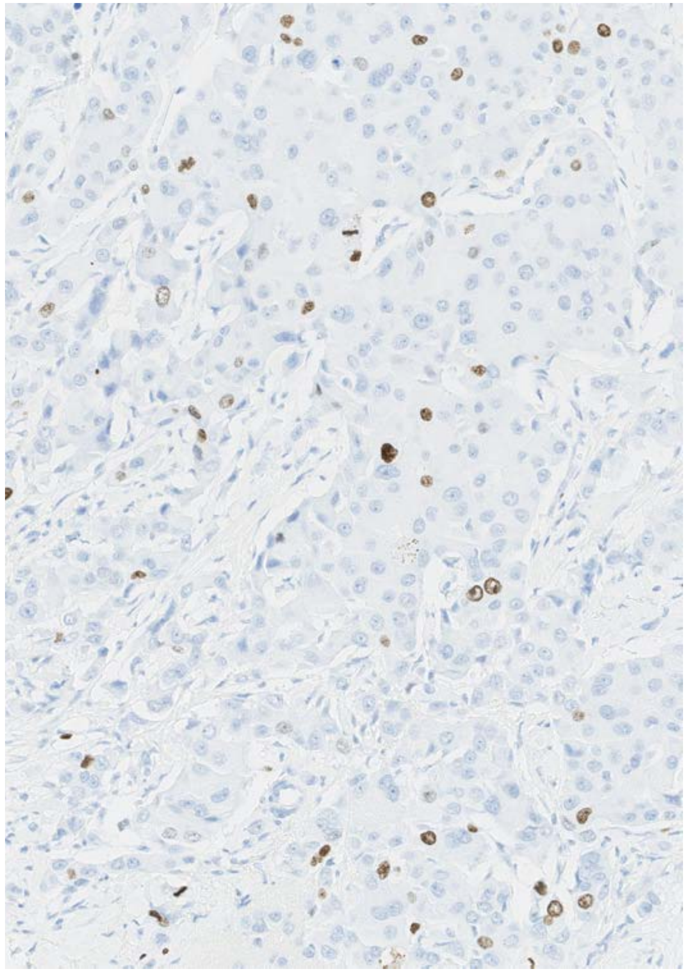
Image analysis in IHC - overview, considerations and applications

Pitfalls - artefacts



Counter staining





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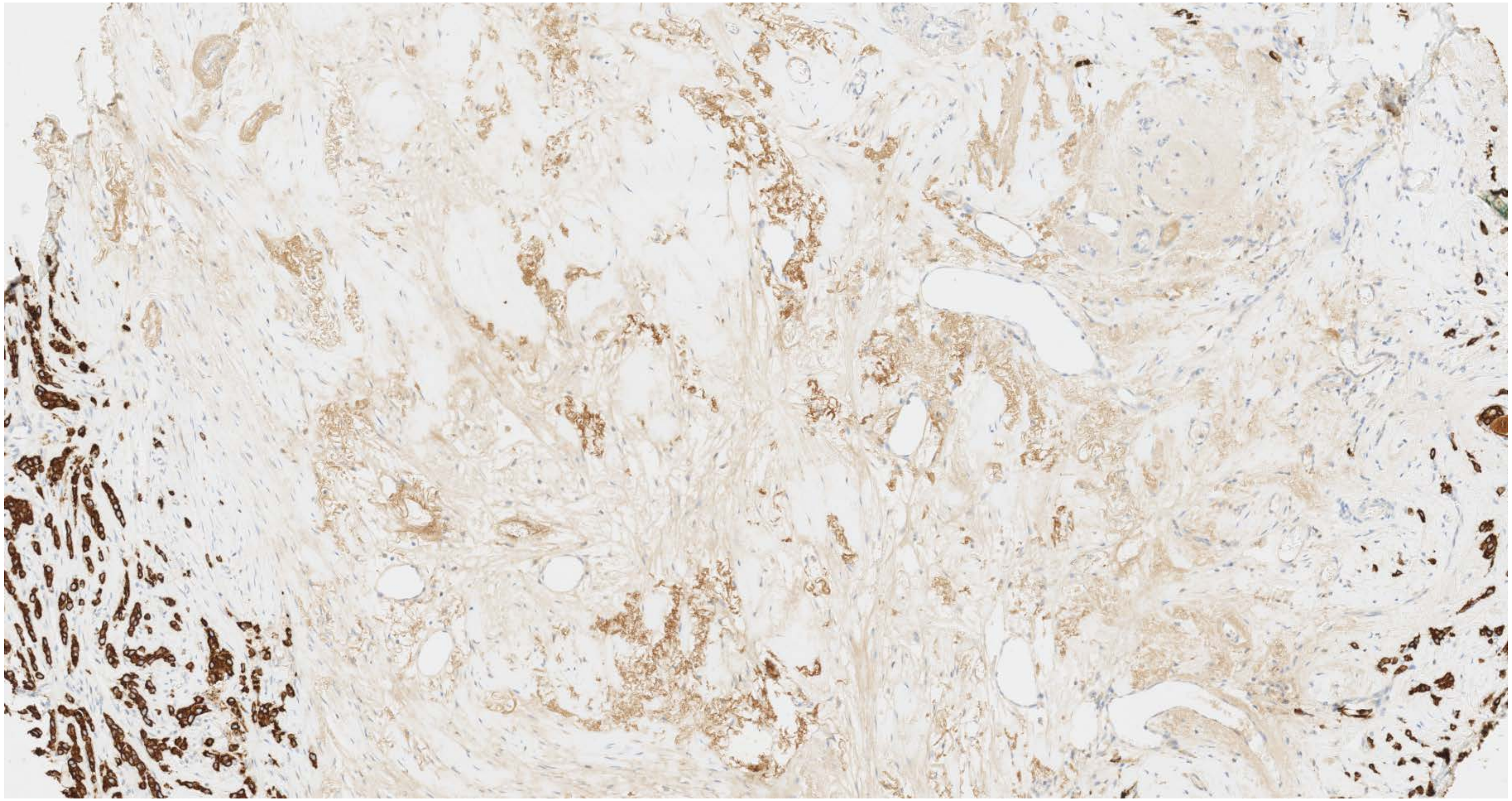
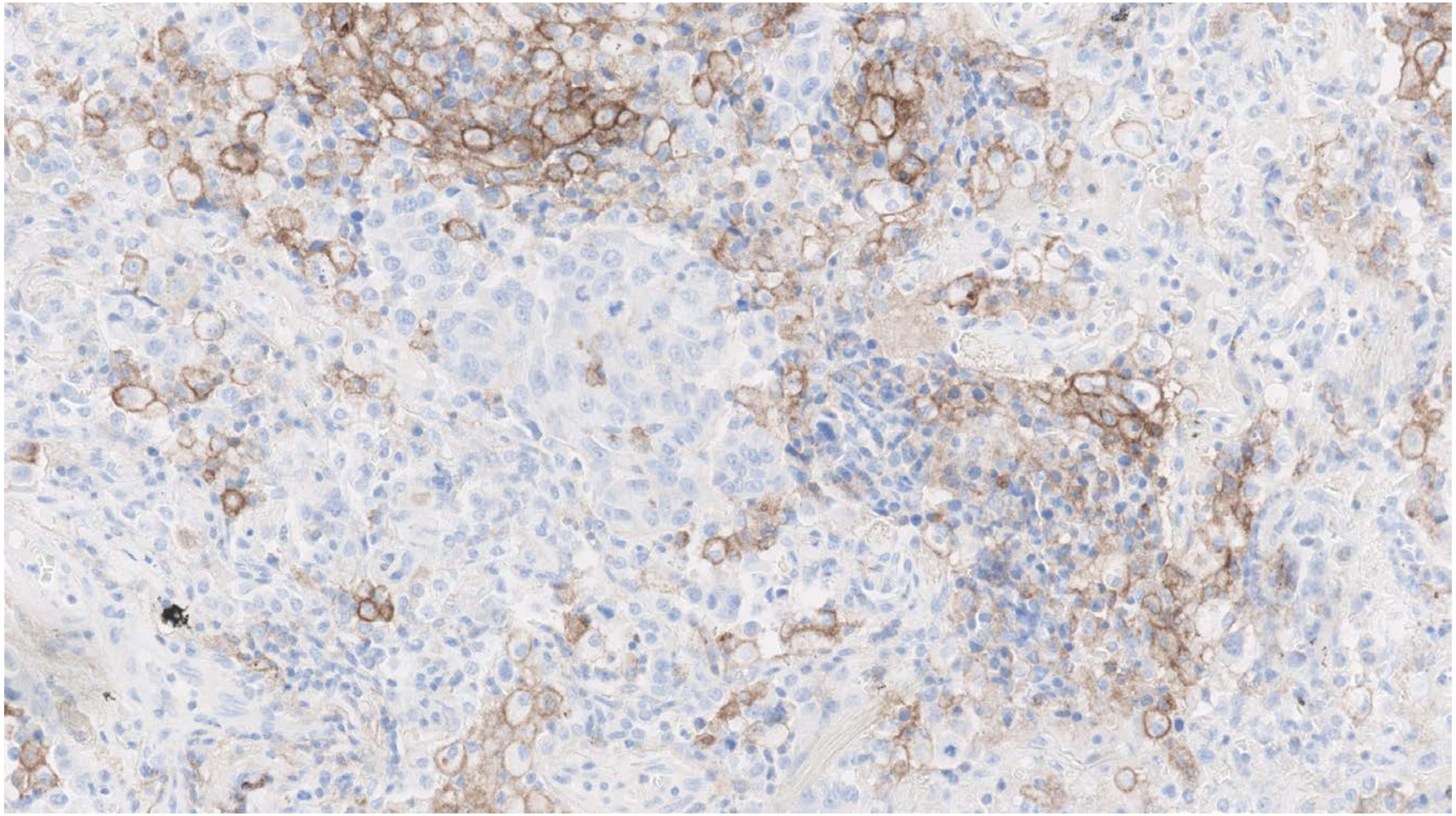
Unspecific / Background staining



Staining of other cells

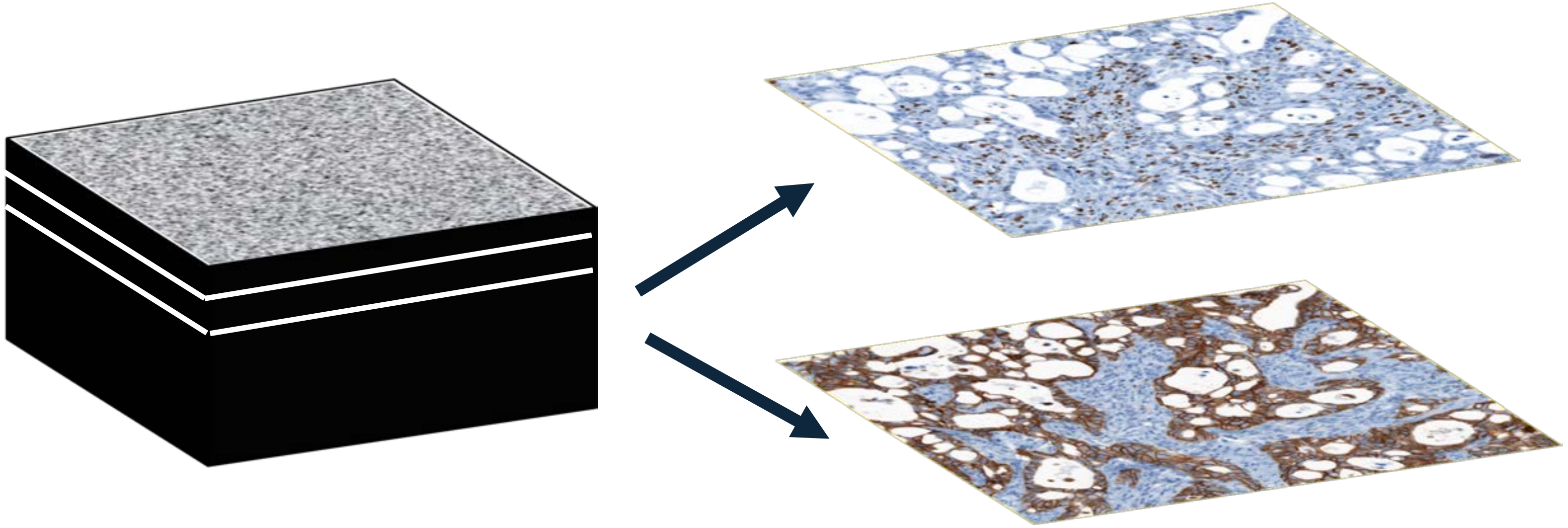


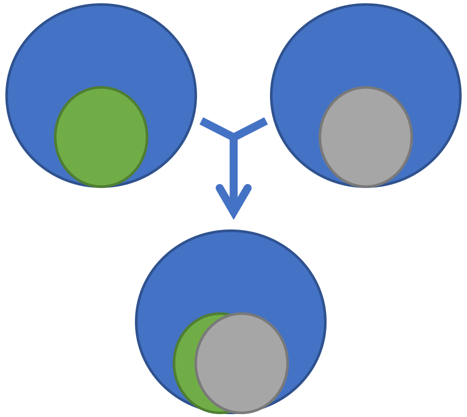
Scanning - background

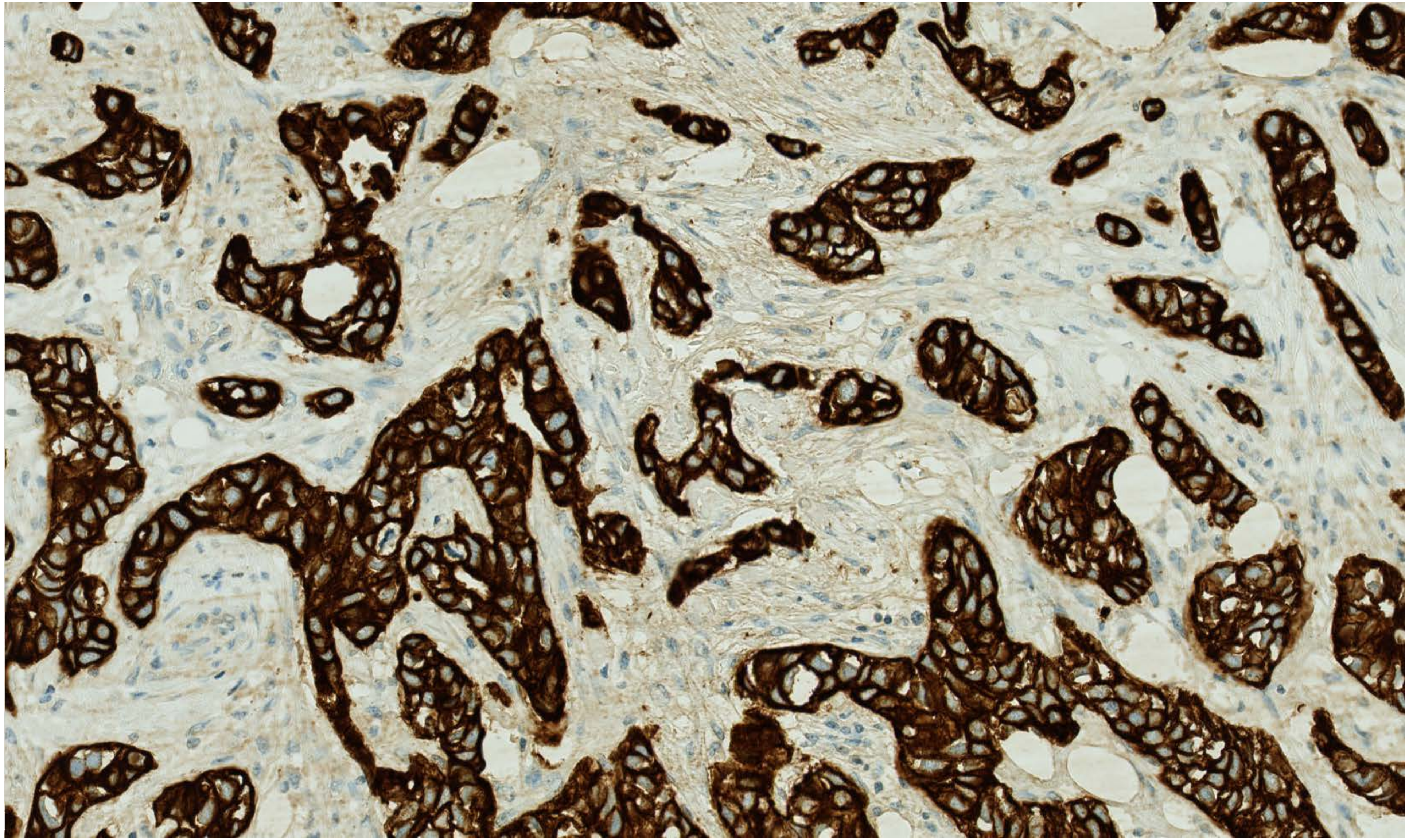
Validation of alignment

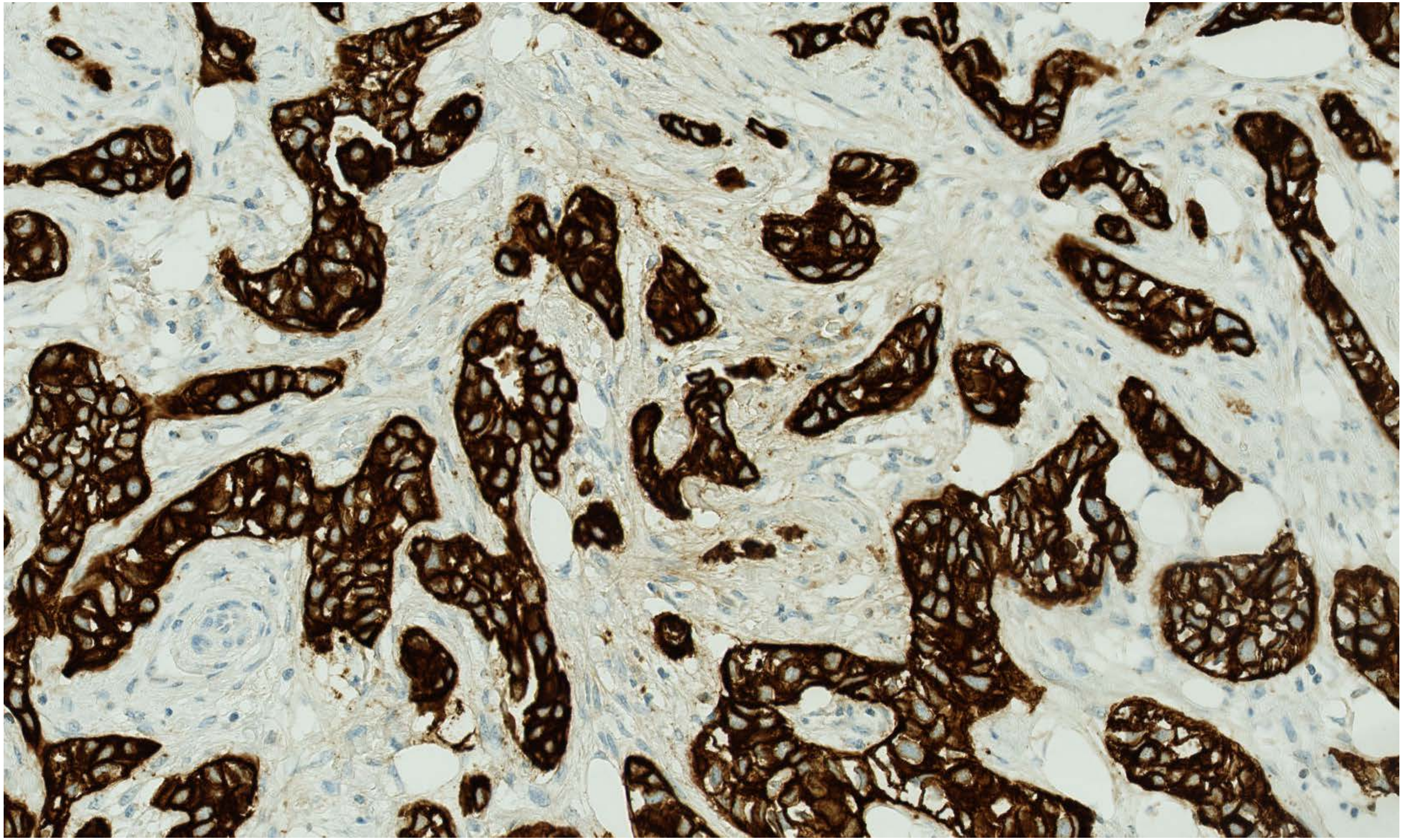
Digital Image Analysis – Ki67

Validation of alignment

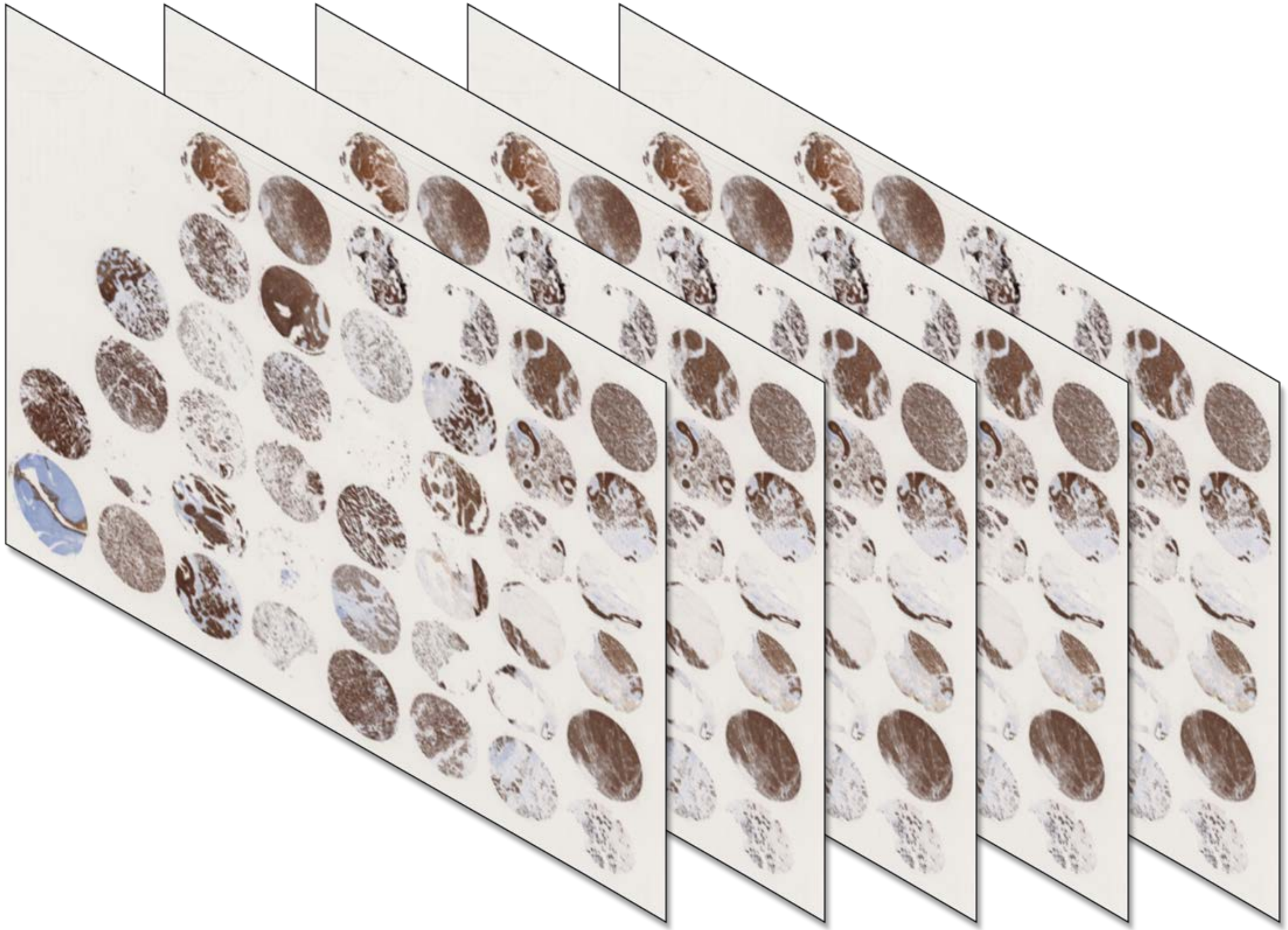








Five parallel slides of PCK



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

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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

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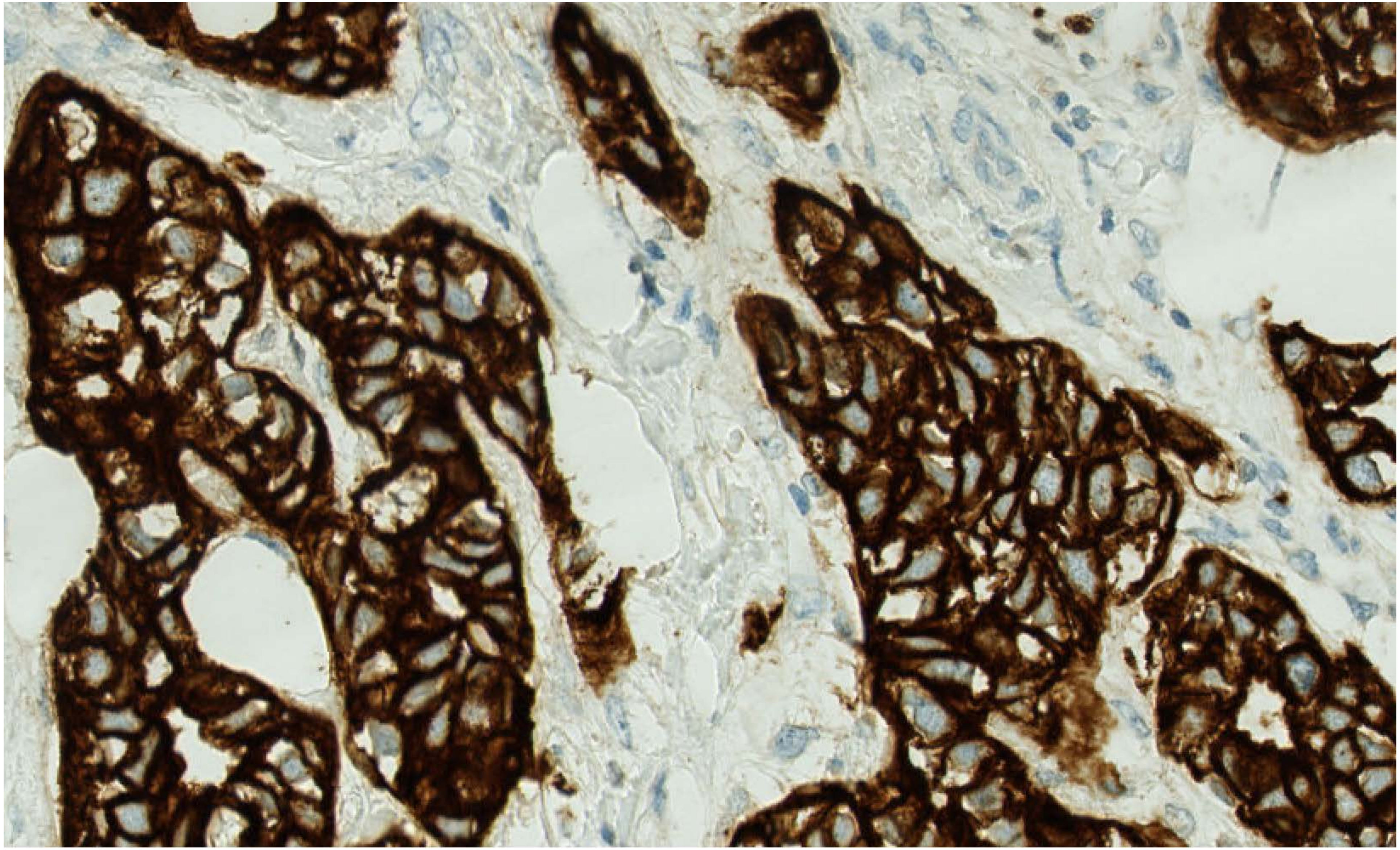
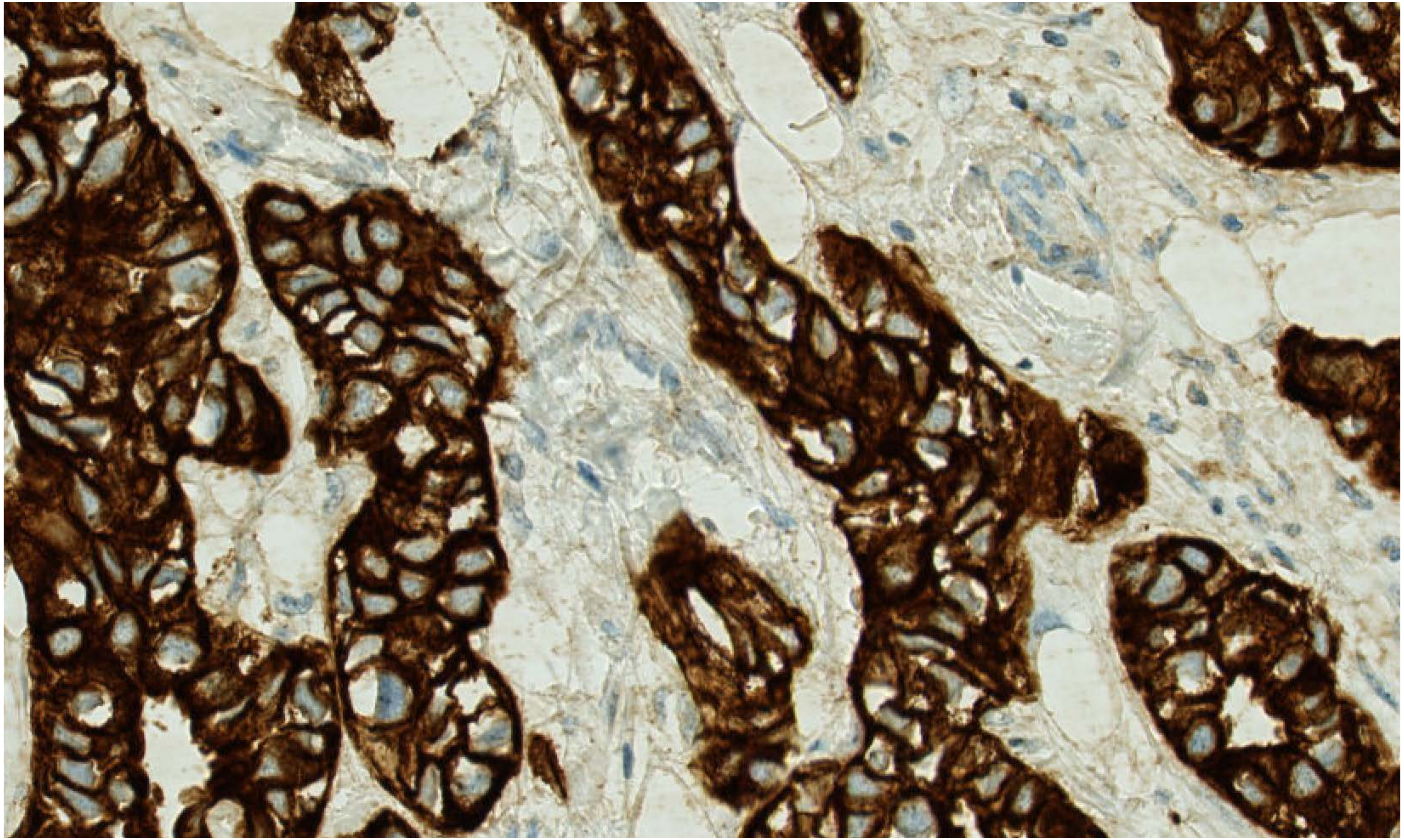
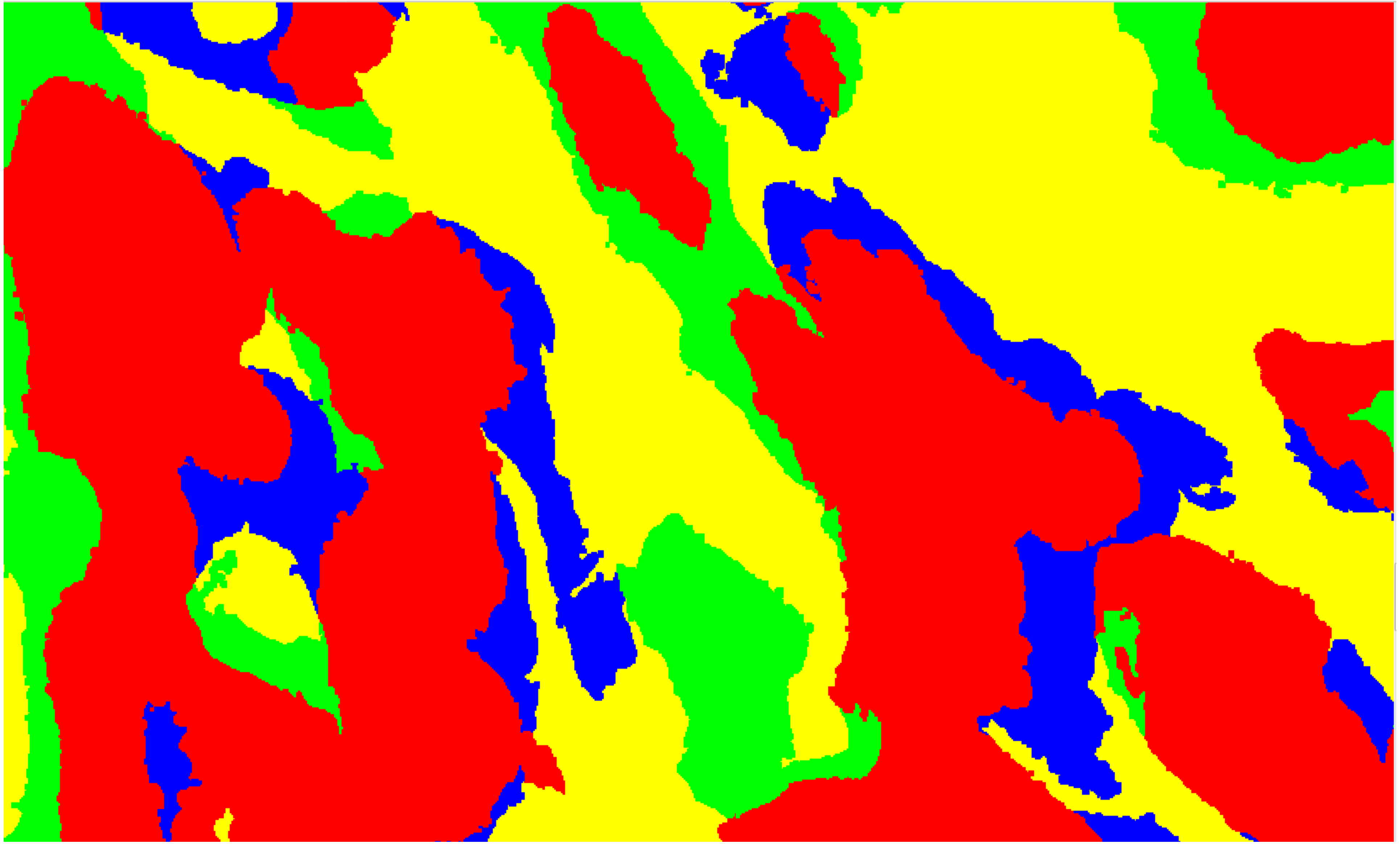






Overlap/agreement (%)

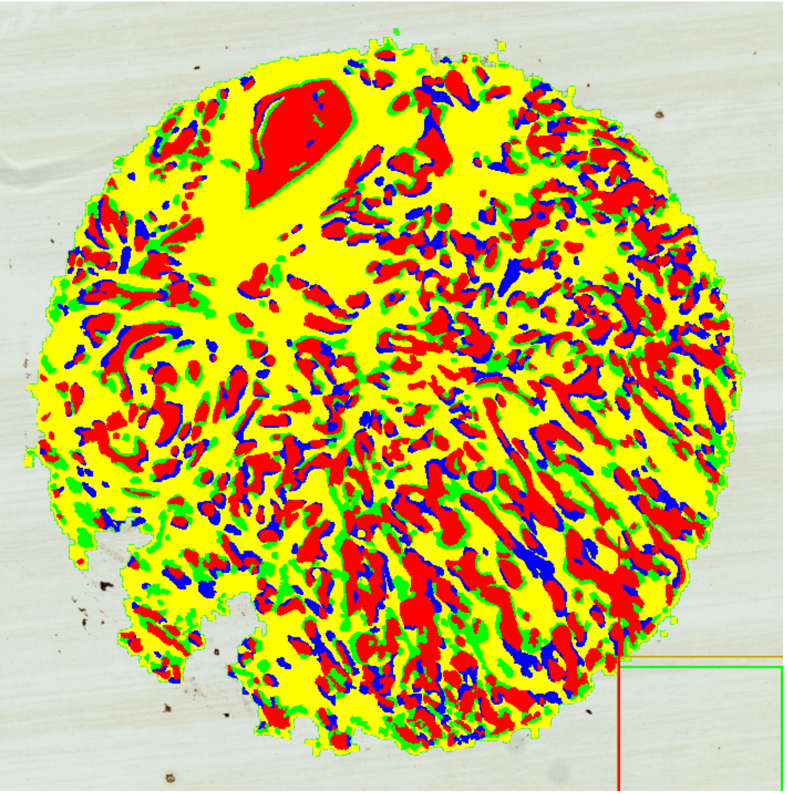
- Calculated as:

PCK positive area in both slides +

PCK negative area in both slides

Divided by total area

+ + +



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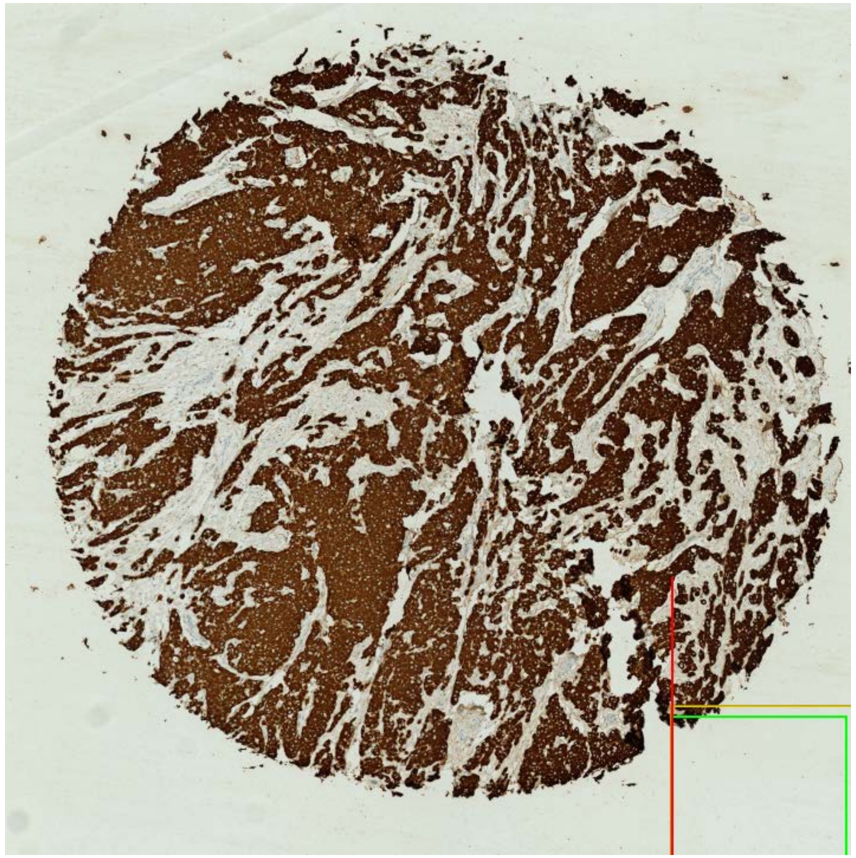
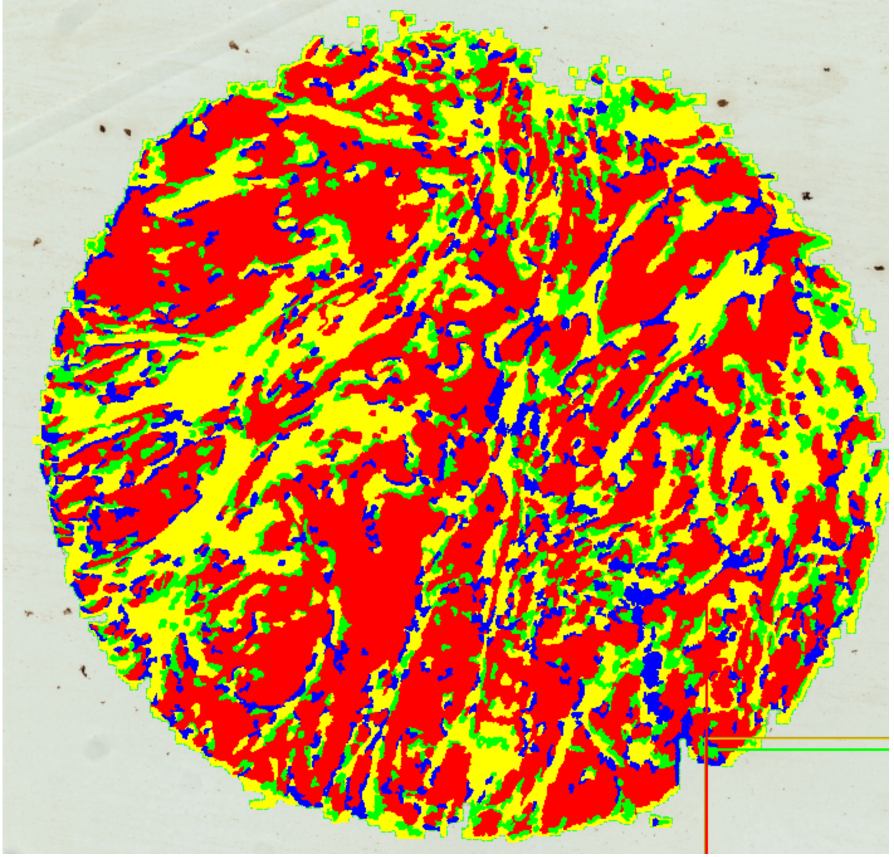
Good agreement (>90 %)





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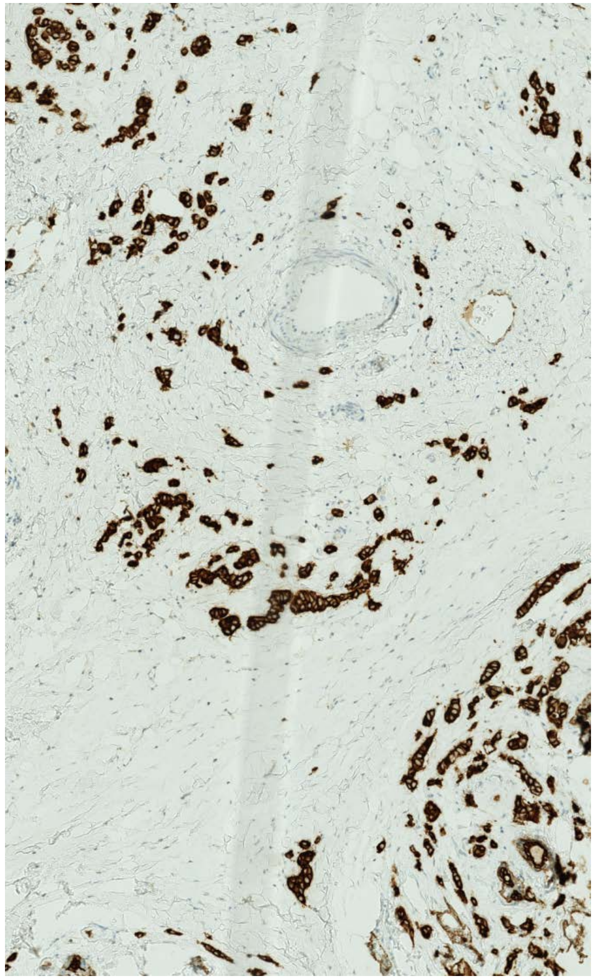
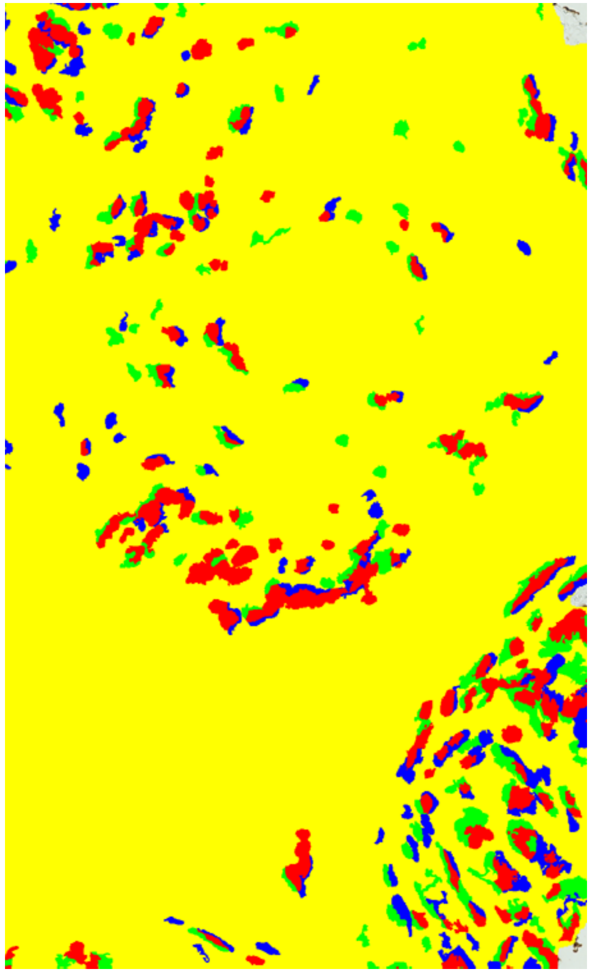
Less good agreement





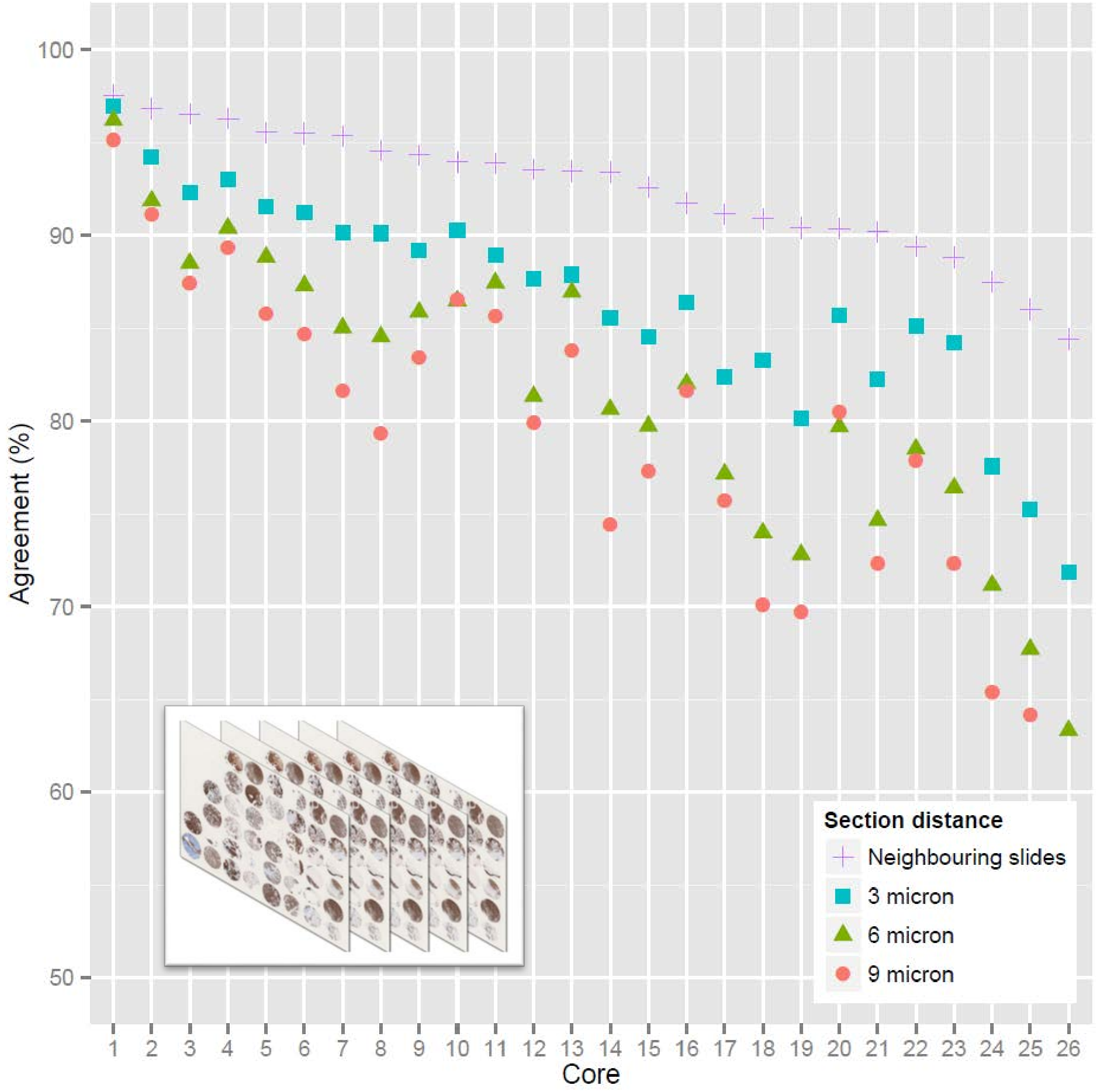

100
90
80
70
60
50
Agreement (%)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
Core
Section distance
Neighbouring slides
3 micron
6 micron
9 micron

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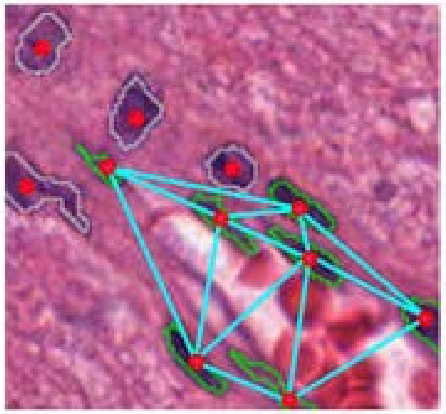
Image analysis – advanced algorithms

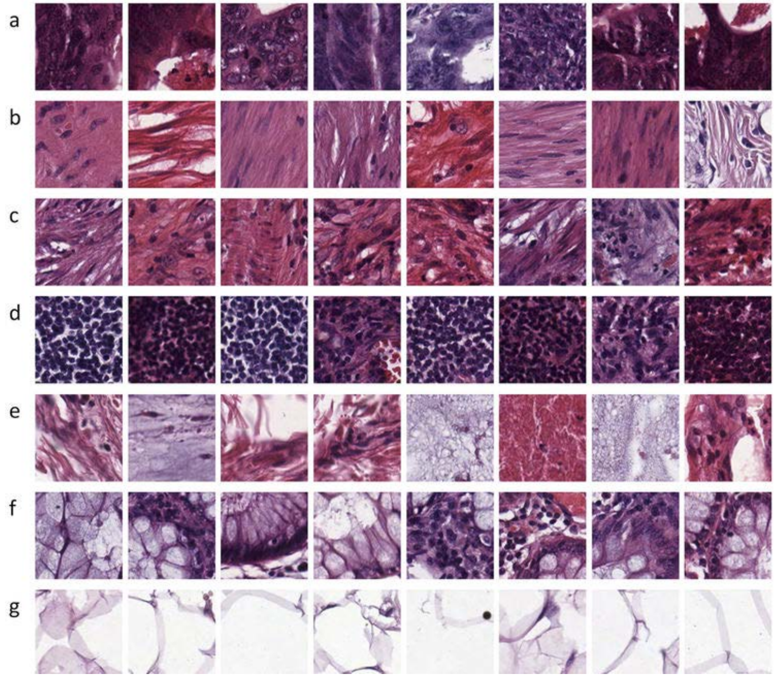
Image analysis in IHC - overview, considerations and applications

Advanced algoritms

- More complex algorithms
- Successive application of several algorithms
- Not only thresholds
- Texture-based
- Architecture-based
- Feature-based training
    - Feature may be selected statistically and unsupervised



Quantitative phenotyping



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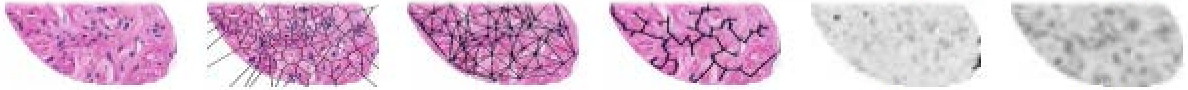
Advanced algorithms – architectural and texture

AUTOMATED GRADING OF PROSTATE CANCER USING ARCHITECTURAL AND TEXTURAL IMAGE FEATURES

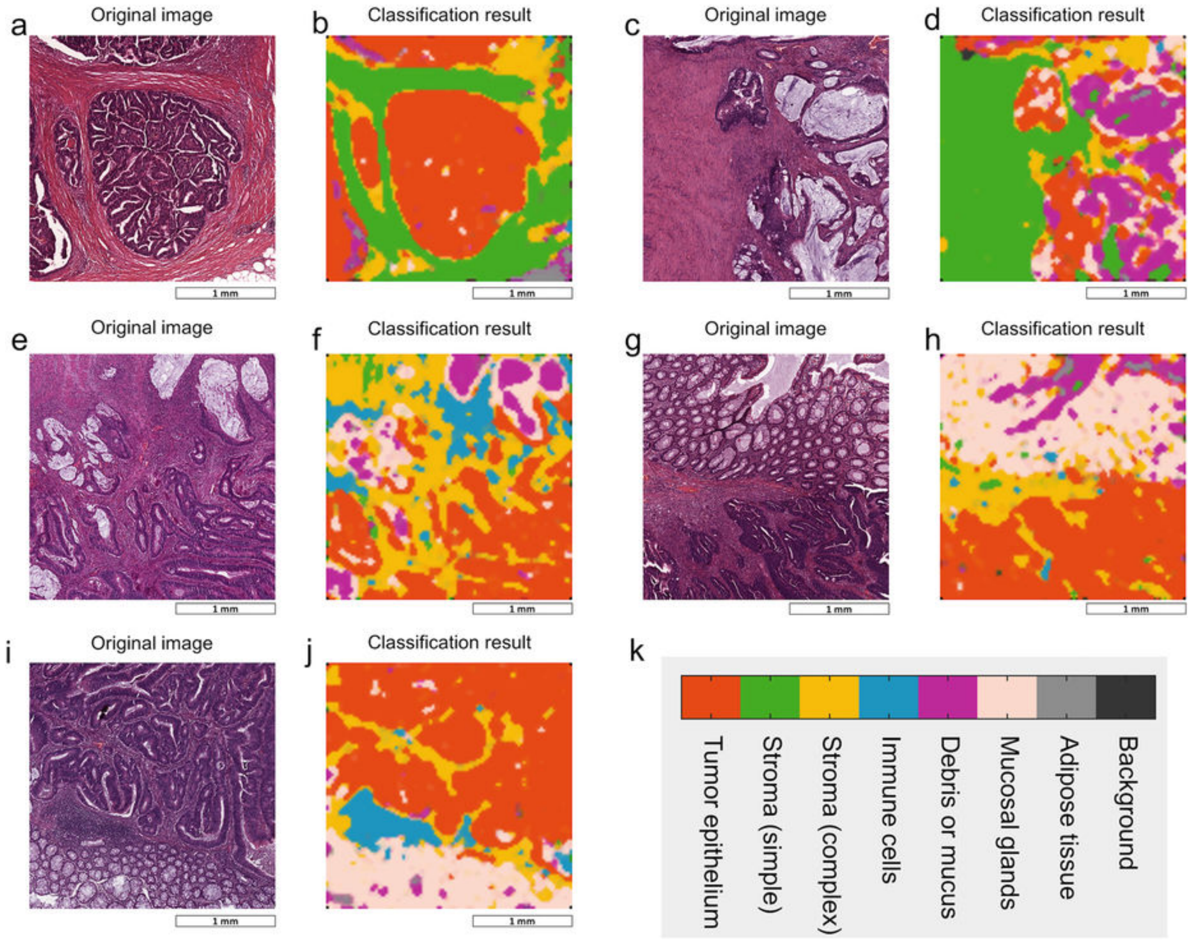
of four different tissue classes: benign epithelium, benign stroma, Gleason grade 3 adenocarcinoma, and Gleason grade 4 adenocarcinoma. The SVM classifier was able to distinguish between all four types of tissue patterns, achieving an accuracy of 92.8% when distinguishing between Gleason grade 3 and 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-

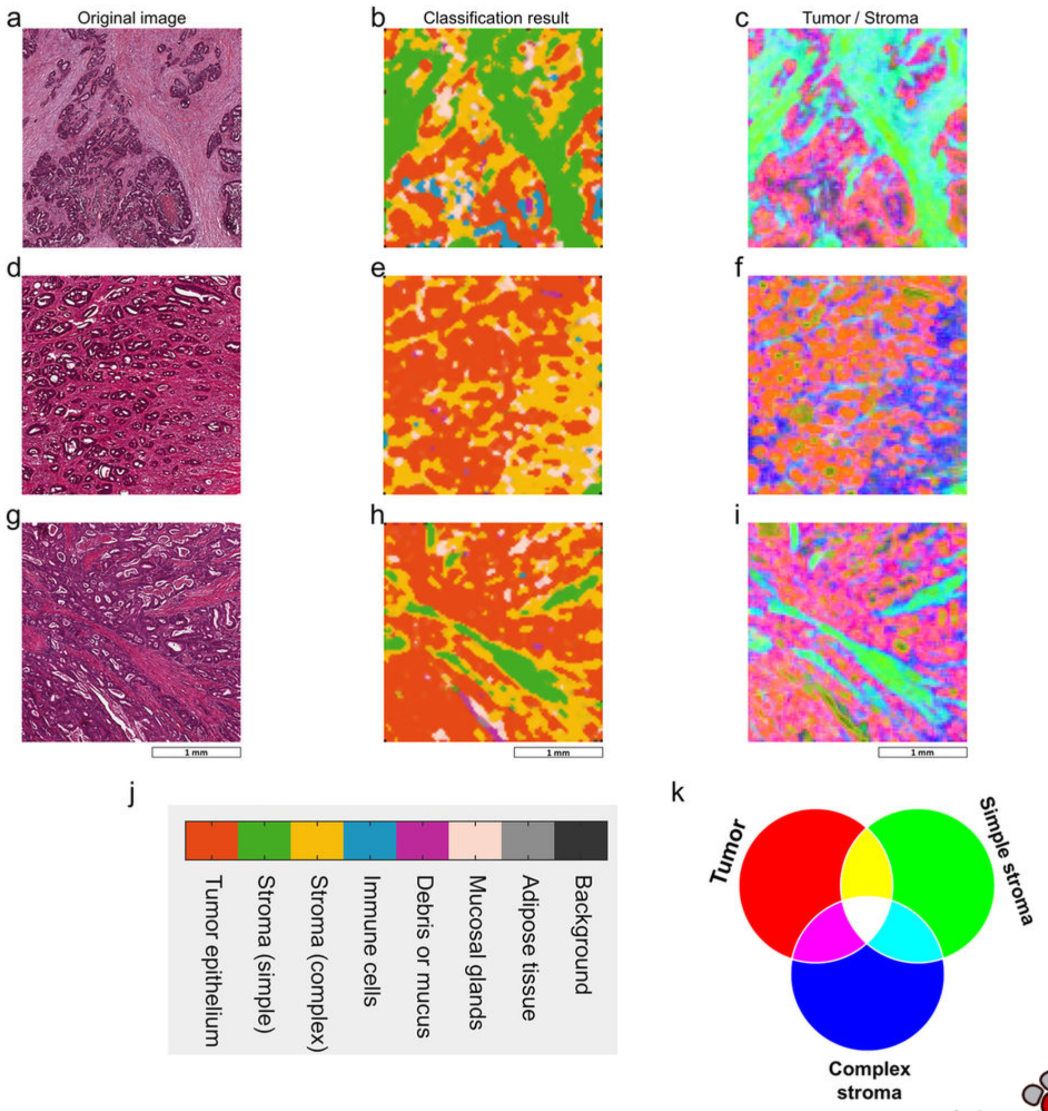


(s) (t) (u) (v) (w) (x)

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 ($s = 3$, $\theta = \frac{5\pi}{8}$).

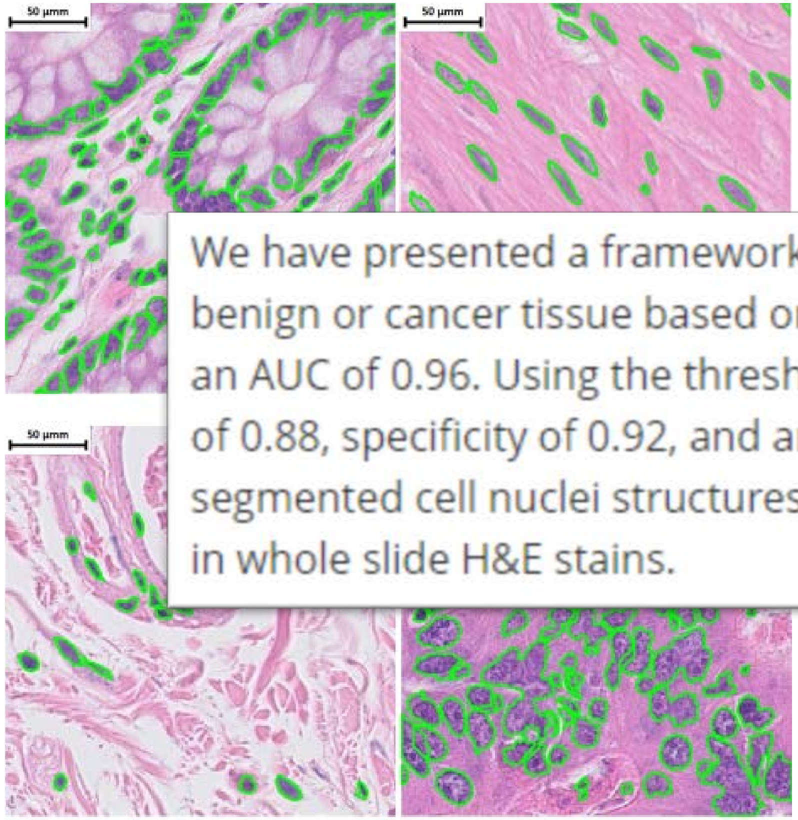
Advanced algoritms - texture





Kather, J. N., Weis, C. A., Bianconi, F., Melchers, S. M., Schad, L. R., Gaiser, T., ... & Zöllner, F. G. (2016). Multi-class texture analysis in colorectal cancer histology. *Scientific reports*, *6*, 27988.

Advanced algorithms – cell nuclei texture



C D

Table 1. The nine most important features selected for the ROI classifier

Feature number	Feature name	Feature image	Variable (Gini) importance
1	Maximum intensity	Saturation	16.27
7	Mean long rum emphasis	Blue	6.85
8	Maximum intensity	Hue	6.25
9	Long run low gray level emphasis	Blue	5.82

We have presented a framework for classification of ROI's within whole slide H&E stains as containing benign or cancer tissue based on features extracted from segmented cell nuclei structures obtaining an AUC of 0.96. Using the threshold maximizing sensitivity and specificity simultaneously, a sensitivity of 0.88, specificity of 0.92, and an accuracy of 0.91 was obtained. The results indicate that features from segmented cell nuclei structures can be used to discriminate between benign or cancerous colon tissue in whole slide H&E stains.

Jørgensen, A. S., Rasmussen, A. M., Andersen, N. K. M., Andersen, S. K., Emborg, J., Røge, R., & Østergaard, L. R. (2017). Using cell nuclei features to detect colon cancer tissue in hematoxylin and eosin stained slides. *Cytometry Part A*, *91*(8), 785-79

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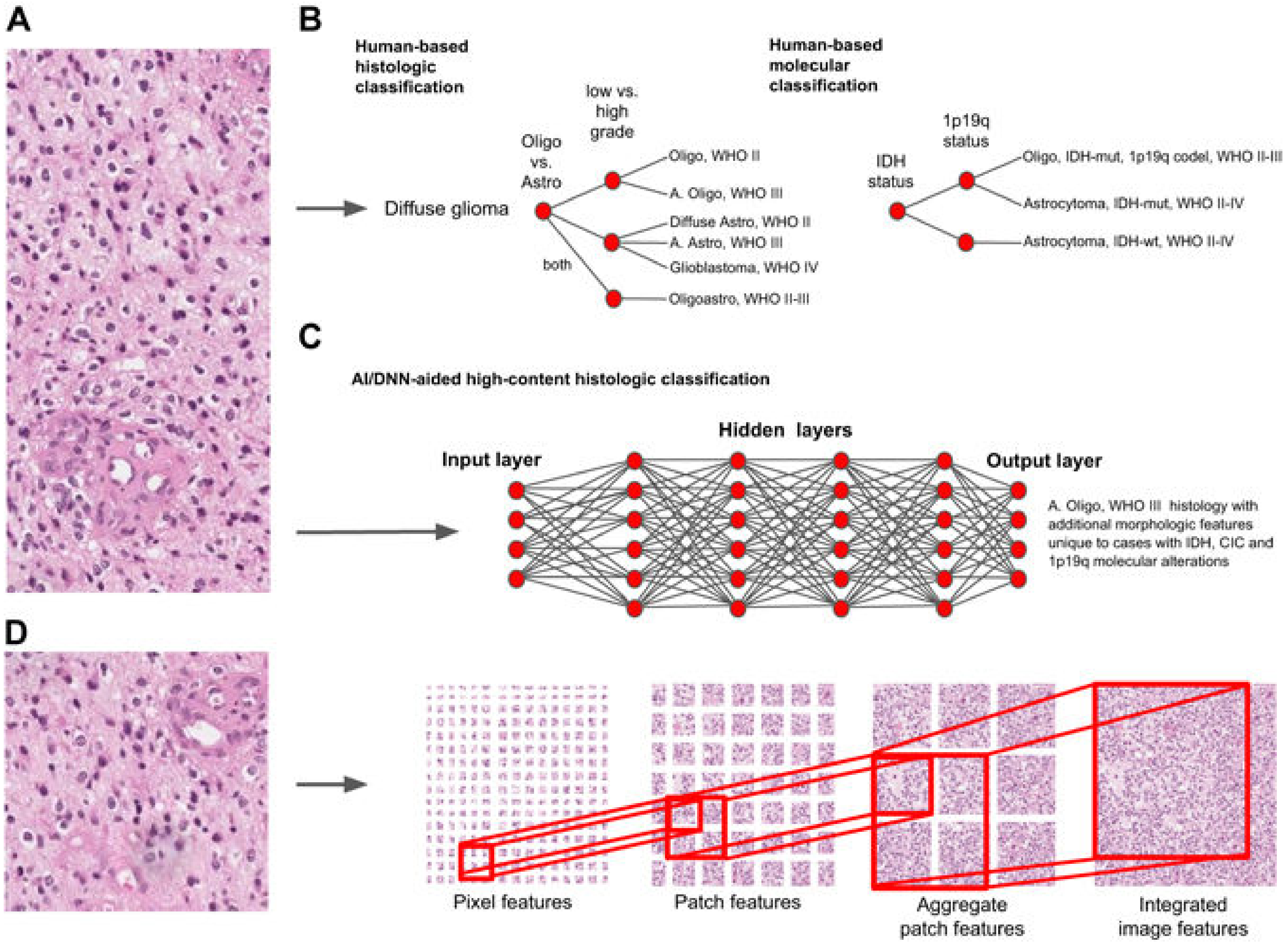
AI



Djuric, Ugljesa, et al. "Precision histology: how deep learning is poised to revitalize histomorphology for personalized cancer care." npj Precision Oncology 1.1 (2017): 22.