Quantitative image analysis in breast cancer
Virtual Double Staining of Ki67

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2nd NordiQC Conference on Applied Immunohistochemistry
Disclosures: none
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 – 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

<table>
<thead>
<tr>
<th></th>
<th>2001</th>
<th>2007</th>
<th>2009</th>
<th>2012</th>
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</thead>
<tbody>
<tr>
<td>Participants</td>
<td>42</td>
<td>100</td>
<td>124</td>
<td>229</td>
</tr>
<tr>
<td>Sufficient</td>
<td>71%</td>
<td>73%</td>
<td>77%</td>
<td>89%</td>
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## Performance marks in Run B13 (2012)

<table>
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<th>Good</th>
<th>Borderline</th>
<th>Poor</th>
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<tbody>
<tr>
<td>Total</td>
<td>166</td>
<td>39</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>Proportion</td>
<td>72%</td>
<td>17%</td>
<td>8%</td>
<td>3%</td>
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</table>
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

Bar chart showing counts for different methods:
- Eyeball estimate
- Image analysis method
- Manual count
- Overall average of all tumour cells
- Tumour hot spots area examined
- Other
Influence of experience
Influence of method

[Box plot showing the distribution of results for different methods (Eyeball estimate vs. Manual count) across various cores.]
Digital Image Analysis

Criteria

• Identify nuclei
• Distinguish Ki67 positive and negative nuclei
• Exclude non-tumour cells from analysis
Virtual Double Staining (VDS)
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
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 Digitital 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
Bland-Altman

Difference in PI (VDS - Manual) (%)

Average PI by VDS and Manual Counting
Systematic Random Sampling

- Manually counted Proliferation Indices (%) were counted in areas selected by Systematic Random Sampling

- Therefore, results can be used as an estimate of the whole core
VDS on Whole Core

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

![Bar chart showing controls among NordiQC-participants. The categories are Breast cancer, Lymphoid tissue (Control tissue), and No control. The chart indicates a higher frequency of control tissue compared to other categories.]
Ki67 in lymphoid tissue

Figure 4.1: Tonsil control 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
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.
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 Hartman2,6 & Märten Fernö2

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. Zabaglo1, L. Zabaglo2, J. Salter1, J. Salter2, H. Anderson1, H. Anderson2, M. Hills1, R. A’Hern2, M. Dowsett1, and M. Dowsett2

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
Preliminary results

The graph shows the relative proliferation index for different staining platforms (Dako, Leica, Ventana) and clones (30.9, Mib1, MM1, SP6) for both concentrate and ready-to-use solutions.
SP6 concentrate, Ventana platform

Proliferation Index: 38%

MM1 RTU, Leica platform

Proliferation Index: 12%
DISCUSSION & FUTURE PERSPECTIVES
Discussion

• Still experimental, algorithm 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
Which cells were considered positive?

Moderately stained

Weakly stained
Digital Image Analysis – Ki67

IMAGE ANALYSIS IN NORDIQC ASSESSMENTS
Image analysis in EQA?
Image analysis in EQA?
Pilot experiment

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

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• 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
**H-score:**
1 x (% Weakly) +
2 x (% Moderate) +
3 x (% Strong)
Strongly stained nuclei
Discussion

• Still experimental, algorithm not yet optimized for variance in staining protocols/platforms

• Challenged when nuclei overlap or cell borders are blurry
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