What kind of material should we use for ICC in our daily routine

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Diversity of preparing cytological material

• Cell block
• Direct smears
  – air dried
  – In CO2 ice
  – wet fixed
• Liquid material (effusions, urine, spinal fluid)
• Liquid based preparations
  • Cytospin
  • ThinPrep/SurePath
Cell block

- Main disadvantage availability of enough material
- Main advantages
  - very similar to histology in protocol for fixation, embedding, AB concentration and evaluation of immunostaining
  - Possible to cut several sections an investigate multiple AB

- Cellient
- Histogel
- Thrombin, blood clot or equivalent
- Other
Direct smears

- Wet fixed in ethanol
- **Air dried** and post fixed
  - Ethanol
  - Methanol
  - Aceton
  - Formalin
  - Sequential fixation

“Specific” but disturbing background staining due to disruption of cells resulting in cytoplasmic content lying as a granular debris in the background

synaptophysin
Advantages and disadvantages direct smears

• Limited number of spare slides
• Few AB’s can be investigated
• Unwanted background staining due to necrosis of tumour cells
• Insufficient blocking of endogen peroxidase in RBC
• “specific” but disturbing staining of disrupted cytoplasmic content
• Both air dried and wet fixed unstained smears can be used (but require different protocols!)
• Prestained smears may be used, but QC with controls problematic
  – Ethanol fixed and PAP stained smears may be stained with the same protocol as histology in most cases
Protocols for ICC and SISH on air dried and Giemsa stained smears


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Protocols for immunocytochemical staining (ICC) and in situ hybridization (ISH) of air-dried Diff-Quick or May-Grünwald Giemsa (MGG)-stained smears have been difficult to establish. An increasing need to be able to use prestained slides for ICC and ISH in specific cases led to this study, aiming at finding a robust protocol for both methods. Materials and Methods: The material consisted of MGG- and Diff-Quick-stained smears. After diagnosis, one to two diagnostic smears were stored in the department. Any additional smear(s) containing diagnostic material were used for this study. The majority were fine needle aspirates (FNAC) from the breast, comprising materials from fibroadenomas, fibrocystic disease, and carcinomas. A few were metastatic lesions (carcinomas and malignant melanomas). There were 64 prestained smears. Ten smears were Diff-Quick stained, and 54 were MGG stained. The antibodies used for testing ICC were Ki-67, ER, and PgR, CK MNF116 (pancytokeratin) and E-cadherin. HER-2 Dual SISH was used to test ISH. Citrate, TRS, and TE buffers at pH6 and pH9 were tested, as well as, different heating times, microwave powers, and antibody concentrations. The ICC was done on the Dako Autostainer (Dako*, Glostrup, Denmark), and HER-2 Dual SISH was done on the Ventana XT-machine (Ventana / Roche®, Strasbourg, France). Results: Optimal results were obtained with the TE buffer at pH 9, for both ICC and ISH. Antibody concentrations generally had to be higher than in the immunohistochemistry (IHC). The optimal microwave heat treatment included an initial high power boiling followed by low power boiling. No post fixation was necessary for ICC, whereas, 20 minutes post fixation in formalin (4%) was necessary for ISH. Conclusions: **Microwave heat treatment, with initial boiling at high power followed by boiling at low power and TE buffer at pH 9 were the key steps in the procedure. Antibody concentrations has to be adapted for each ICC marker. Post fixation in formalin is necessary for ISH.**
Liquid based preparations (LBC = ThinPrep: Cytolyt + Preservcyt)

- Commercial
  - Surepath
  - ThinPrep
  - others
- Home made
- Type of fixative in LBC
  - Ethanol
  - Methanol
  - Formalin
  - Others
- Physiological saline with/without calf serum or equivalent medium
**Advantages (and disadvantages) of LBC based ICC (=ThinPrep)**

- *Cytolytic* agent in the fluid will remove the RBC
- Transfer from preliminary fixative with cytolytic agent (Cytolyt) to long term fixative (Preservcyt)
- *Removes practically all unwanted background that might disturb the interpretation of ICC*
- Possible to make several preparations (cytospin(!) or other method), but restricted when material is limited
- Long time storage of preparations and residual material in liquid for weeks and months (-4°C, -20°C and -80°C, respectively) without loss of anticenicity
  - Liquid based material from fine needle aspirates from breast carcinomas offers the possibility of long-time storage without significant loss of immunoreactivity of estrogen and progesterone receptors. Torill Sauer, Kristin Ebeltoft, Mette Kristin Pedersen, Rolf Kåresen Cytojournal 2010

• Stored, residual material suitable as positive/negative controls
Specimen handling prior to ICC from LBC (ThinPrep- methanol based fixation)

• 1-2 direct smears for morphological diagnostics FNAC
• Aspirated material is rinsed in Cytolyt or equivalent. This ensures a mild but adequate fixation and hemolysis.
• Fixation for at least 2-3 hrs, overnight/weekend fixation is OK
• Centrifugation of liquid cell material; remove supernatant and immerse the rest in Preservcyt
• Cells are left in Preservcyt (-4°C or RT) until the day of ICC and cytospins or equivalent are prepared shortly before immunostaining

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What about SurePath?

- SurePath® (Becton Dickinson Pty Ltd) is an ethanol based liquid fixative
  - originally developed for liquid based preparations of cervical cytological specimens
  - suited for automated screening
- The morphology using Papanicolaou’s staining method (PAP) is excellent, and mucus, blood and inflammatory cells are to a large extent removed
- Today it is used for all kinds of cytological material, namely gynecological and non-gynecological exfoliative material as well as fine-needle aspirations
- SP contains a small amount of formaldehyde (< 0.2 %)
  - Effect of formaldehyde in ICC?
Effect of formaldehyde in SP in ICC of effusion (CK7) (Pilot study-Bachelor degree)

- Rest material from unfixed effusions containing either carcinoma cells or a fair number of mesothelial cells
- The cells were transferred to Surepath and left in the liquid for 1, 5 and 12 days before immunostaining.
- Day 0 equaled a staining protocol not using Surepath for fixation.
- CK7 was used as a test marker because it is “always” positive in effusions.
- Immunostaining was done at several time points
  - varying protocols for pretreatment (=3)
  - varying incubation time of CK7 AB.
  - Adding or omitting neutral buffered formalin (NBF) as post fixation was also recorded.
- The cytoplasmic staining intensity of carcinoma cells and/or mesothelial cells was evaluated.
Results

• the small amount of formaldehyde in SurePath influences the immunoreactivicty of cells in the liquid

• Reduced staining intensity may be seen within 5 days of storage in the SurePath liquid

• HRT is essential and the pretreatment must reflect the routine storage time of cells in Surepath liquid before eventual immunostaining

• Post fixation with NBF should be omitted.

• Should probably not be used for long time storage (?)

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Common causes of FN and FP findings in ICC

- Misinterpretation of neoplastic cell population
- Insufficient peroxidase blocking
- Necrotic cells
- Inappropriate handling during staining procedure
  - (dried preparations) (steroid receptors!!)
  - HRT (too much or not enough)
- AB cross reactivity, AB not specific
- Nonspecific AB binding
- Inappropriate fixation (too short- prolonged formaline fixation)
- Antigen diffusion
- AB concentration to low/high
Validation of ICC procedures for prognostic and predictive markers

- Results of prognostic and predictive markers that are scored in any way (not just pos/neg) have treatment consequences and must be validated towards histology.
- The markers must be validated against the histological protocol and have a concordance of at least 95% with the histological results.
- ER and PgR have a very robust cut-off and are easily validated.
- HER-2/neu status can be optimised for cell blocks, very difficult for smears or LBC.
ICC of Ki-67

- An easy marker!?
- Can be determined on
  - Direct smears
  - Prestained smears
  - LBC preparations
- No specific technical problems
- IHC interpretation is problematic, no universal agreement
- ICC validation and optimization a problem due to non-agreement of how and where to count as well as cut offs
- ICC might give a mean, but never “hot spots”
Internal/intralaboratory QC; positive and negative controls in ICC procedures

– Cell material with known immunophenotype
  • Cell lines
  • Direct smears
  • Cell suspensions
  • Effusions
– Xtra slide/specimen for negative control, omitting primary AB
– If using cell block, QC as in IHC: parafin embedded histological sections of tumour tissue
– Use of prestained smears for ICC should be an exception, an “emergency” procedure

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As to FN results of steroid receptors (ER/PgR)

- What happens when we air dry the smears?
  - Ruptures in the cytoplasmic membrane
  - Ruptures in the nuclear “membrane”

- Do we also cause ruptures and changes in the tertiary structure of large molecules as f.ex. ER/PgR?
  - Random sites
  - Predilection sites?
  - Irreversible
We do not know for sure what causes the ICC/IHC discrepancy, but

- Probably the old Abbott recommendation is still good advice:
- So, whatever procedure you use for ER/PgR ICC:
  - ”Do not let the slides dry out at any time”
Use LBC to minimize FN ER/PGR

- The cytological literature favour ER/PgR ICC based on cell material in liquid suspensions
- Methanol based fixative seems more effective than ethanol for investigating steroid receptors
- Concordance between cytology and histology generally good
- ER/PgR have a very robust cut off as to positivity/negativity
- Evaluate percentage of positive tumour cell nuclei
- Staining intensity irrelevant
External/interlaboratory QC in immunocytochemistry (ICC)

– Circulation of smears/slides from liquid suspensions containing cells with known immunophenotype
  • commercial cell lines
  • Cancer cells from actual patients (FNAC material)
    – Marinsek et al. Multinational study of oestrogen and progesterone receptor immunocytochemistry on breast carcinoma fine needle aspirates. Cytopathology 2013; 24(1):7-20
  • Scrape material from the cut surface of fresh, unfixed tissue
  • effusions

– Optimal storage conditions for control material, both suspensions and slides:
  • How long before the reactivity is significantly reduced?
  • Optimal storage temperature (RT? -20°C? -80°C?)
So, we have several options as to specimen and preparation. What should we choose for our daily routine?

Key question:

how robust eventually vulnerable is (are) my chosen method(s) and preparation(s)?
Conclusions

1. Direct smears, LBC preparations, cytospins, cell blocks are all suitable for ICC, but **LBC +cytospin is recommended (it is VERY robust)!**
2. ICC staining protocols must be optimised for the different preparations and/or fixations
3. Methanol is an excellent fixation for ICC
4. Post fixation in NBF is recommended
5. Any amount/percentage of formaldehyde requires HRT
6. Determination of prognostic and predictive markers on cytological material must be optimized and validated against histology and the results will have treatment consequences
7. QC as in IHC; positive/negative control material is the challenge, but all “ordinary” lymphoid, epithelial and mesothelial markers would be obtainable from effusions

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Thank you for your attention!
Diagnostic immunocytochemistry

• Presence/lack of myoepithelial markers in papillary tumours
  – p63, CK5/6

• Markers for metaplastic carcinomas/carcinosarcomas and sarcomas
  – vimentin
  – myoepithelial markers (p63, CK5/6)
  – HMW markers (squamous differentiation)
  – endothelial markers

• Neuroendocrine differentiation in breast carcinomas
  – chromogranin
  – synaptophysin

• Metastases
  – MM
  – Carcinomas from lung, GI tract, genitalia interna etc