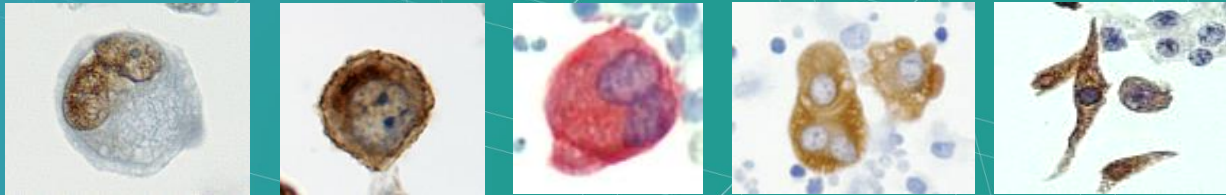

Immunocytochemistry – overview, considerations and applications

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INSTITUTE OF PATHOLOGY

UNIVERSITY OF LJUBLJANA ♦ FACULTY OF MEDICINE

Evidence Behind This Presentation

EFCS surveys

- Immunocytochemistry — 245 participants from 31 countries
- Cell blocks — 402 participants from 37 countries

Multicenter ICC studies

- Performance and validation of ICC on cytology preparations
- Immunocytochemistry Performance in Lung Cancer Cytology

UK NEQAS ICC

- External quality assessment data

Our studies and experience

- Biomarker preservation and on-slide controls
- ICC validation and performance
- Cell block preparation and cellularity
- Pilot cell block EQA

Immunocytochemistry (ICC): IHC applied to cytology samples

Cytology

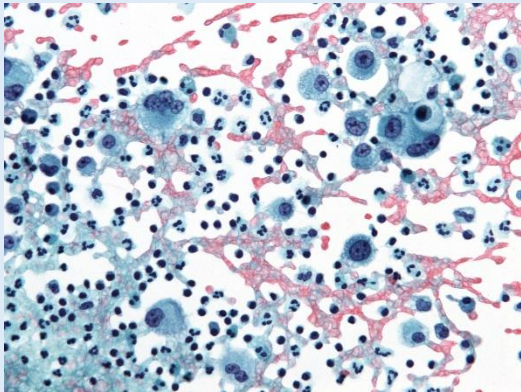
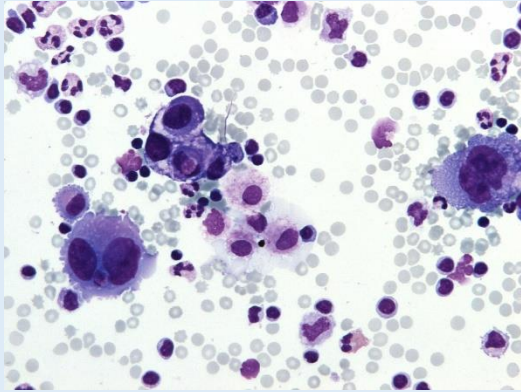
- Minimally invasive diagnostic method
- First line, sometimes ONLY available
- US-FNA, EUS-FNA

ICC

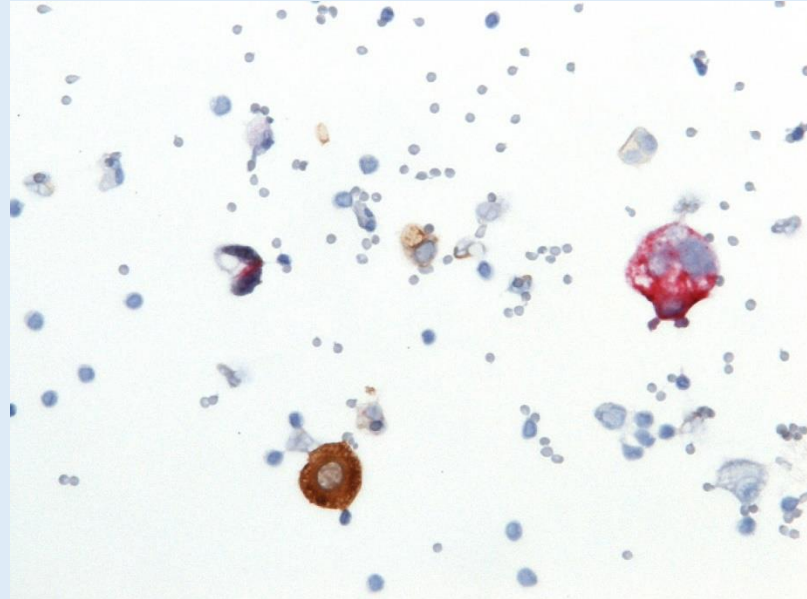
- Tumor typization
- Metastasis origin
- Prognostic/predictive

Value of ICC in a modern cytopathology- effusions

Pleural effusion, M, 80 yrs, ca pancreas

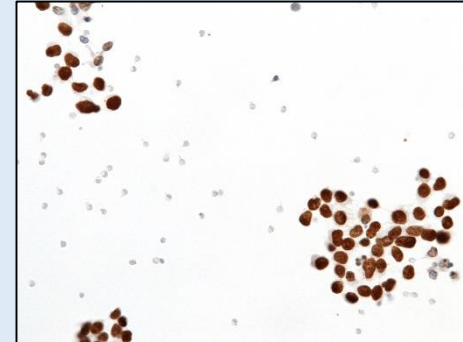
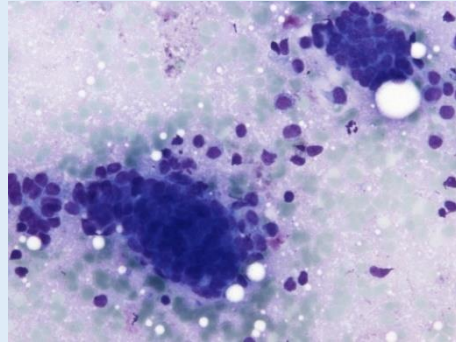
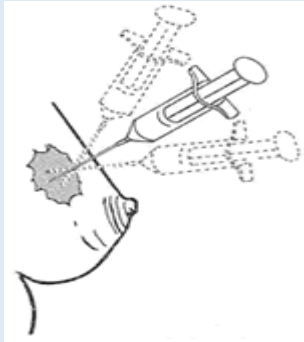


Double ICC – Calretinin/MOC31

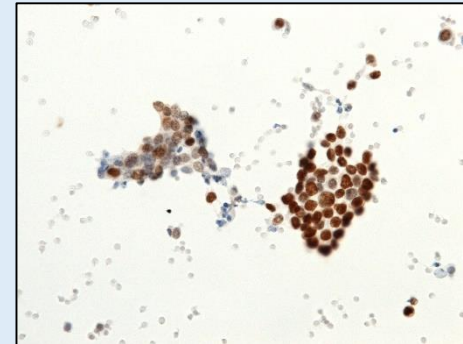


Value of ICC in a modern cytopathology - breast cancer- hormone receptor status

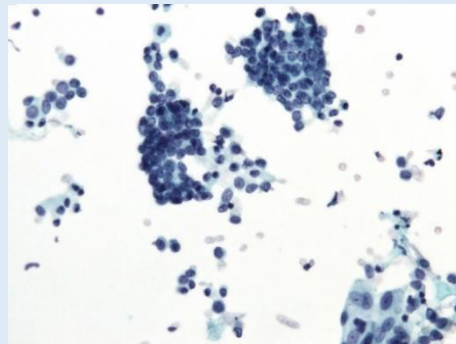
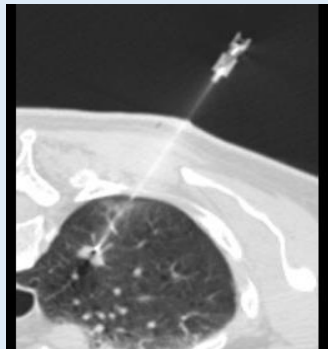
F, 87 yrs, tumour in right breast 4 x 3,5 cm



ER



PR

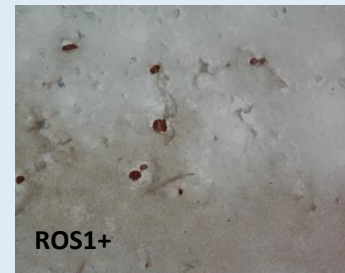
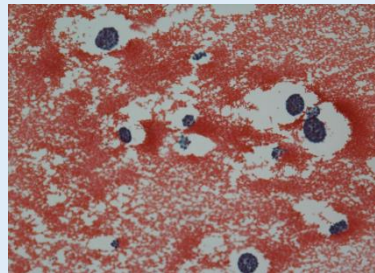
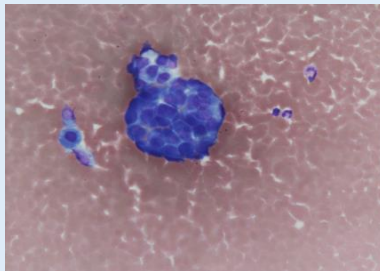


Biomarkers – lung cytology

Thursday afternoon

- Pericardial effusion, M, 38 yrs on intensive care
- Smoker, cough, fatigue, weight loss
- Infiltrates in lung
- Same-day ICC on cytospins → ROS1 +
- Immediate ROS1 TKI treatment

Thursday – same-day cytospin ICC



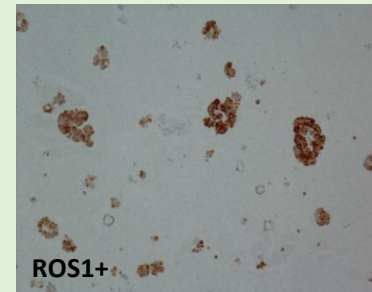
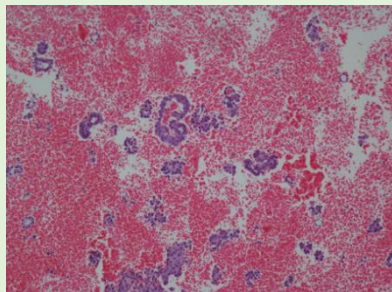
Immediate ROS TK1 treatment

Monday

- Cell block: IHC + FISH confirm ROS1 positivity

Monday – cell block confirmation

Cell block



After 4 years

- ROS1 + metastasis

Immunohistochemistry (IHC)

=

Immunocytochemistry (ICC)

- Principles
- Basic steps
- Antibodies
- Reagents
- Platforms
- General QA/QC principles

Immunohistochemistry (IHC)

≠

Immunocytochemistry (ICC)

Pre-analytical

- Sample management and processing
- Fixation

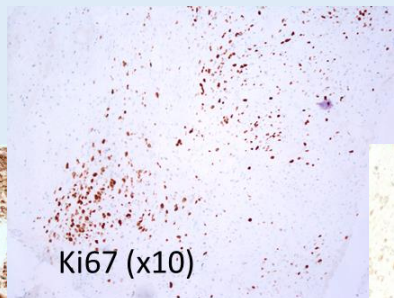
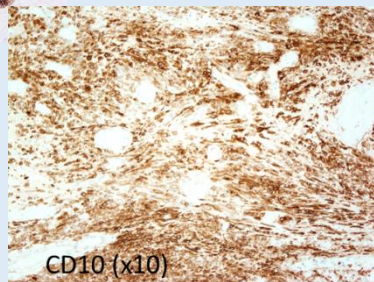
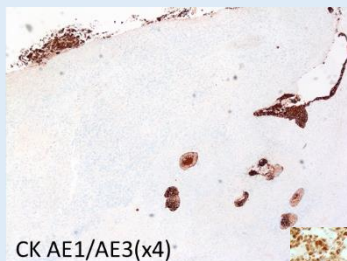
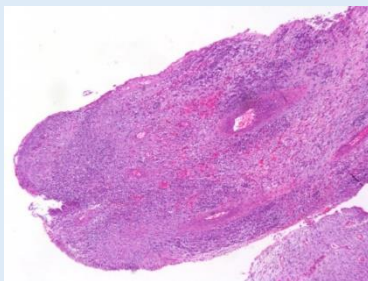
Analytical

- Pretreatment
- Antibody dilutions/protocols
- Detection systems

QA/QC

- Control slides
- Optimization
- Validation

Histology – IHC workflow



Cytology –ICC workflow

On site immediately

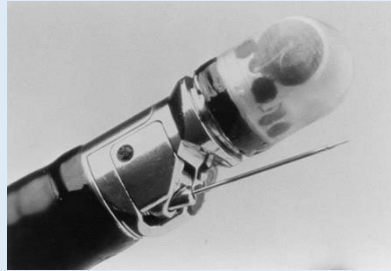
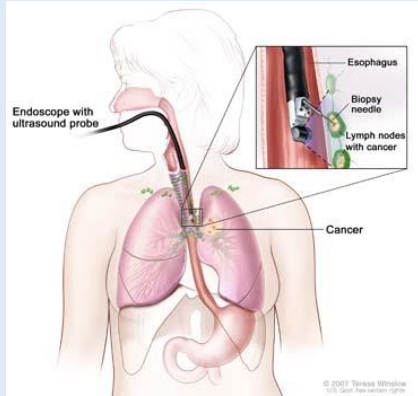
Diagnostic smears

Smear for **R**apid **O**n **S**ite **E**valuation (ROSE)
sample adequacy ? ancillary test ?



Sample for ICC, special stain, flow
cytometry, FISH, ISH, molecular test ?

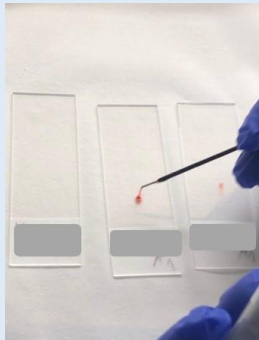
Endoscopic ultrasound guided fine-needle biopsy (EUS-FNBs)



Immediate on site
evaluation of sample
adequacy and
cellularity

Rapid On Site Evaluation - **ROSE**

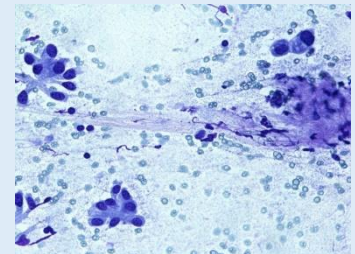
Smears for morphology



Smear for ROSE



Cell medium



Laboratory workflow



Cytomorphology



SY-CB



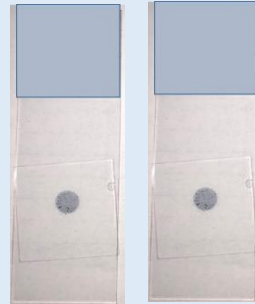
KROM-CB



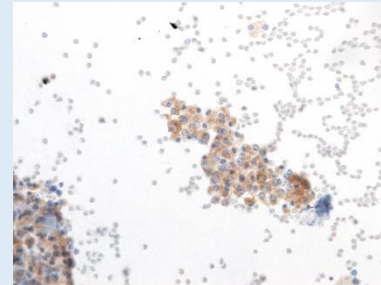
Cell pellet stored at -70°C for potential molecular testing



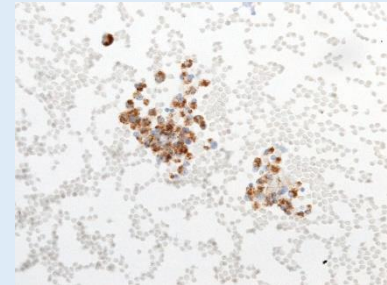
ROSE



SY- cytopsin



KROM- cytopsin



Cytology – **ICC workflow**

- Low and unknown sample volume/cellularity
- Sample adequacy
- Immediate decision for ancillary methods

Cytology sample processing – **slide preparation** options

- Cell blocks
- Direct smears
- Cytospins
- Liquid based cytology – LBC

Cell blocks

FFPE cell blocks $\approx$ FFPE tissue samples



Advantages

- IHC protocols can often be transferred from FFPE tissue
- Established IHC QA/QC approaches can be applied
- May require less extensive additional validation

Cell blocks - disadvantages

Lack of standardized preparation

- medium for sample collection (fixative, PBS, commercial solutions, RPMI, other)
- fixation (formalin and non- formalin based)
- cell pellet preparation (agar, HistoGel, plasma thrombin, Cellient,)
- Cell block method
- **Low-cellularity samples challenges**
- Time consuming (↑ TAT)
- ↑ price

Cell blocks in daily cytology practice

- Preparation methods in use
- Common challenges
- Performance in routine practice
- Comparison of preparation methods

EFCS CB survey 2021

402 participants/37 countries/12 questions

Main reasons for CB preparation

- ICC: 89%
- Molecular testing: 62%
- Morphological diagnosis: 60%

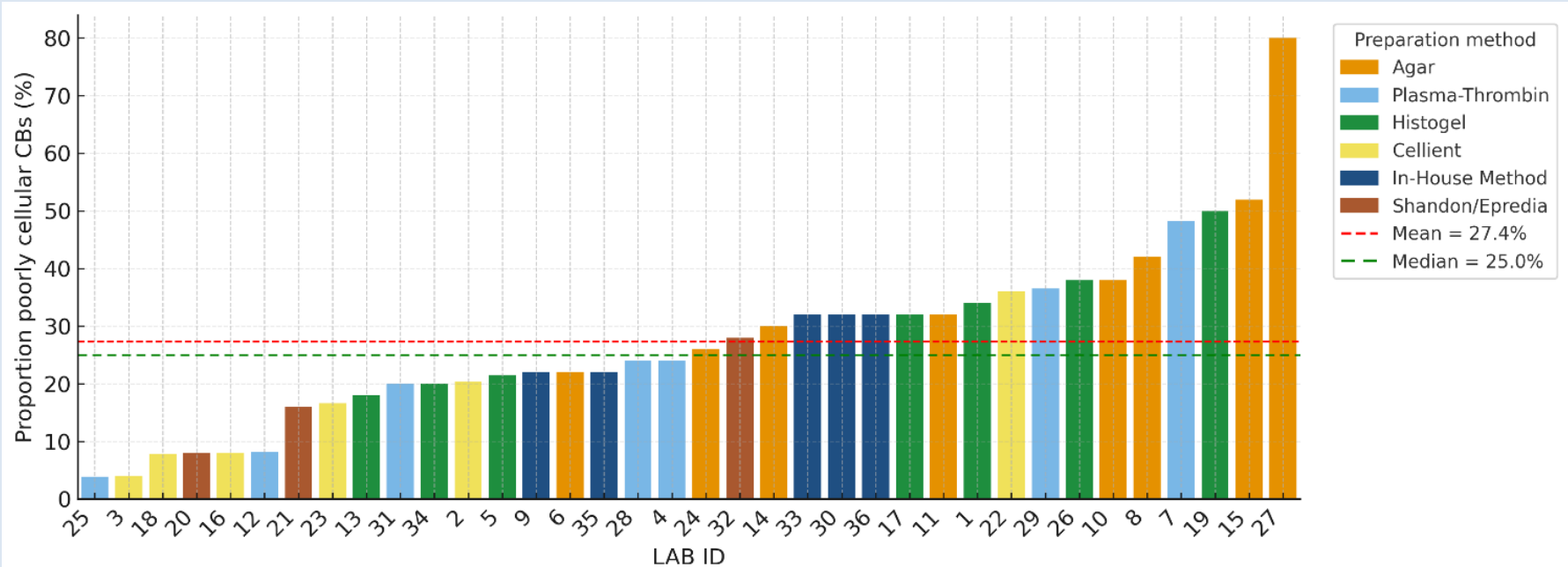
Main reported problems

- Low cellularity: 70%
- Dispersed cells: 26%
- Insufficient sections: 17%

Wide variability in CB preparation practices
Low cellularity is the most commonly reported problem

34 laboratories | 36 CB sets | **1,817** CBs - assessed cellularity

Poorly cellular CBs: 4%–80% across laboratories

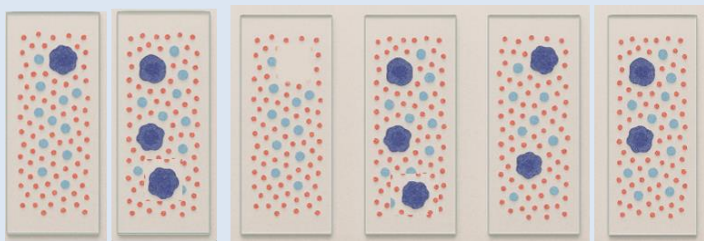
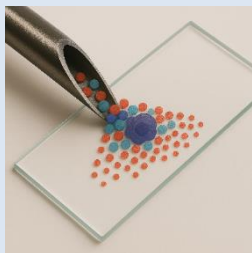


Other cytology preparations for ICC

Smears

Advantages:

- always available
- quick, simple, inexpensive

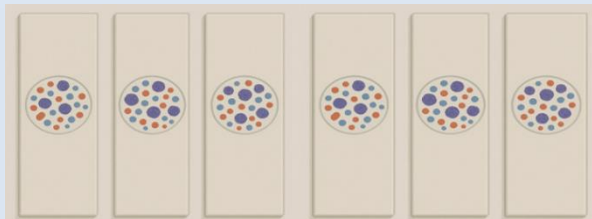


Disadvantages:

- sample triaging: which case/ how many smears
- uneven and uncontrolled distribution of the cells
- background ICC staining
- **unstandardized:**
 - unstained, Papanicolaou stained, MGG, Diff-Quick
 - fixation: drying before or after, acetone, ethanol based, formalin based, combination of fixatives, one step, multi steps
 - storage: freezer, refrigerator, RT, dried, in a fixative, PEG

Cytospins

slides prepared by cytocentrifuge from cell suspension



Advantages

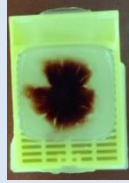
- **multiple slides**
- monolayer, controlled distribution of the cells
- short or long term storage of cell suspension/slides
- postponed decision for ancillary tests

Disadvantages

- cytocentrifuge
- non standardized procedure
- knowledge, experience, cooperation

Suitable for low cellular samples

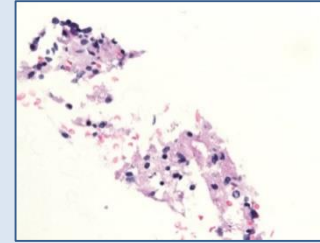
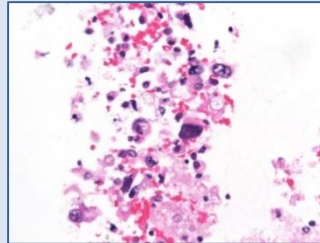
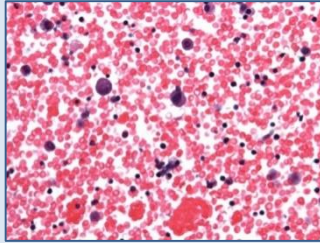
20×10^6 cells



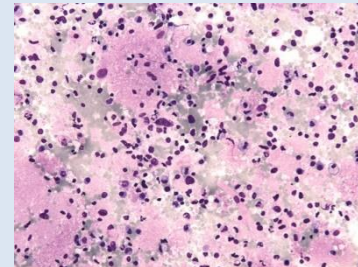
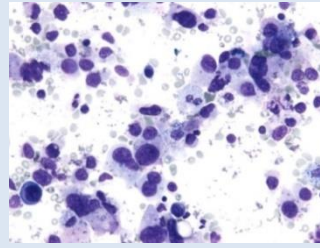
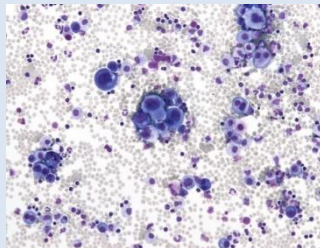
1.2×10^6 cells



0.1×10^6 cells



corresponding cytopins

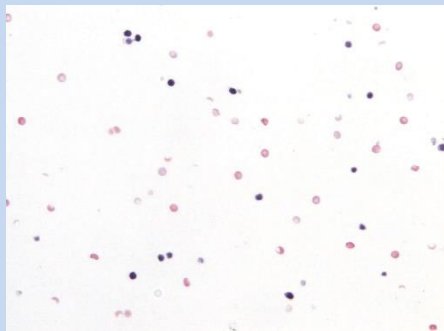


Cytospins

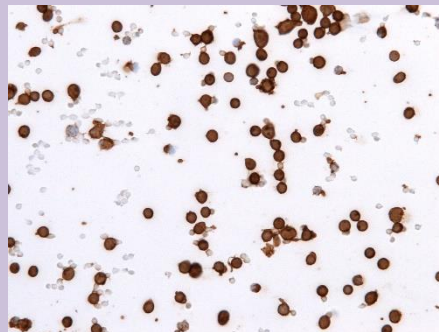
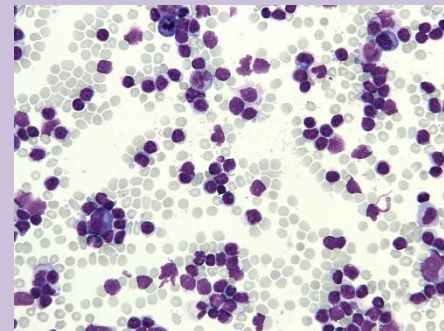
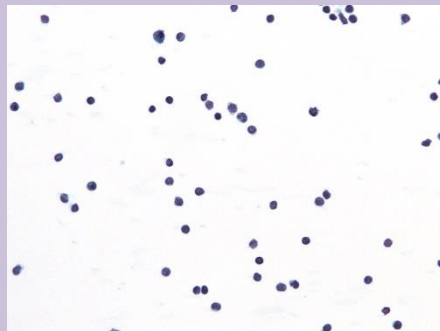
Knowledge, experience, cooperation

Stokes B.O. Principles of cytocentrifugation. *Lab. Med.* 2004;35:434–437.

Cell block



Cytospins



← CD45 →

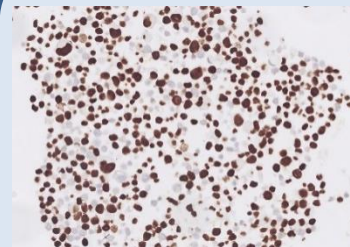
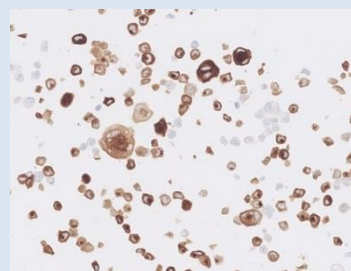
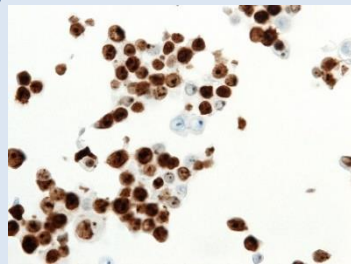
ER

Ki67

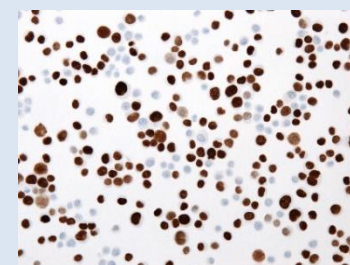
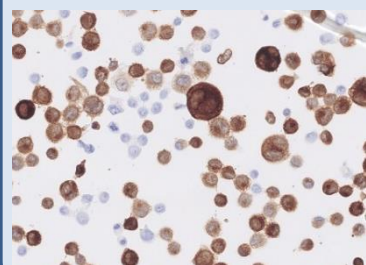
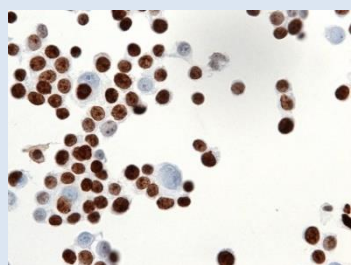
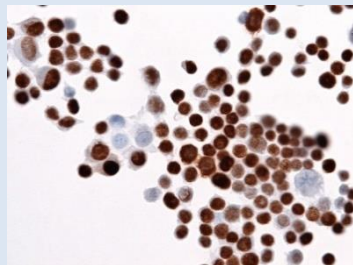
MelanA

p40

CB



Cytospin



Liquid based cytology (LBC)

- sample suspended in commercial transport medium
- automated slide preparation (ThinPrep, SurePath, CellPrep....)
 - membrane filtration
 - gradient centrifugation



LBC

Advantages

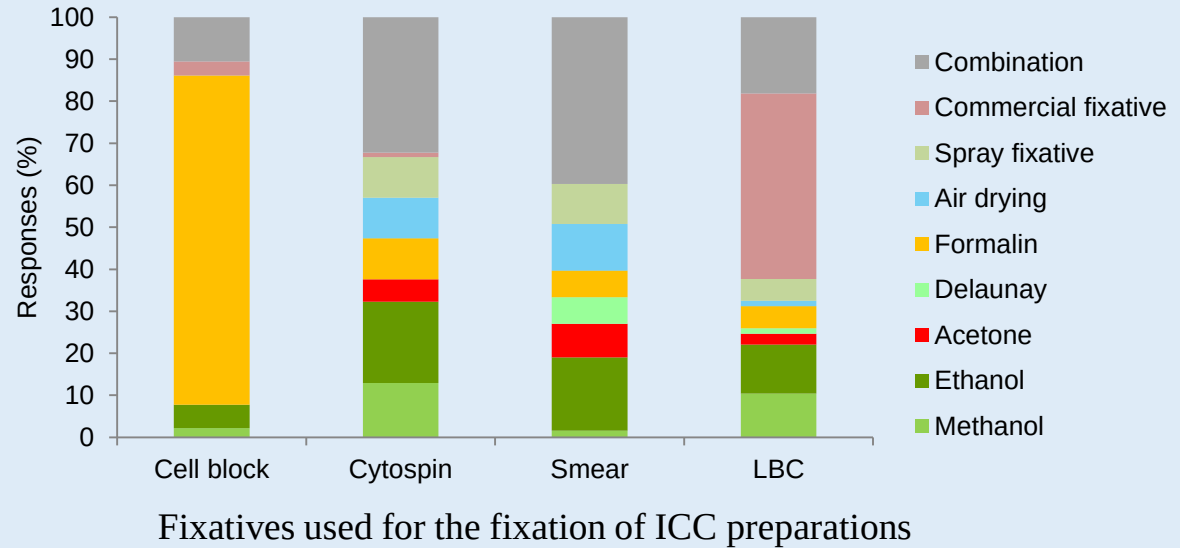
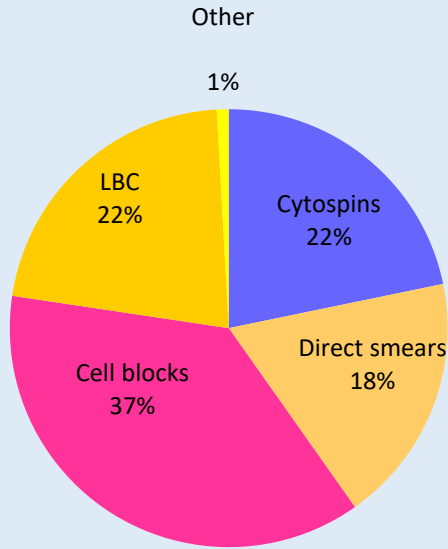
- easy storage of samples
- postpone decision
- monolayer distribution of cells
- multiple slides

Disadvantages

- expensive equipment
- ↑ cost
- Prefixed cells - clumping

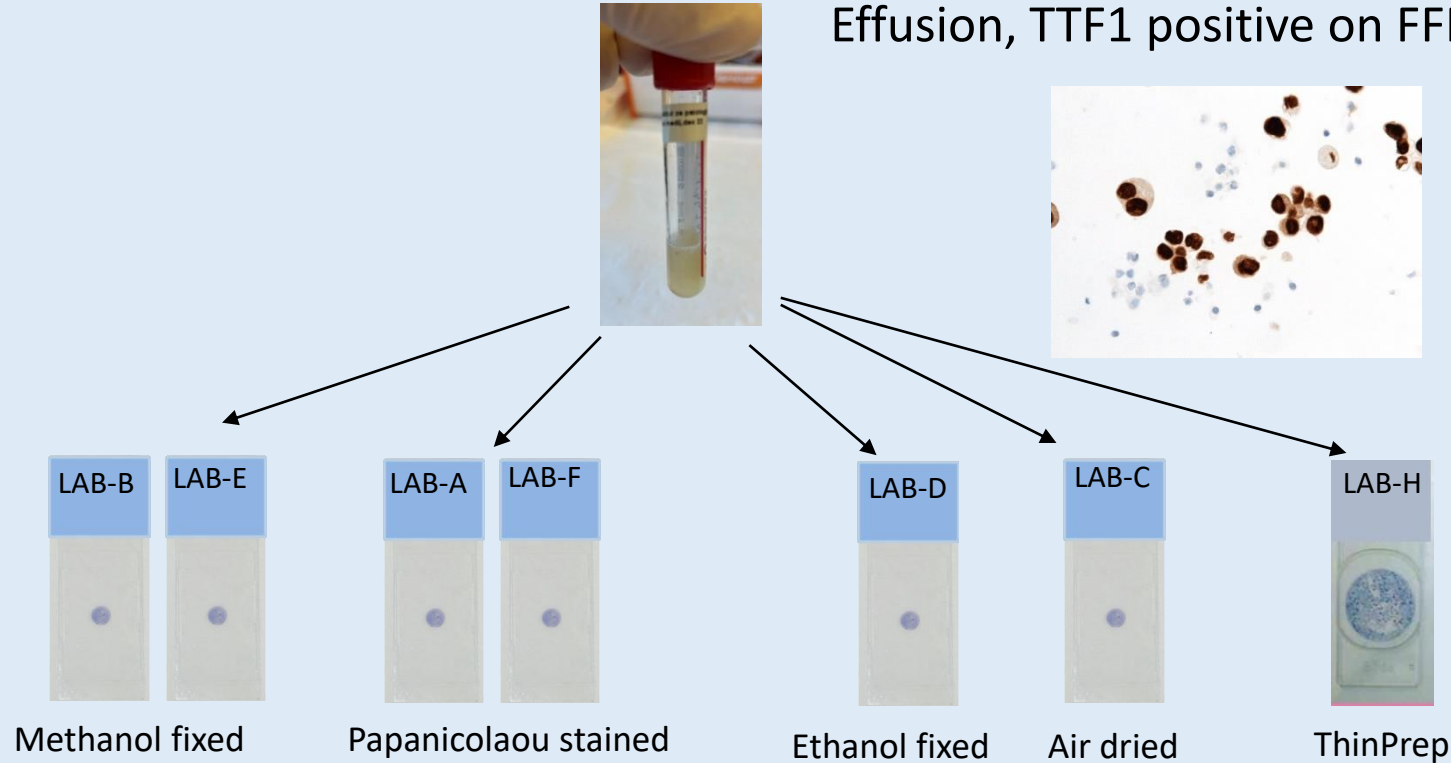


ICC practice extends beyond cell blocks and formalin fixation



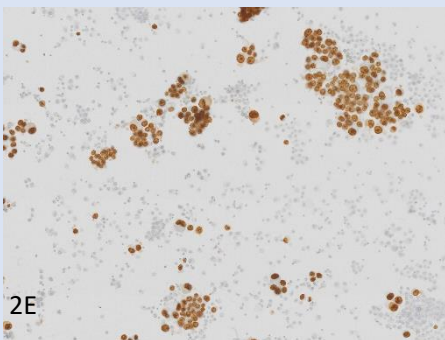
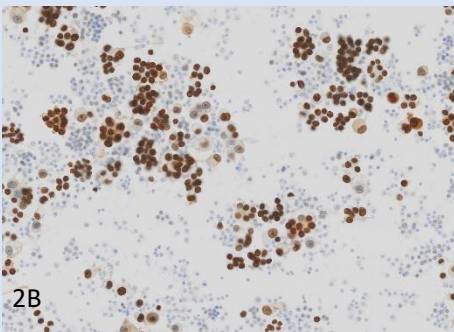
Srebotnik Kirbiš I, Rodrigues Roque R, Bongiovanni M, Strojan Fležar M, Cochand-Priollet B. Immunocytochemistry practices in European cytopathology laboratories-Review of European Federation of Cytology Societies (EFCS) online survey results with best practice recommendations. Cancer Cytopathol. 2020;128(10):757-766.

Multicenter ICC comparison: same sample, different preparations

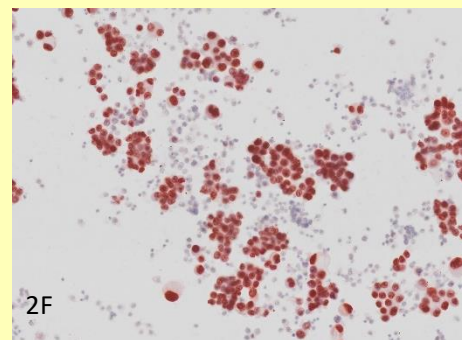
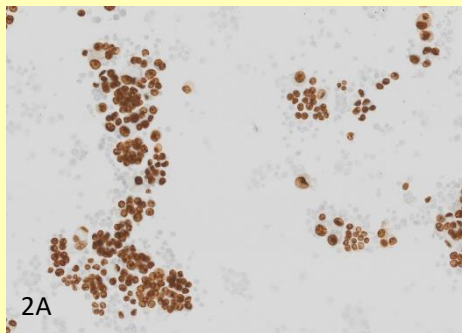


TTF-1 immunoreactivity was well preserved in alcohol-based fixed preparations and was comparable to FFPE

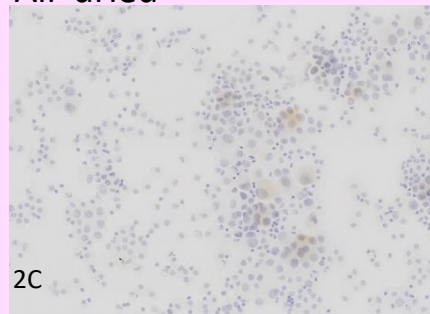
Methanol



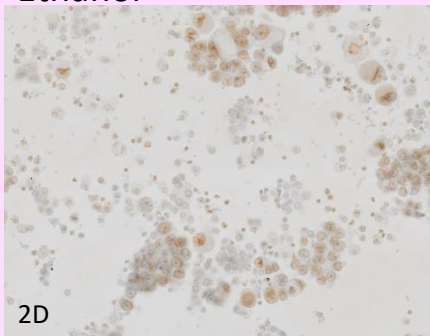
Papanicolaou



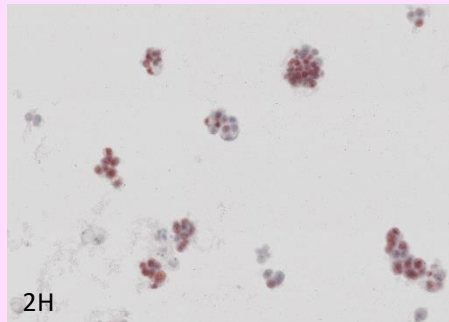
Air-dried



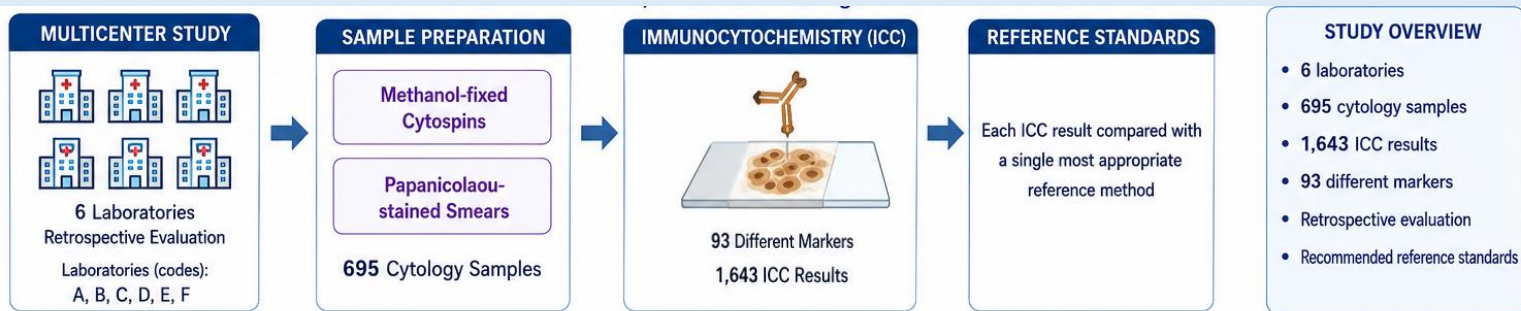
Ethanol



ThinPrep



Multicenter Study: Excellent Performance of ICC on Non-Cell Block Cytology



OVERALL PERFORMANCE (ALL ICC RESULTS, $n = 1,643$)

SENSITIVITY

96.3%

(95% CI: 95.2–97.1%)

SPECIFICITY

98.6%

(95% CI: 97.9–99.1%)

ACCURACY

97.4%

(95% CI: 96.7–97.9%)

COHEN'S κ

0.944

Almost perfect agreement

Cohen's κ of 0.944 indicates almost perfect agreement between ICC and reference methods

PERFORMANCE BY PREPARATION

Fixation	N (ICC results)	Sensitivity (%)	Specificity (%)	Cohen's κ (Agreement)
Methanol	1,278	96.46	98.95	0.951
Papanicolaou	365	95.80	96.85	0.916

KEY TAKEAWAY



ICC performed on both methanol-fixed cytospins and Papanicolaou-stained smears demonstrates excellent and comparable performance, with high sensitivity (>95%), specificity (>96%), and Cohen's $\kappa > 0.90$, indicating almost perfect agreement with reference standards.

Challenges of ICC

- Cytology sample processing for ICC is **not standardized**
- Considerable variability in all aspects of ICC on cytology samples
- High quality ICC can be achieved on different cytology preparation
- But how do we ensure reliable ICC results?

Quality assurance/quality control (QA/QC)

Why?

- Reliable ICC results (correct, accurate, repeatable)
- Accreditation

How?

- Control slides
- ICC optimization and validation
- External quality control (EQA)

- Institute CLS. Quality assurance for design control and implementation of immunohistochemistry assays: approved guideline, second edition. CLSI Document I/LA28-A2: Clinical and Laboratory Standards Institute; 2011.
- Hardy LB, Fitzgibbons PL, Goldsmith JD, Eisen RN, Beasley MB, Souers RJ, et al. Immunohistochemistry validation procedures and practices: a College of American Pathologists survey of 727 laboratories. Arch Pathol Lab Med. 2013;137(1):19-25.
- Torlakovic EE, Riddell R, Banerjee D, El-Zimaity H, Pilavdzic D, et al. Canadian Association of Pathologists-Association canadienne des pathologistes National Standards Committee/Immunohistochemistry: best practice recommendations for standardization of immunohistochemistry tests. Am J Clin Pathol. 2010;133(3):354-65.

Control slides

Positive control slides

- Sample with known expression of antigen
- Prepared as patients sample

Check:

- staining procedure
- antibody reactivity

Negative control slides

- Additional slide from diagnostic sample
- Replacing primary antibody with diluent buffer

Check:

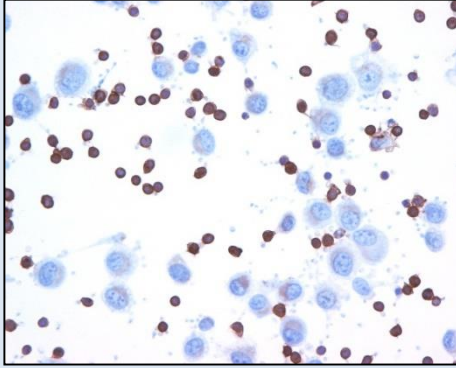
- non-specific staining

Control should be prepared the same as test sample*

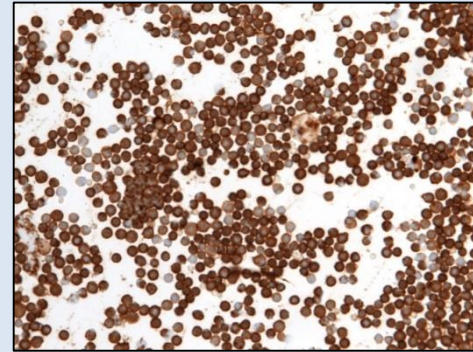
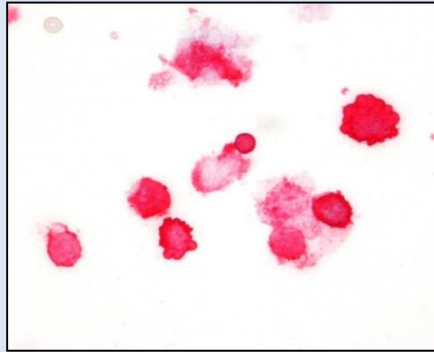
Sample	Control
FFPE tissue	FFPE tissue
Cell blocks -Histogel	Cell blocks -Histogel
Cell blocks - Shandon	Cell blocks - Shandon
Cell block - other	Cell block - other
Cytospins - methanol	Cytospins - methanol
Cytospins - acetone	Cytospins - acetone
LBC - ThinPrep	LBC - ThinPrep
LBC- SurePath	LBC- SurePath
Smear - air dried	Smear - air dried
Smear - formalin	Smear - formalin

- Each step in sample preparation can affect IR
- ICC procedure for FFPE and cytology slides not identical
- * analytical protocol for control and the sample should be the same

Positive control slides



- enough well distributed cells in monolayer
- positive and negative cell population
- good cell morphology



How to prepare enough good control slides from cytology samples?

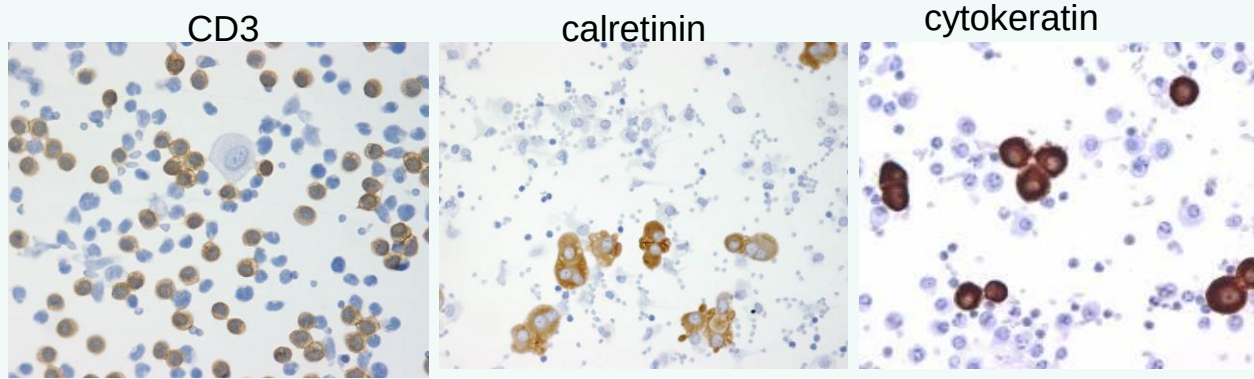
Cytology samples for controls

- leftovers of diagnostic cytology samples
- effusions
- cytology samples (FNA's, brushings) of fresh resected tumours
- human cell lines

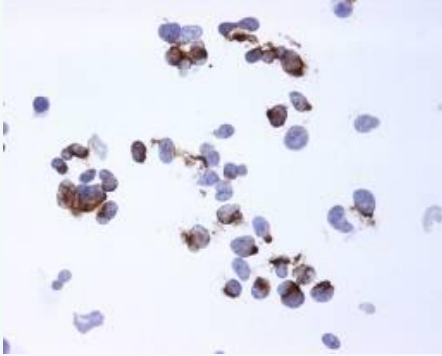


Effusion for controls

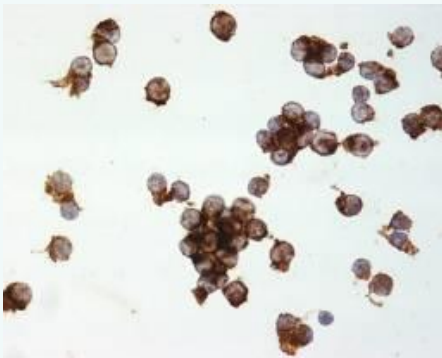
- lymphoid cells (CD3,CD20,CD45)
- mesothelial cells (calretinin, HBME, CK5/6)
- carcinoma cells (cytokeratins, MOC-31)



FNA's of resected tumors

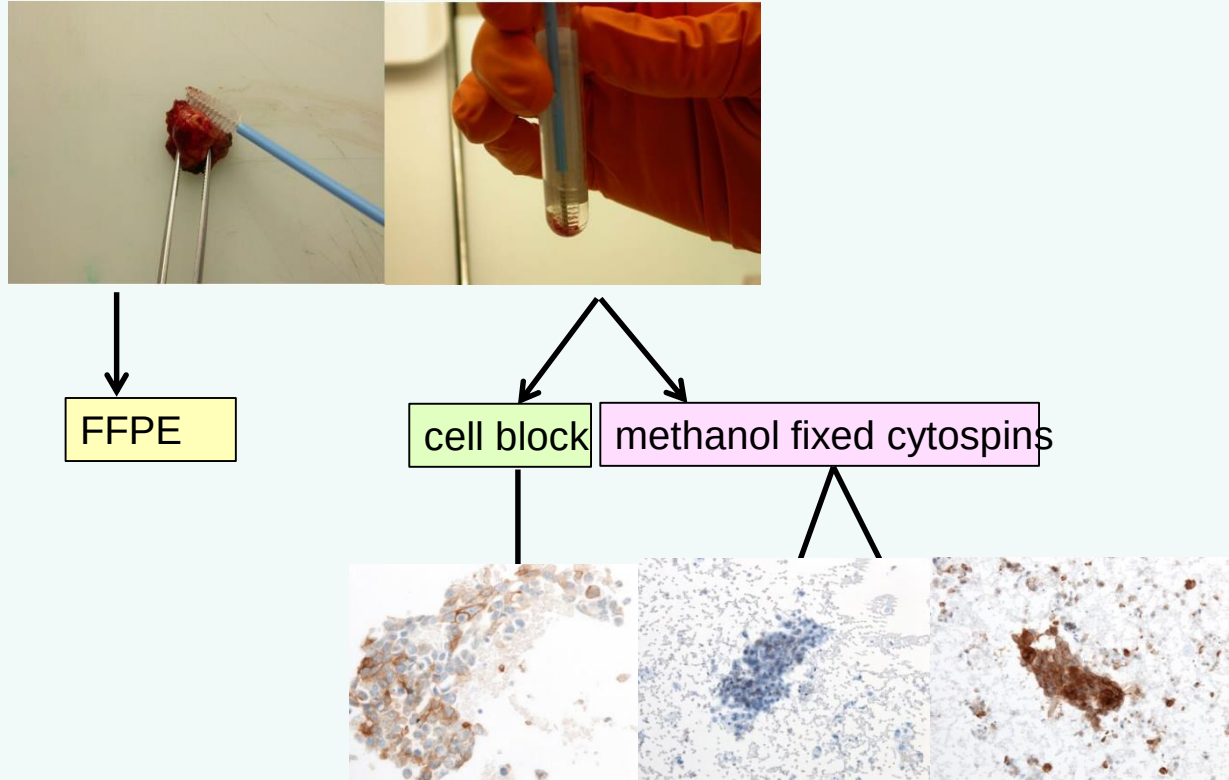


ex-vivo FNAB sample of intra-abdominal **desmoplastic small cell tumour**; **desmin** on Papanicolaou stained cytospin

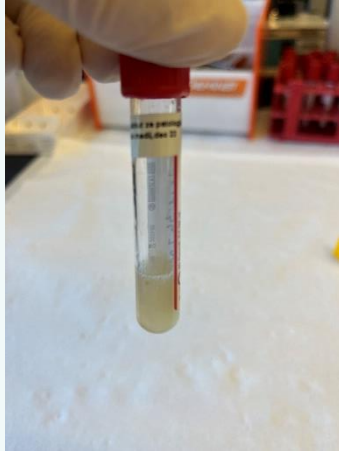


ex-vivo FNAB sample of **thyroid carcinoma**; **thyroglobulin** on Papanicolaou stained cytospin

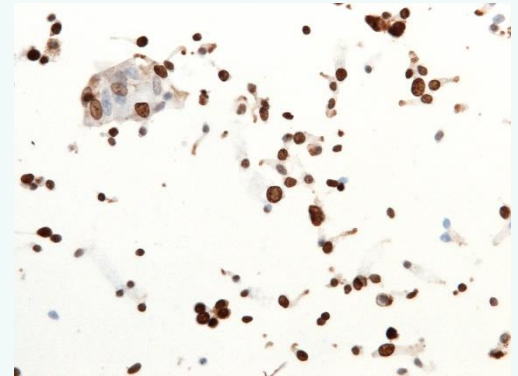
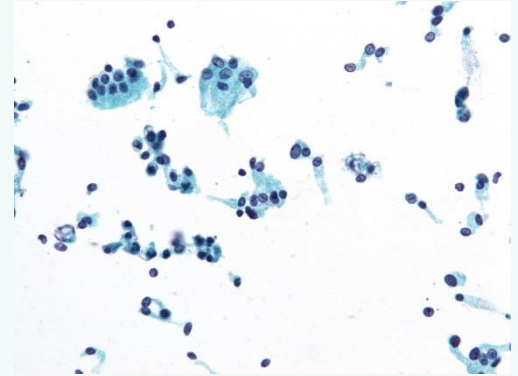
Brushing of resected tumors- PDL1 study



Brushing of fresh tissue – controls SATB2



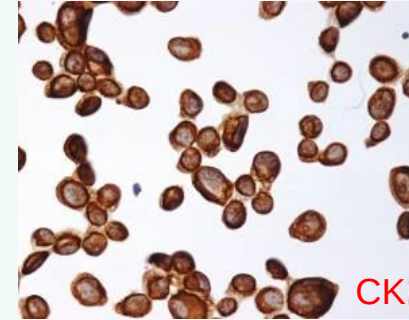
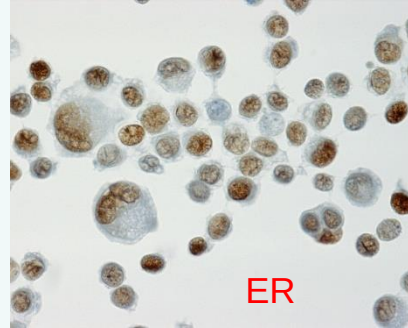
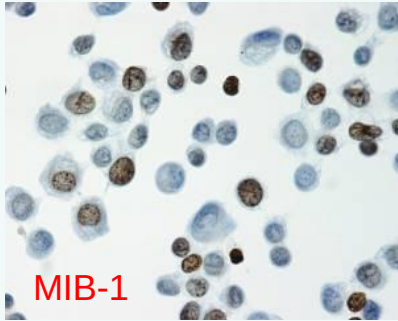
→ 30 cytopsins



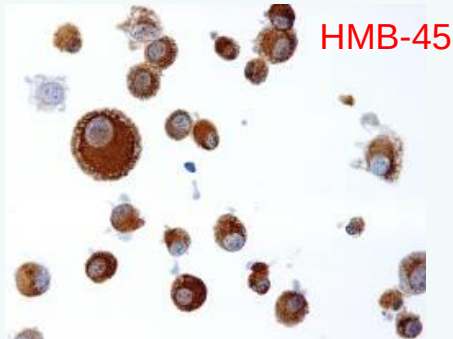
SATB2 on cytopsin

Cell lines for controls

Human breast cancer cell line MCF-7



Human melanoma cell line SK-MEL 28



Good control slides from cytology samples

Teamwork:

- Identify suitable sample
- Prepare and test candidate controls

TIME:

- Slide preparation
- Validation / comparison
- Documentation
- Ongoing monitoring

Negative controls

Negative control slides

- Additional slide from diagnostic sample
- Replacing primary antibody with diluent buffer

Check:

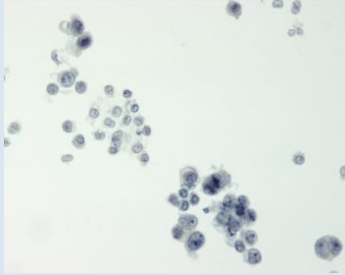
- non-specific staining

Each sample?

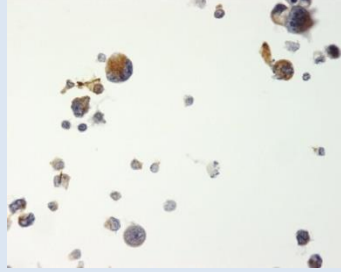
- according to lab experiences
- any change in slide preparation technique
- any change in immunostaining protocol

Negative controls – new detection kit

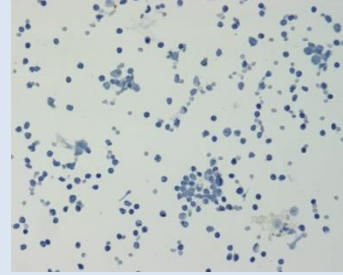
Detection kit 1



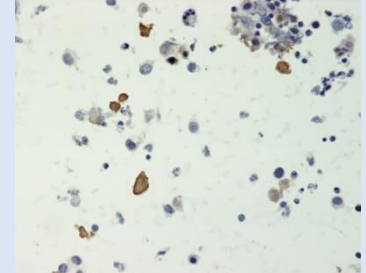
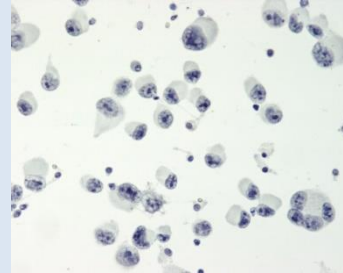
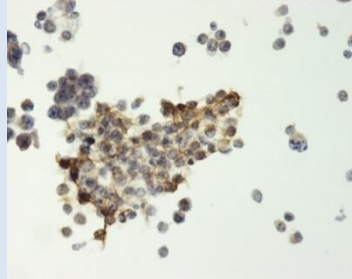
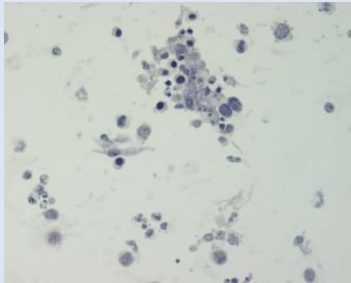
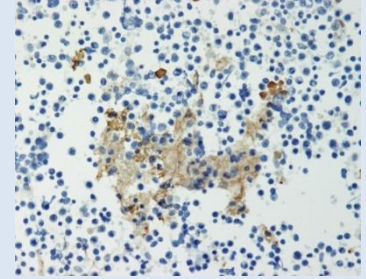
Detection kit 2



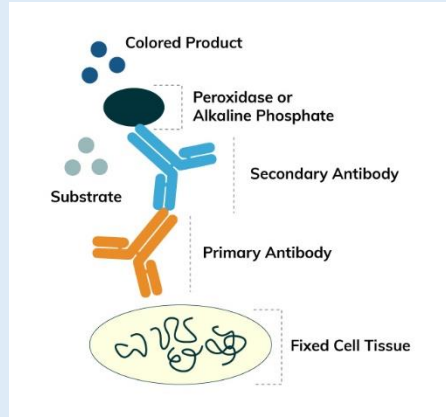
Detection kit 1



Detection kit 2



Optimization and validation



IHC antibodies and protocols are developed and validated for FFPE tissue

But ICC is performed on a variety of preparations with different pre-analytical conditions

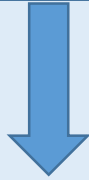
→ Therefore, ICC requires preparation-specific optimization and validation

Optimization

Changes in pre-analytical steps → adjustment of analytical conditions

Validation

Does the optimized method provide reliable and accurate results?

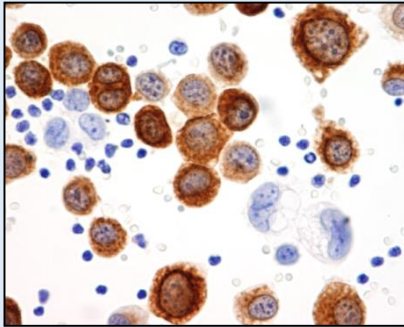


Requires:

- Adequate positive controls
- **Appropriate assessment of ICC results!**

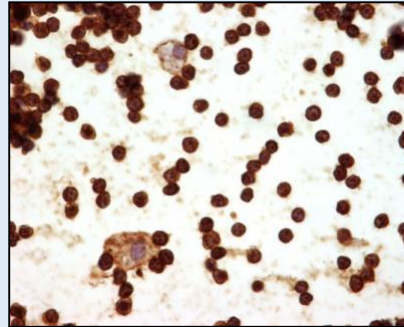
Quality of ICC

Optimal quality ICC

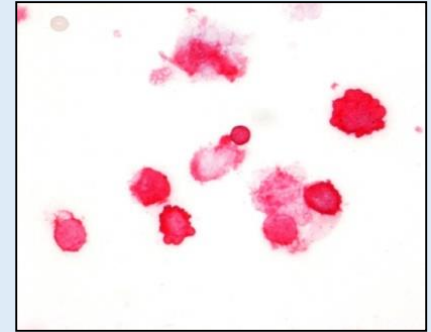


- properly localized
- clearly visible
- specific
- well preserved cell morphology
- no background

Poor quality ICC



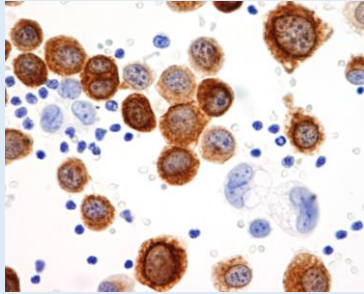
- poor cell morphology
- non specific staining
- background



Discrepancy in perception of immunocytochemical staining quality

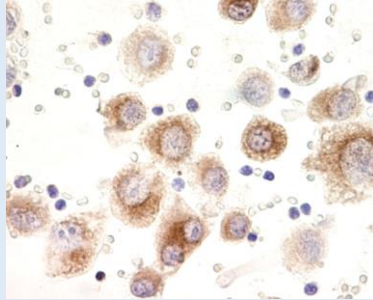
HMB-45 on identical UK NEQAS slides

Lab 1



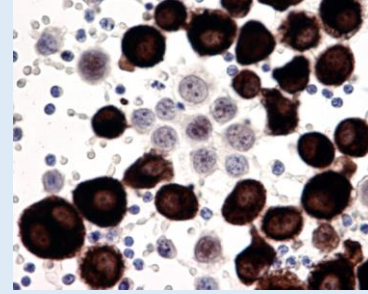
Very good
Very good

Lab 2



Very good
Borderline

Lab 3



Very good
Borderline

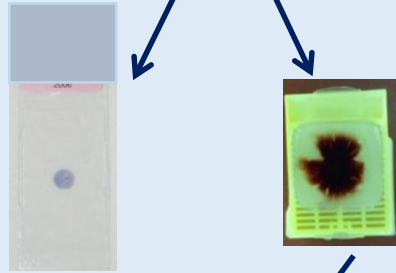
In house assessors
External assessors

UK NEQAS ICC assessment scores(1-5)

Score	Description	Interpretation
5	Excellent	No improvements are required.
4	Good	Minor improvements are possible.
3	Adequate Sub-optimal- clinically safe	Deficiencies are present, but the result remains interpretable • weak demonstration of antigen, below expected level • non-specific and/or inappropriate staining, but not preventing interpretation • some morphological damage from excessive pretreatment • poor tissue/section quality • excessive or very weak hematoxylin counterstain
2	Poor Sub-optimal -clinically unsafe	Clinically uninterpretable.
1	Inadequate	Clinically uninterpretable.

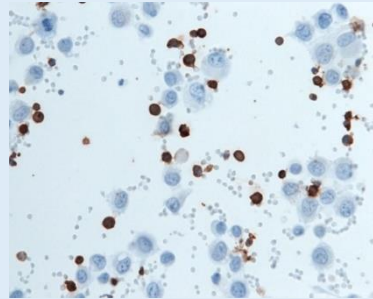
Run 108 – CD45, melanoma

- Human melanoma cell line SK-MEL28
- Effusion with carcinoma cells, few mesothelial cells, Erci
- FNAB of lymph node

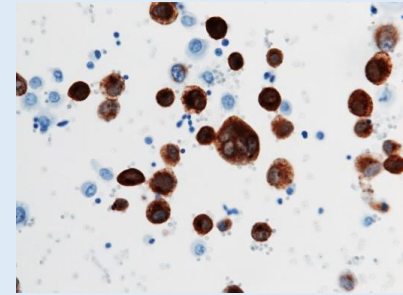


CD45, HMB-45, MelanA, S-100

CD45



HMB-45



Optimization of ICC protocols

Optimization – adjusting staining conditions to achieve the best balance between specific and non-specific staining

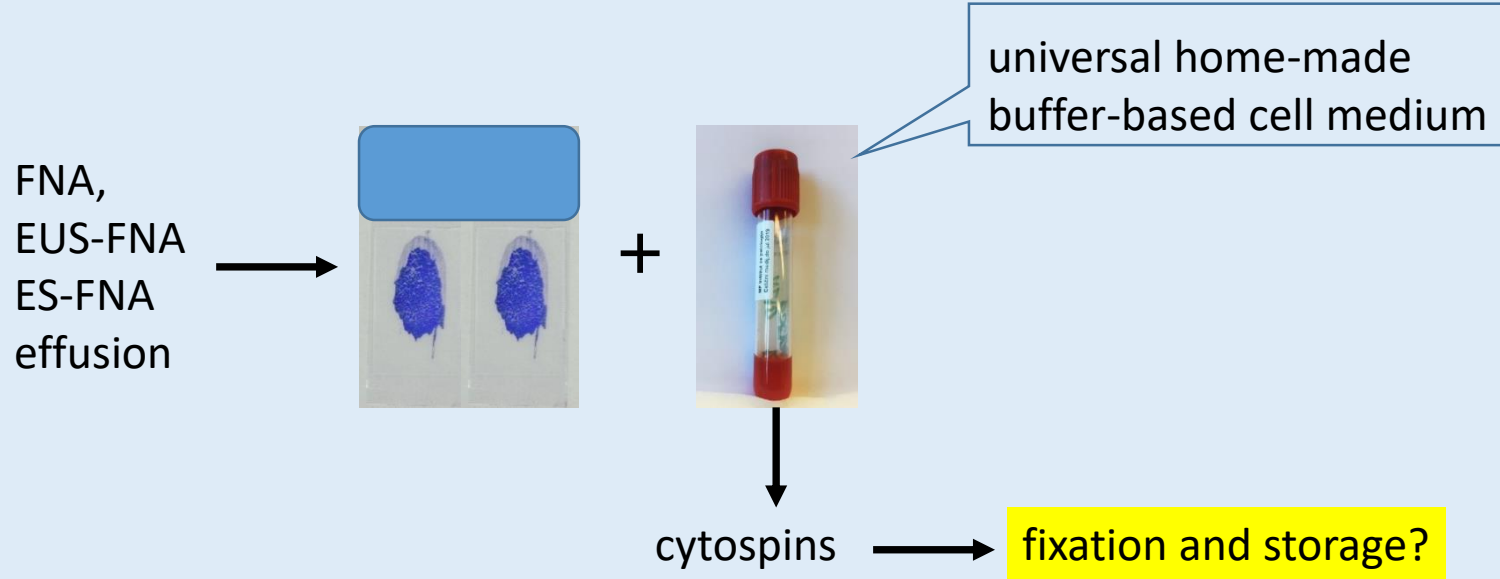
ICC protocols $\neq$ IHC protocols

ICC protocols ≠ IHC protocols

- Cellient cell blocks - adapted IHC protocol for **15/30** antibodies
 - LBC: **10 % Ab** non reactive/inconsistent on LBC using IHC protocol
 - Thrombin CB : Cellient CB - modified FFPE protocol required for **43%** of cases/assays on Cellient CB
-
- Sauter et al. Validation and Optimization of Immunohistochemistry Protocols for Use on Cellient Cell Block Specimens. Cancer (Cancer Cytopathol) 2016;124:89-99.
 - Sauter JL, Ambaye AB, Mount SL. Increased utilization, verification, and clinical implications of immunocytochemistry: Experience in a northern New England hospital. Diagn Cytopathol 2015;43(9):688-95.
 - Sauter JL, Grogg KL, Vrana JA, Law ME, Halvorson JL, Henry MR. Young investigator challenge: Validation and optimization of immunohistochemistry protocols for use on cellient cell block specimens. Cancer Cytopathol. 2016;124(2):89-100.

Validation = objective evidence that an ICC assay performs reliably for its intended use

Validation of ICC on cytopins – our approach



Validation of ICC

- Optimal fixation for **CD markers** (ICC : IHC: flow cytometry)
- Optimal fixation for **Ki67** (ICC: S-phase)
- Optimal fixation for **ER** (MCF-7 cell line, ICC:IHC)

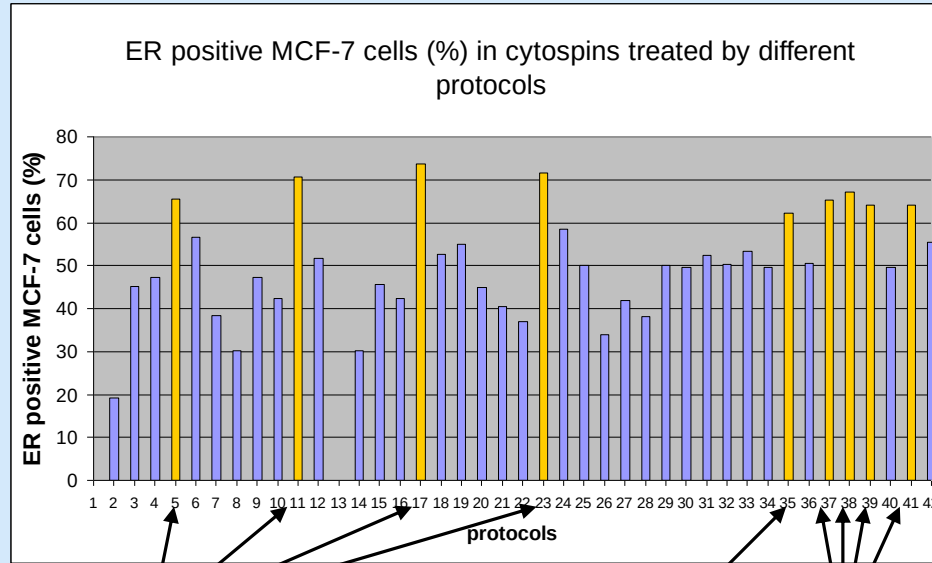
ER optimization and validation

Optimal protocol set-up on MCF-7 cell line

Evaluation of protocols on ex-vivo FNAB samples

Introduction of automated immunostaining

Follow up - response to hormonal treatment



Papanicolaou stained, **Mw10, D100**

5: Delaunay 1hr

11: 96 % ethanol 1hr

17: Delaunay 12 hrs

23: 96 % ethanol 12 hrs

CellFix
Mw10, D100

Methanol

37: Mw 0, D100

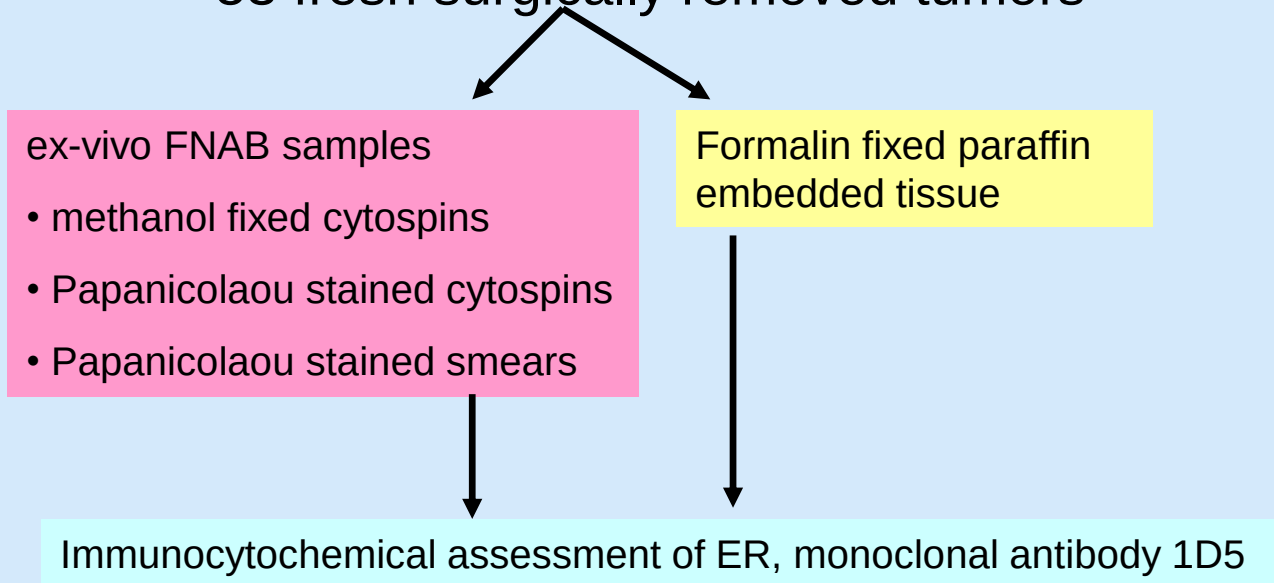
38: Mw 0, D200

39: Mw 5, D100

41: Mw 10, D100

Protocol evaluation on ex-vivo FNAB samples

53 fresh surgically removed tumors



ER on ex-vivo FNAB samples - concordance with corresponding tissue sections

	concordance	kappa
Papanicoalou stained smears	92 %	0.75
Papanicoalou stained cytopspins	94 %	0.84
methanol fixed cytopspins	100 %	1.00

Validation of ICC on cytopins

- Optimal fixation for **CD markers** (ICC : IHC: flow cytometry)
- Optimal fixation for **Ki67** (ICC: S-phase)
- Optimal fixation for **ER** (MCF-7 cell line, ICC:IHC)

} Methanol

Kirbis IS, Flezar MS, Krasovec MU. MIB-1 immunostaining on cytological samples: a protocol without antigen retrieval. Cytopathology. 2004;15(3):154-159. doi:10.1111/j.1365-2303.2004.00146.x

Srebotnik Kirbiš I, Us Krašovec M, Pogačnik A, Strojan Fležar M. Optimization and validation of immunocytochemical detection of oestrogen receptors on cytopins prepared from fine needle aspiration (FNA) samples of breast cancer. Cytopathology. 2015;26(2):88-98. doi:10.1111/cyt.12143

Srebotnik Kirbis I, Prosen L, Strojan Flezar M. Time-related changes in cell morphology and biomarker immunoreactivity for cells stored in a buffer-based cell medium. Cytopathology. 2021;32(4):513-518. doi:10.1111/cyt.12980

Validation of ICC

38 other markers:

- positive controls with known/expected expression
- methanol preserve all tested antigens

Validation of ICC

Initial validation

- 50 routine diagnostic cytology samples
- 146 paired ICC–IHC results
- **97% concordance**
- **$\kappa = 0.93$**

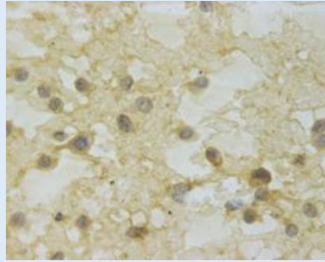
10 years later – Routine performance in a multicenter study

- 260 ICC tests
- **Sensitivity 98.6%**
- **Specificity 96.5%**
- **Accuracy 97.7%**
- **$\kappa = 0.95$**

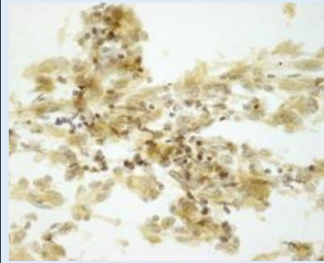
Evolution of ICC on cytology samples

1988
Direct smears

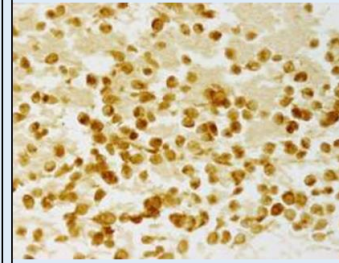
CK



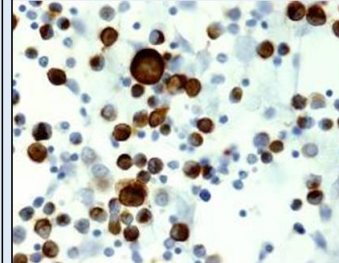
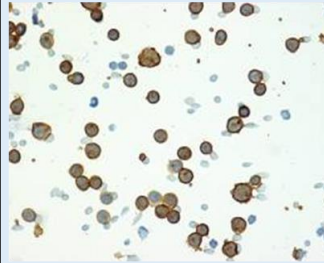
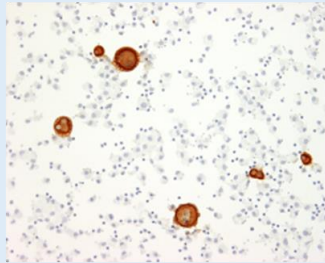
CD45



DES



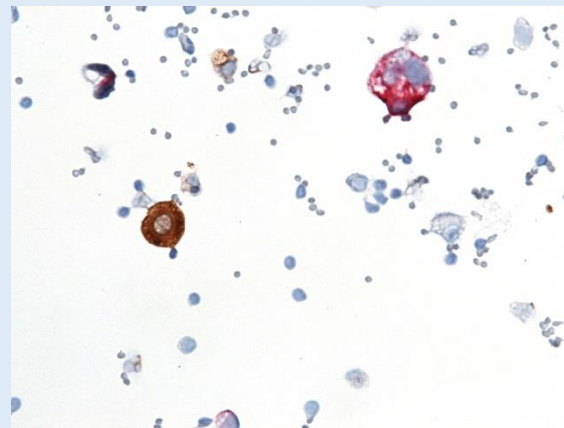
2008
Cytospins



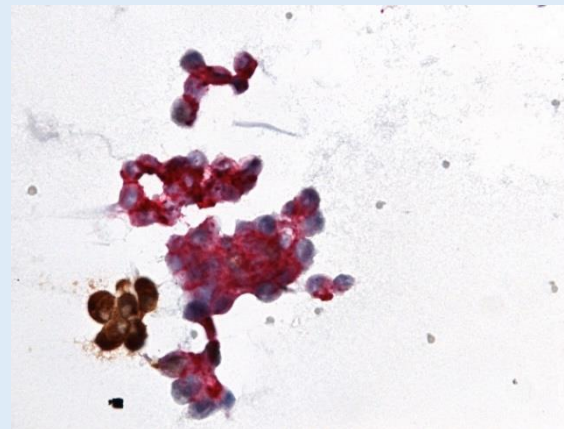
Integrated on-slide controls



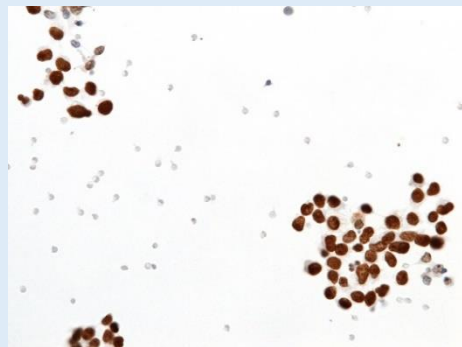
Patient sample



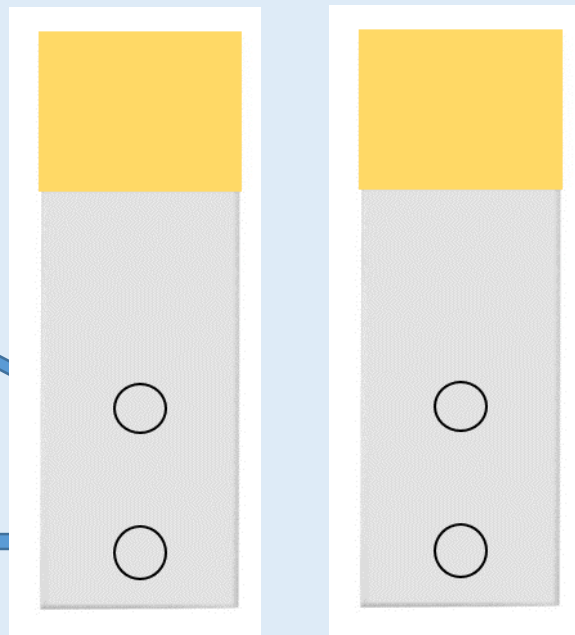
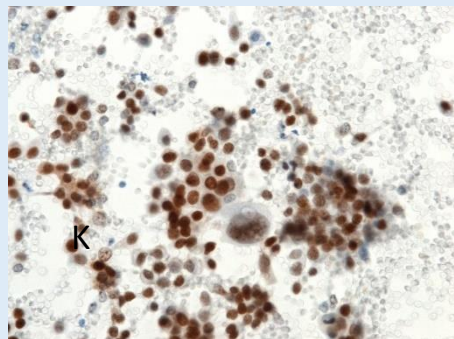
Integrated control



ER

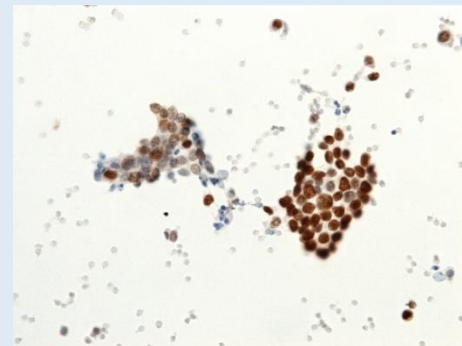


Patient
sample

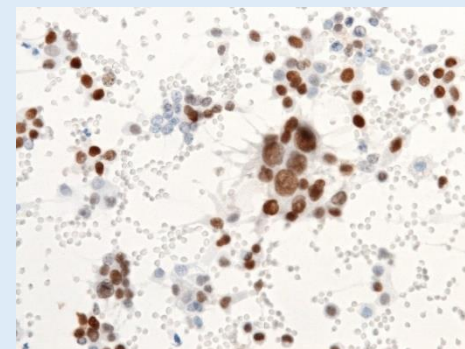


Integrated positive control

PR



Patient
sample



Conclusions

The value of cytology in modern personalized medicine

- Quick, efficient, minimally invasive
- First line diagnosis and monitoring
- Access to deep-seated lesions

Specific features of cytology samples

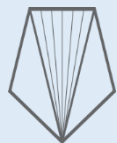
- Small samples, non-standardized processing

Biomarker testing on cytology samples

- Optimization, validation , EQA, recommendations

Thank you





dr. Irena Srebotnik Kirbis



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