IN SITU SEQUENCING FOR RNA ANALYSIS IN PRESERVED TISSUE AND CELLS

Journal Club

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  - In situ hybridization, FISH
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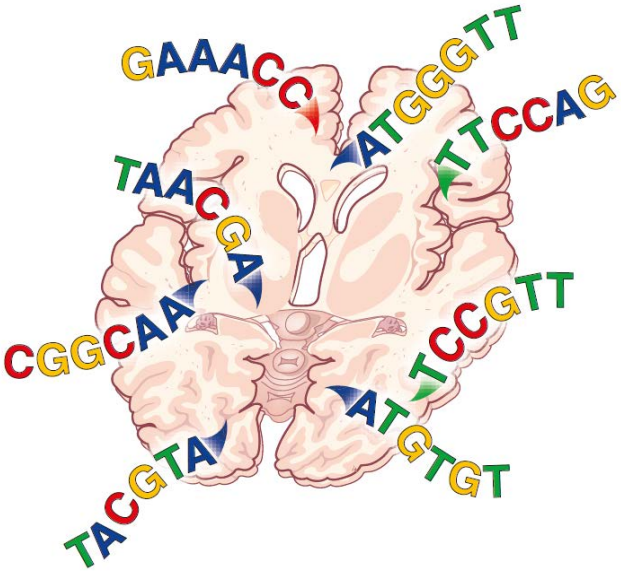
Introduction

- Information gained directly from pathological tissue
- High spatial resolution and histological context
- specific sequencing methods in mixed populations in different cell types
- potentially useful in diagnostics



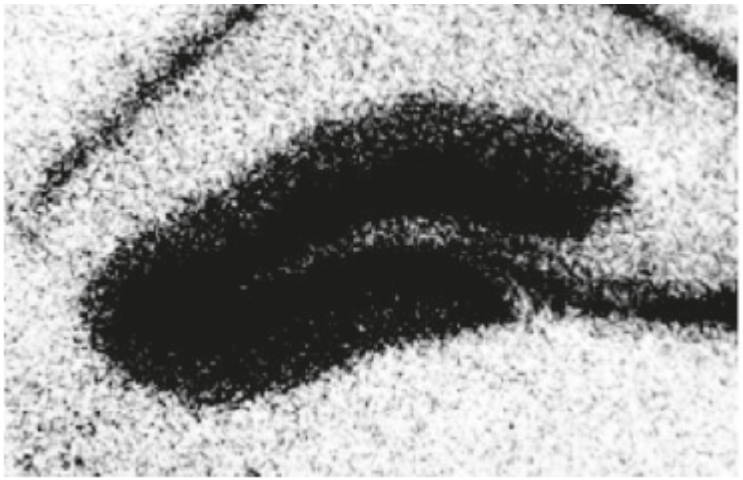
Katie Vicari 2014

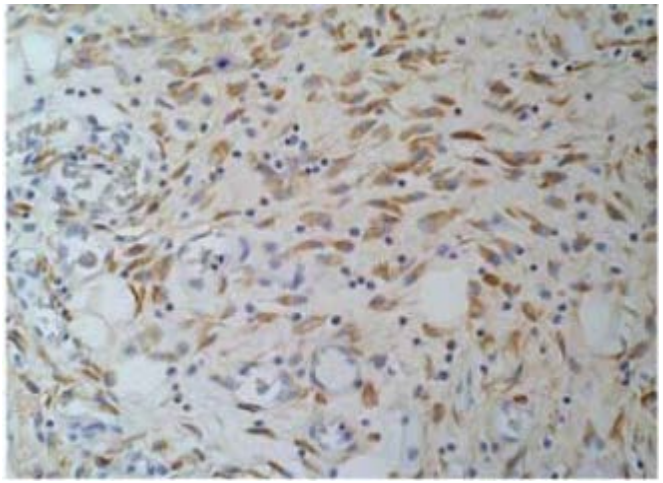
Overview – established methods

- In situ hybridization, FISH
- CISH
- Laser-capture microdissection

In situ hybridization

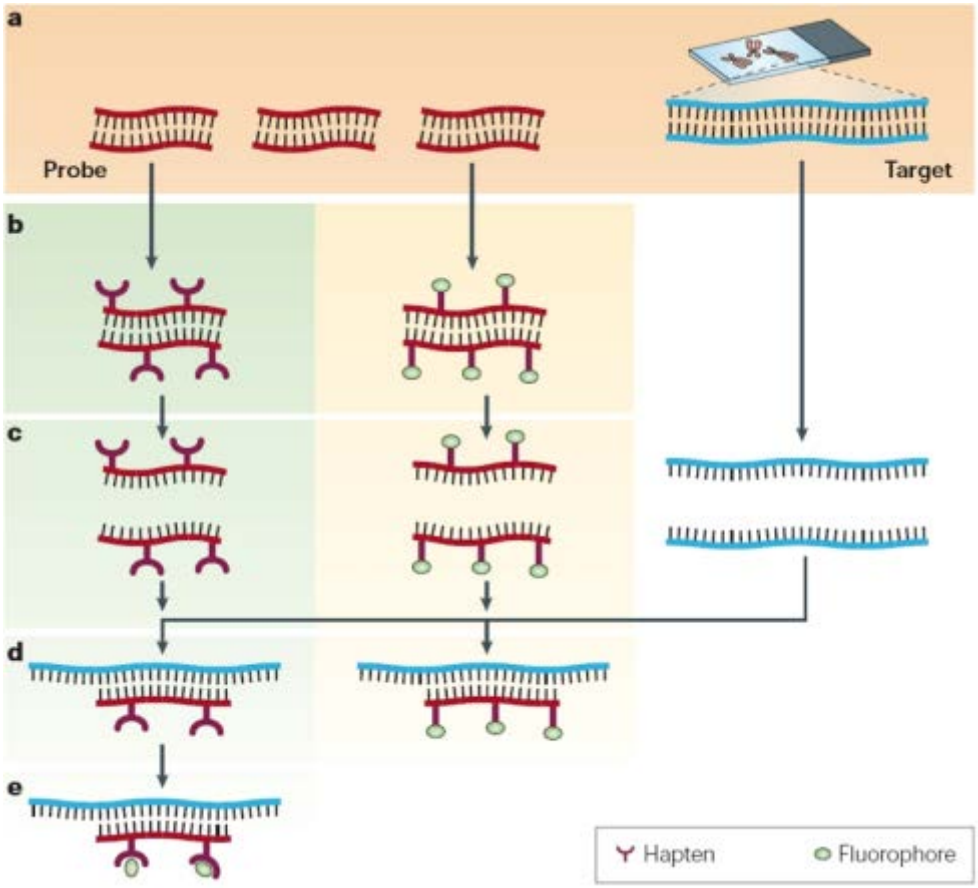
- DNA/RNA antisense probe labeled with either radioactive labeled nucleotides or haptens (e.g. digoxigenin, biotin)
- Hybridization to target sequence
- DIG-labeled probe ist detected by anti-DIG-antibody conjugated with alkaline phosphate
- Catalytically converts the hybridization signal

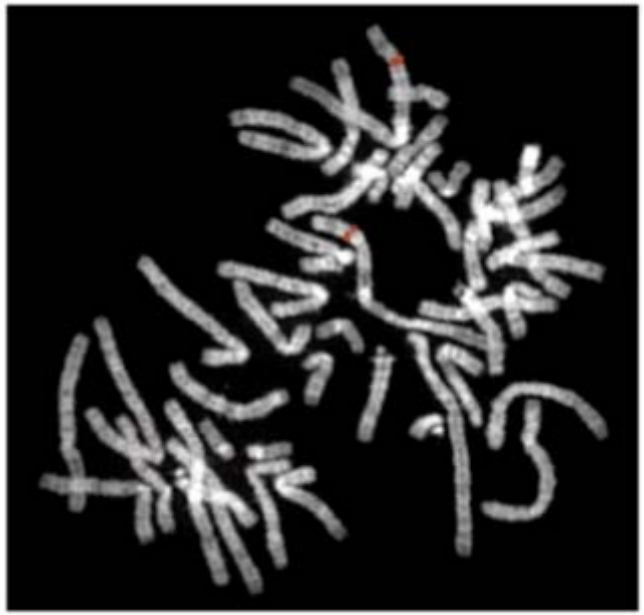




Fluorescence *in situ* hybridization (FISH)

- (in)direct-labeled DNA/RNA probes with fluorophores

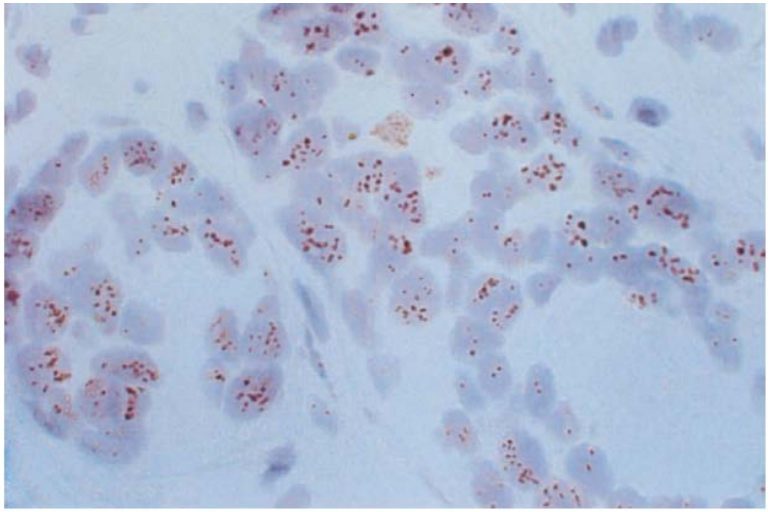




O'Connor, 2008

Chromogenic *in situ* hybridization (CISH)

- Modification of ISH using conventional peroxidase-reaction
- + detectable with light-microscope
- + no fading



- Her3/neu: multiple individual gene copies

Tanner et al., 2000

ISH, FISH

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- Simple, useful for diagnostics
- Morphology based
- Dual or multicolor analyses
- High sensitivity

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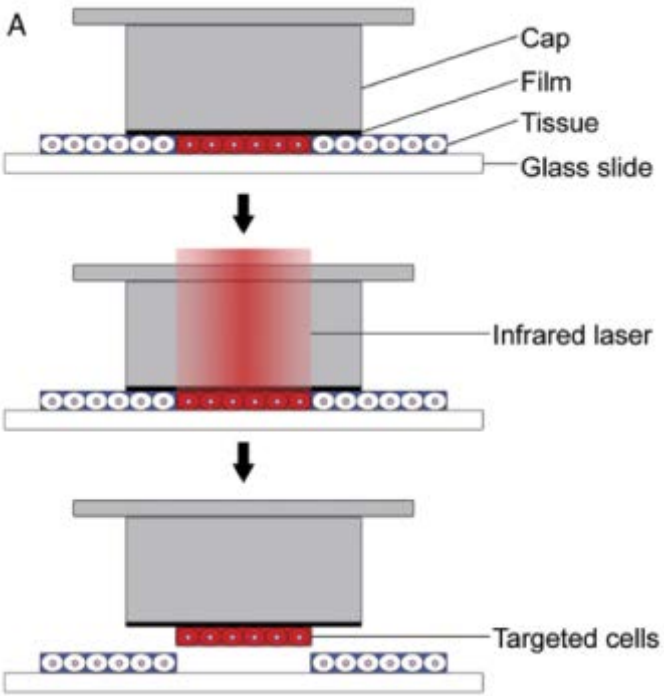
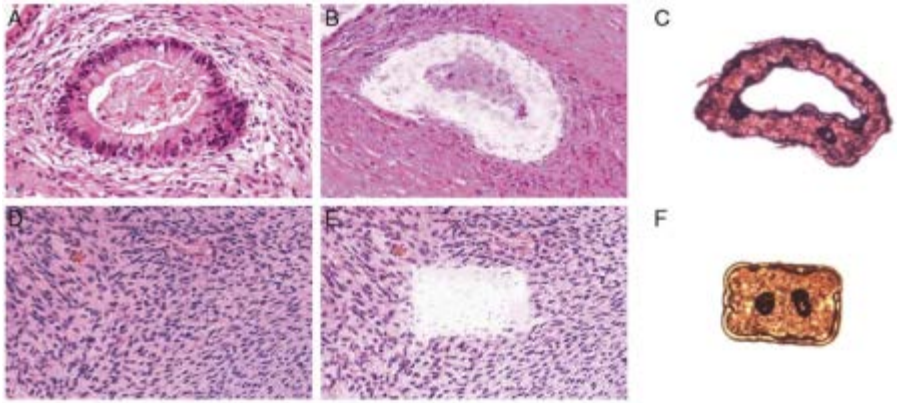
- unspecific binding
- Not a genetic screening tool
- Signal fading (FISH)
- Cytological artifacts
- semi-quantitative
- limited to detecting large alterations

Laser-capture microdissection (LCM)

- Isolation of specific cells out of a heterogeneous tissue by cutting away unwanted cells
- Analyzation by PCR, RT-PCR, microarray, Western blotting etc.





Cheng et al., 2013

LCM

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- Fast and precise isolation of cell population
- Suitable for DNA, RNA, Protein detection
- Formalin-fixed parafin-embedded tissue section, frozen tissue, cytology preparaions

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- Cell identifiaction underlies biased investigator
- Contamination of close cells possible
- Failure during liftoff method
- Fixation issues: cross-linking between formalin and proteins

- Fluorescence-activated cell sorting (FACS)
- Single cell sequencing
- In situ PCR
- Quantum dots

Padlock Probes: Circularizing Oligonucleotides for Localized DNA Detection

Mats Nilsson, Helena Malmgren, Martina Samiotaki, Marek Kwiatkowski, Bhanu P. Chowdhary, Ulf Landegren*

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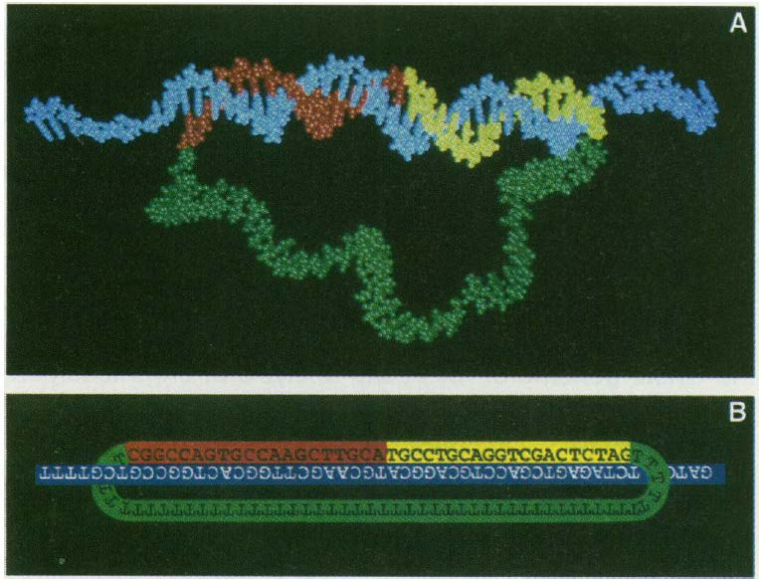
«padlock» probe

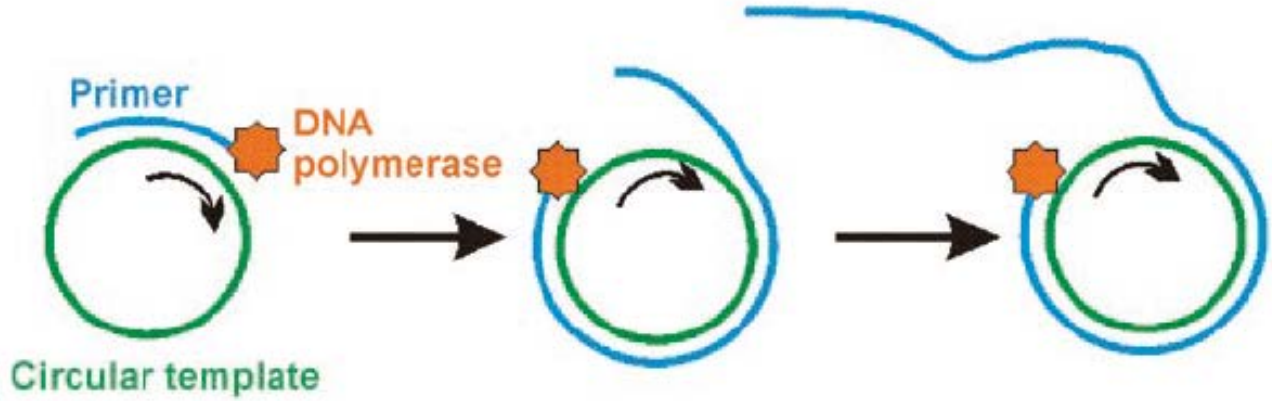
- Linear oligonucleotides of approximatley 70-100nt
- Two target-complementary segments (20-25nt)
- Connected by a linker (50nt) that carrys detectable function
- Brought in juxtaposition by hybridization to a target sequence
- Segments covalently joined by DNA ligase



Nilsson et al., 1994

Rolling-circle amplification

- Unidircetional nucleic acid replication of DNA or RNA
- Occurs in genoms of bacteriophages, viroids



Weian et al., 2008

In situ detection and genotyping of individual mRNA molecules

Chatarina Larsson[1,2], Ida Grundberg[1,2], Ola Söderberg[1] & Mats Nilsson[1]

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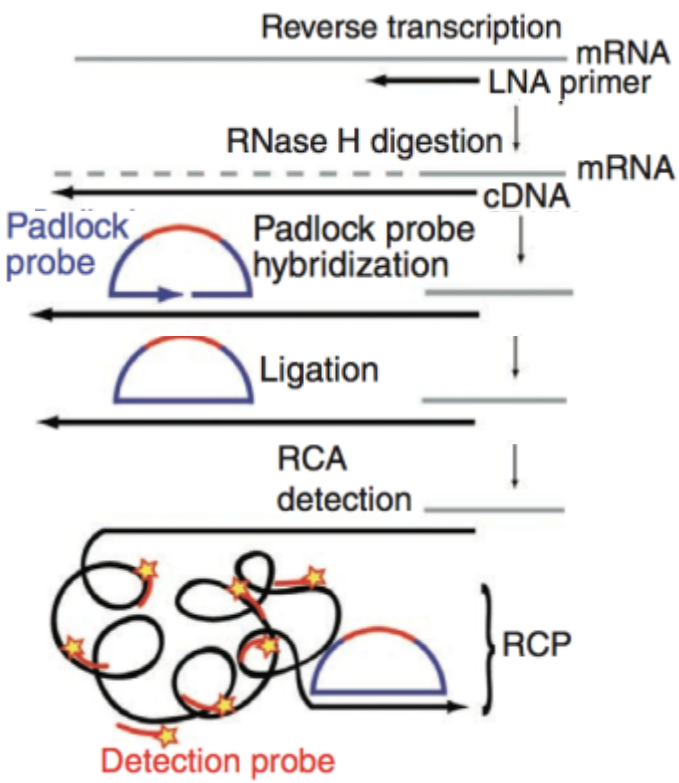
Detection of individual transcripts with padlock probes and target-primed rolling-circle amplification (RCA)



Main steps

a) LNA primer binds , reverse transcription

b) RNase H digestion

c) Padlockprobe hybridization

d) DNA ligase

e) DNA Polymersa

f) Rolling-circle amplification

Larsson et al., 2010

padlock probes

a mRNA sequence *ACTB*

5′ CGCCCCGCGAGCACAGAGCCTCGCCTTTGCCGATCCGCCGCCCGTCCACACCCGCCGCCAGCTCACCATGGATGATGATATCG 3′

b cDNA sequence *ACTB*

5′ GTGGACGGGCGGCGGATCGGCAAAGGCGAGGCTCTGTGCTCGCGGGGCG 3′

c Padlock probe target site

5′ GGCAAAGGCGAGGCTCTGTGCTCGCGGGGCG 3′

d Padlock probe target-specific part

CCGTTTCCGCTCCGAGACACGAGCGCCCCGC

3′ 5′ 3′ 5′

e Complete linear padlock probe including detection sequence and filler sequence

5′ AGCCTCGCCTTTGCCTTCCTTTTACGACCTCAATGCACATGTTTGGCTCCTCTTCGCCCCGCGAGCACAG 3′

f Primer with LNA-modified bases

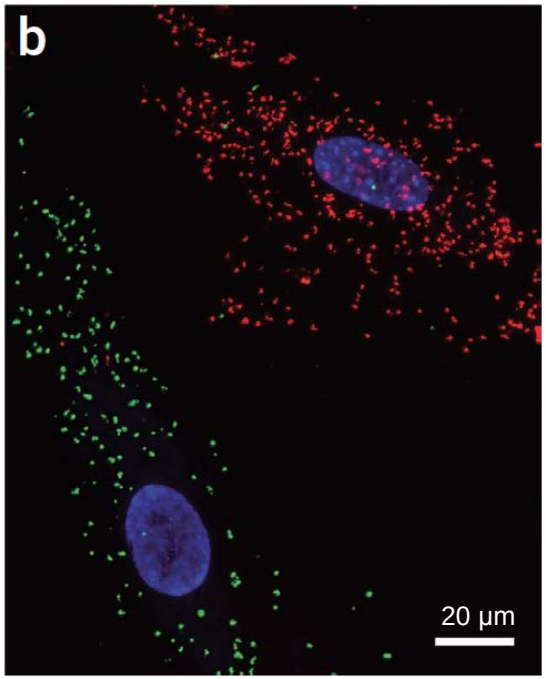
5′ GTGGACGGGCGGCGGATCGGCAAAG 3′

Weibrecht et al., 2013

What now?

- Can different transcripts be distinguished?
- Can they be quantified?

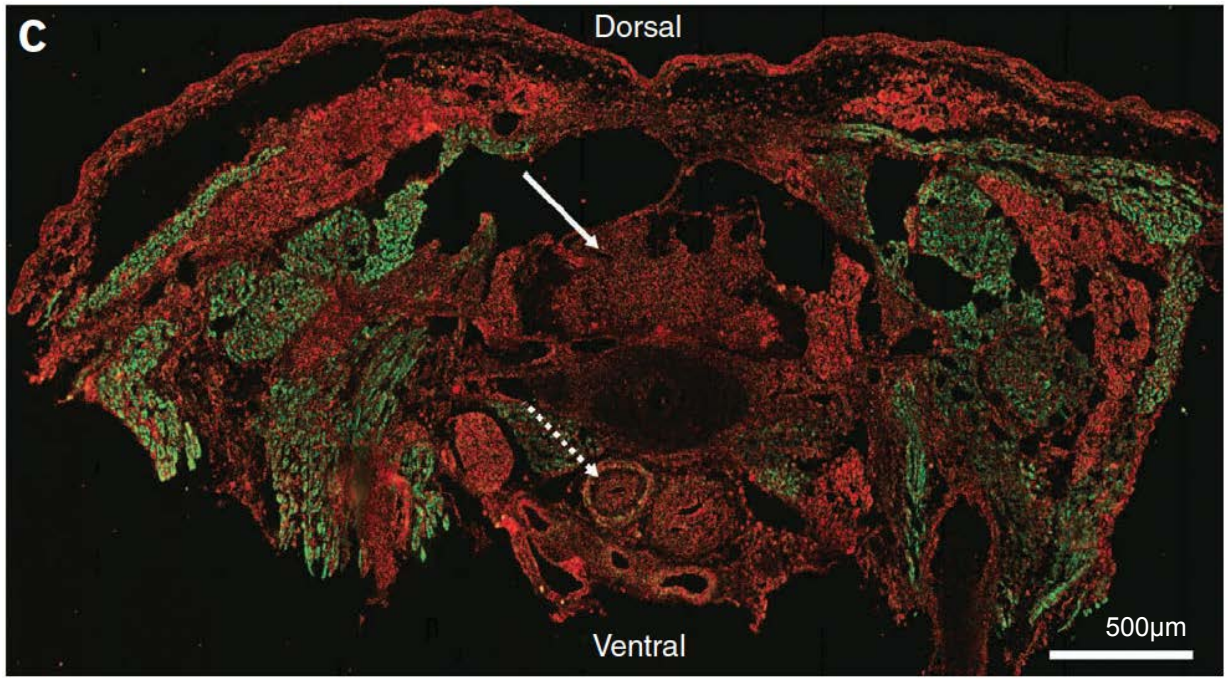
Detection of singel nucleotide differences in actin transcripts



Cocultured human and mouse fibroblast cells

Green: human β-actin

Red: mouse β-actin

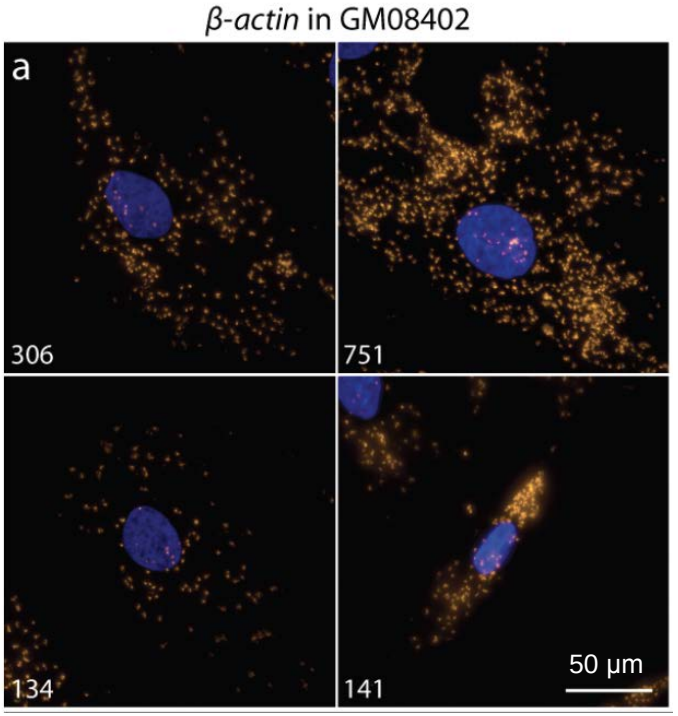


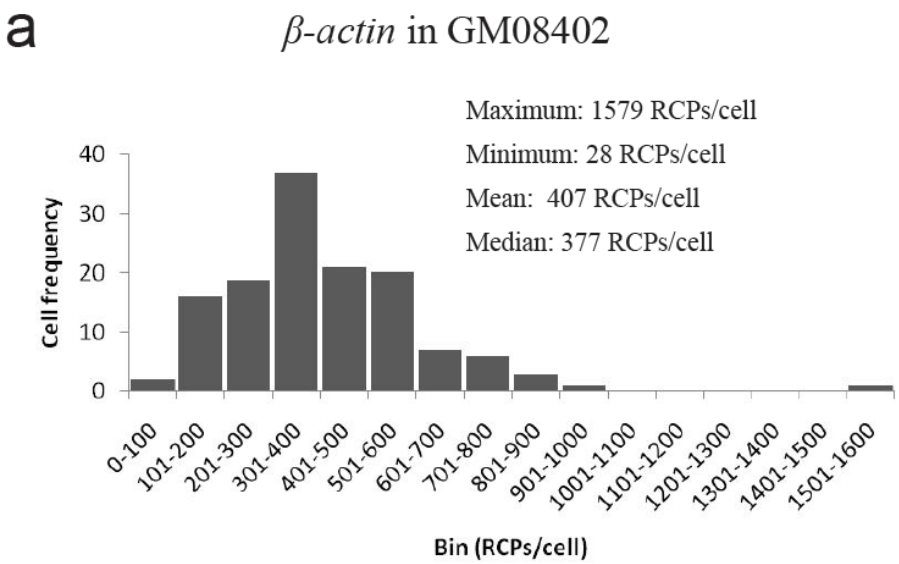
Fresh frozen mouse embryonic tissue (E14.5)

α1-actin (green), β-actin (red)

Larsson et al., 2010

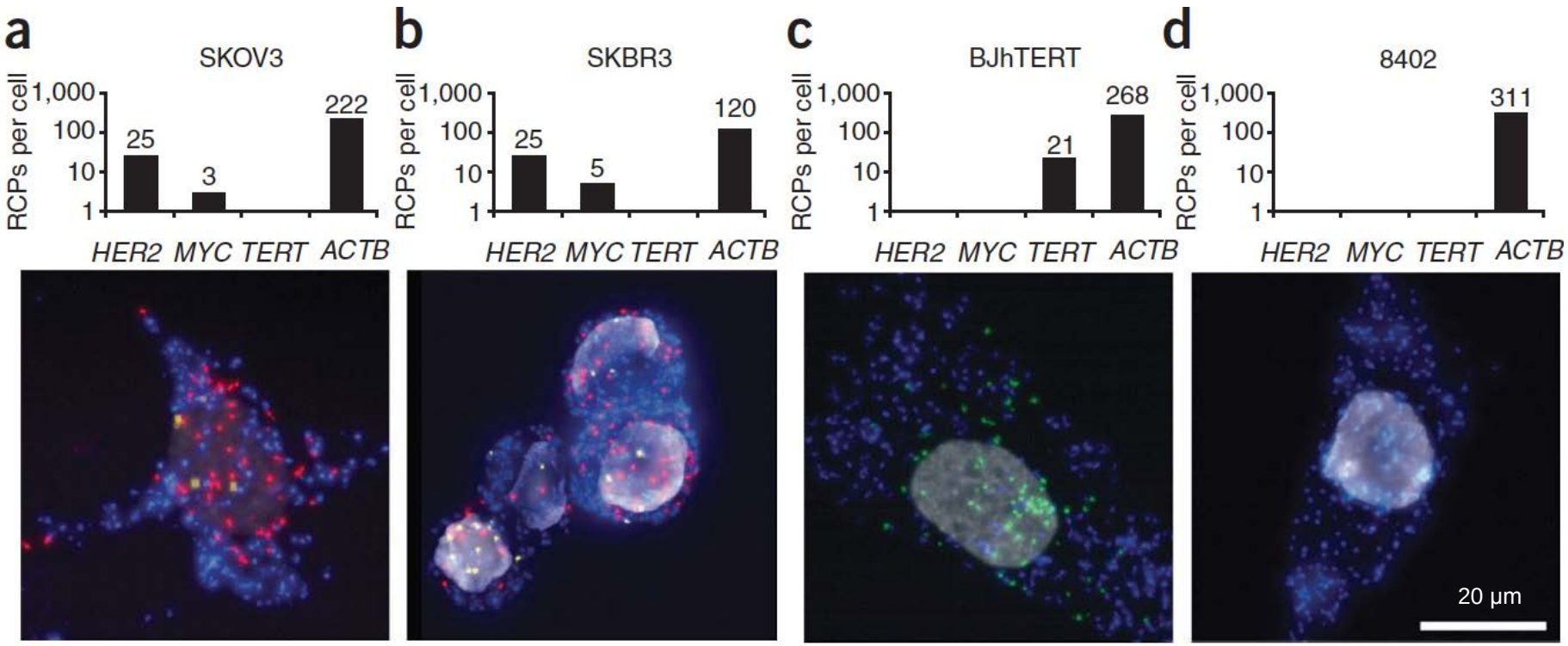
Cell-to-cell variation in expression





Larsson et al., 2010

Multiplex *in situ* detection of cancer-related transcripts in cancer and primary human cell lines



ovarian carcinoma cells

breast carcinoma cells

TERT immortalized fibroblast cells

primary fibroblast culture

Larsson et al., 2010

Summary

- Multiplex detection of expressed single nucleotide sequence variants in human and mouse cells and tissue
- Fresh frozen human tissue sections of 4μm thickness
- Detection probes visualized with Cy3, Cy 5, Texas red and FITC
- Quantification counted digitally using BlobFinder software
- compared in situ data with qPCR
  - ~30% detection efficiency for one assay (β-actin)
  - ~15% detection efficiency in multiplex measurements
- Lower detection rates due to interactions between padlock probes and/or cDNA primers

However,

- Only known sequences can be targeted, and
- Only point mutations detected

- Missing is a technique to detect larger transcripts and unknown sequences

In situ sequencing for RNA analysis in preserved tissue and cells

Rongqin Ke[1,2,5], Marco Mignardi[1,2,5], Alexandra Pacureanu[3], Jessica Svedlund[1], Johan Botling[2], Carolina Wählby[3,4] & Mats Nilsson[1,2]

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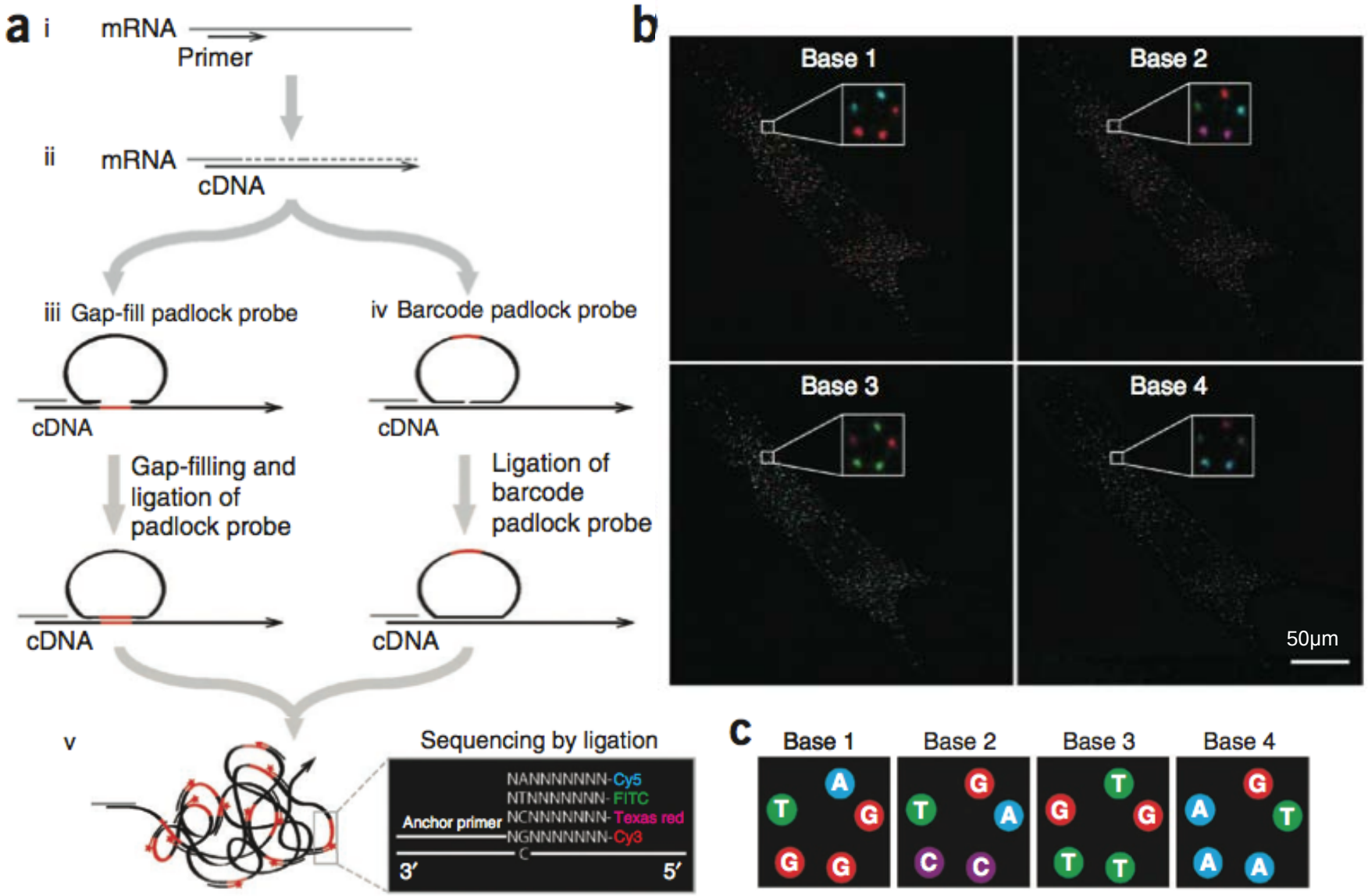


Rongqin et al., 2013

1st cycle

1st cycle
2nd cycle
3rd cycle
4th cycle

A – N – N – N- Yellow

G – N – N – N- Red

T – N – N – N- Blue

C – N – N – N- Green

T – A – N – N- Blue

T – T – N – N- Blue

T – C – N – N- Blue

T – G – N – N- Blue

A – C – T – G

Sequence of interest

1st cycle

1st cycle blue
2nd cycle
3rd cycle
4th cycle

A – N – N – N- Yellow

G – N – N – N- Red

C – N – N – N- Green

only this probe binds and gives a blue signal

T – N – N – N- Blue

A – C – T – G

Sequence of interest

2nd cycle

1st cycle blue
2nd cycle
3rd cycle
4th cycle

N – A – N – N- Yellow

N – T – N – N- Blue

N – C – N – N- Green

N – G – N – N- Red

A – C – T – G

Sequence of interest

2nd cycle

2nd cycle

1st cycle blue
2nd cycle red
3rd cycle
4th cycle

N – C – N – N- Yellow

N – T – N – N- Blue

N – C – N – N- Green

N – G – N – N- Red

only this probe binds and gives a red signal

A – C – T – G

Sequence of interest

3rd cycle

1st cycle blue
2nd cycle red
3rd cycle yellow
4th cycle

N – N – G – N- Red

N – N – T – N- Blue

N – N – C – N- Green

N – N – A – N- Yellow

only this probe binds and gives a yellow signal

A – C – T – G

Sequence of interest

4th cycle

1st cycle blue
2nd cycle red
3rd cycle yellow
4th cycle green

N – N – N – G- Red

N – N – N – T- Blue

N – N – N – A- Yellow

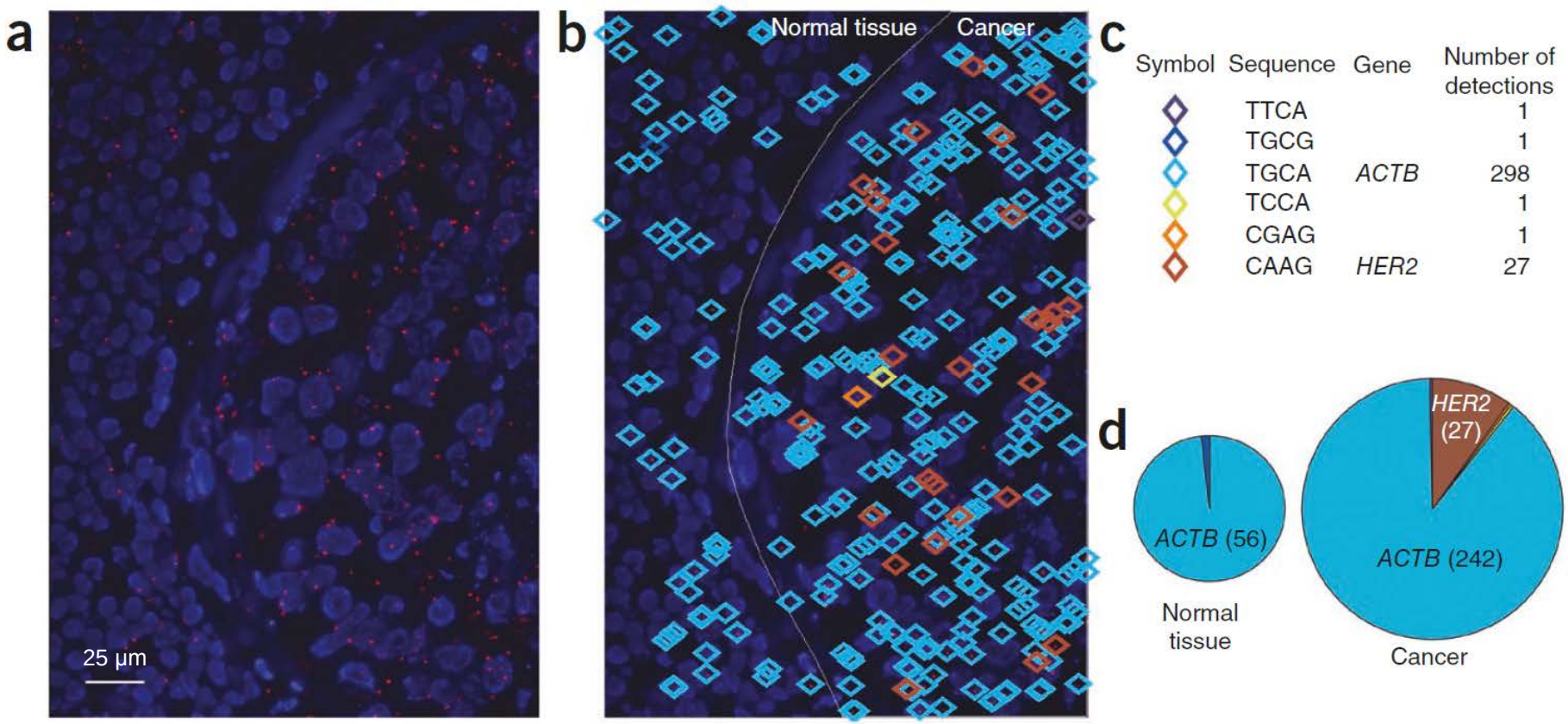
N – N – N – C- Green

only this probe binds and gives a green signal

A – C – T – G

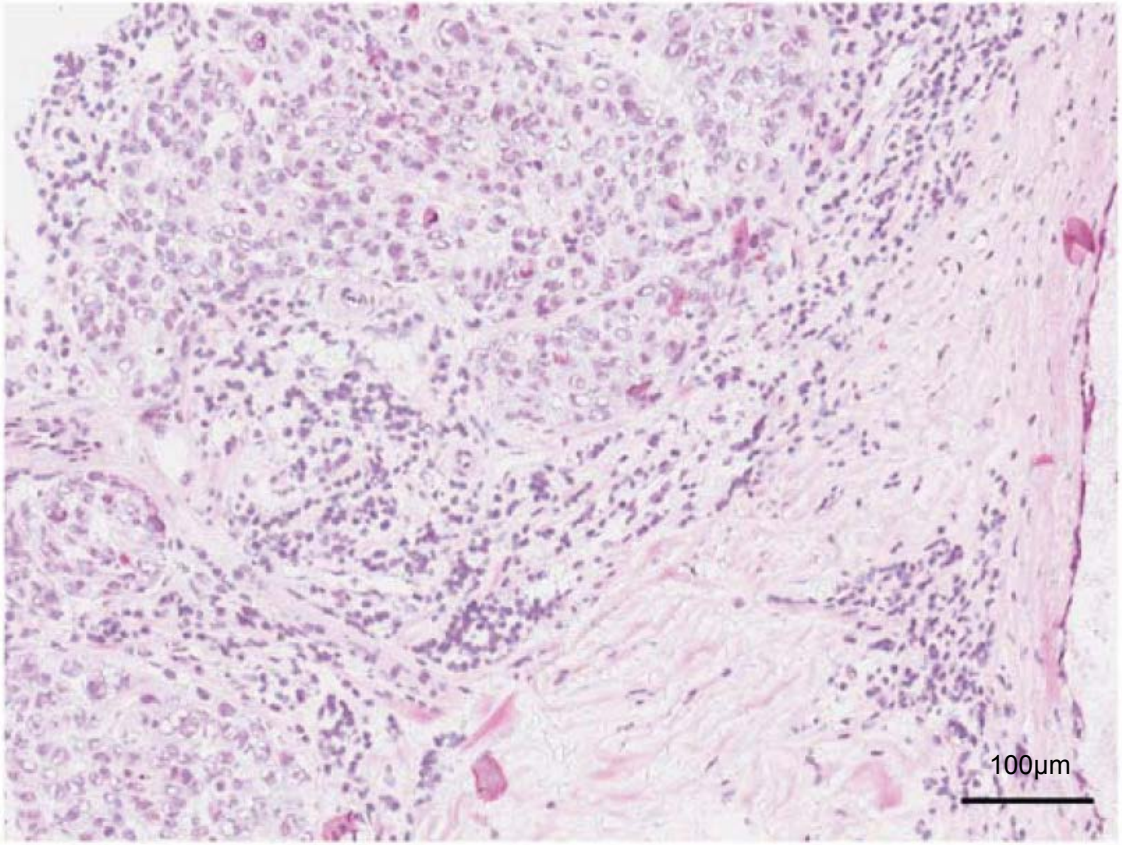
Sequence of interest

- HER2 positive fresh-frozen breast cancer tissue section
- gap-fill padlock probe



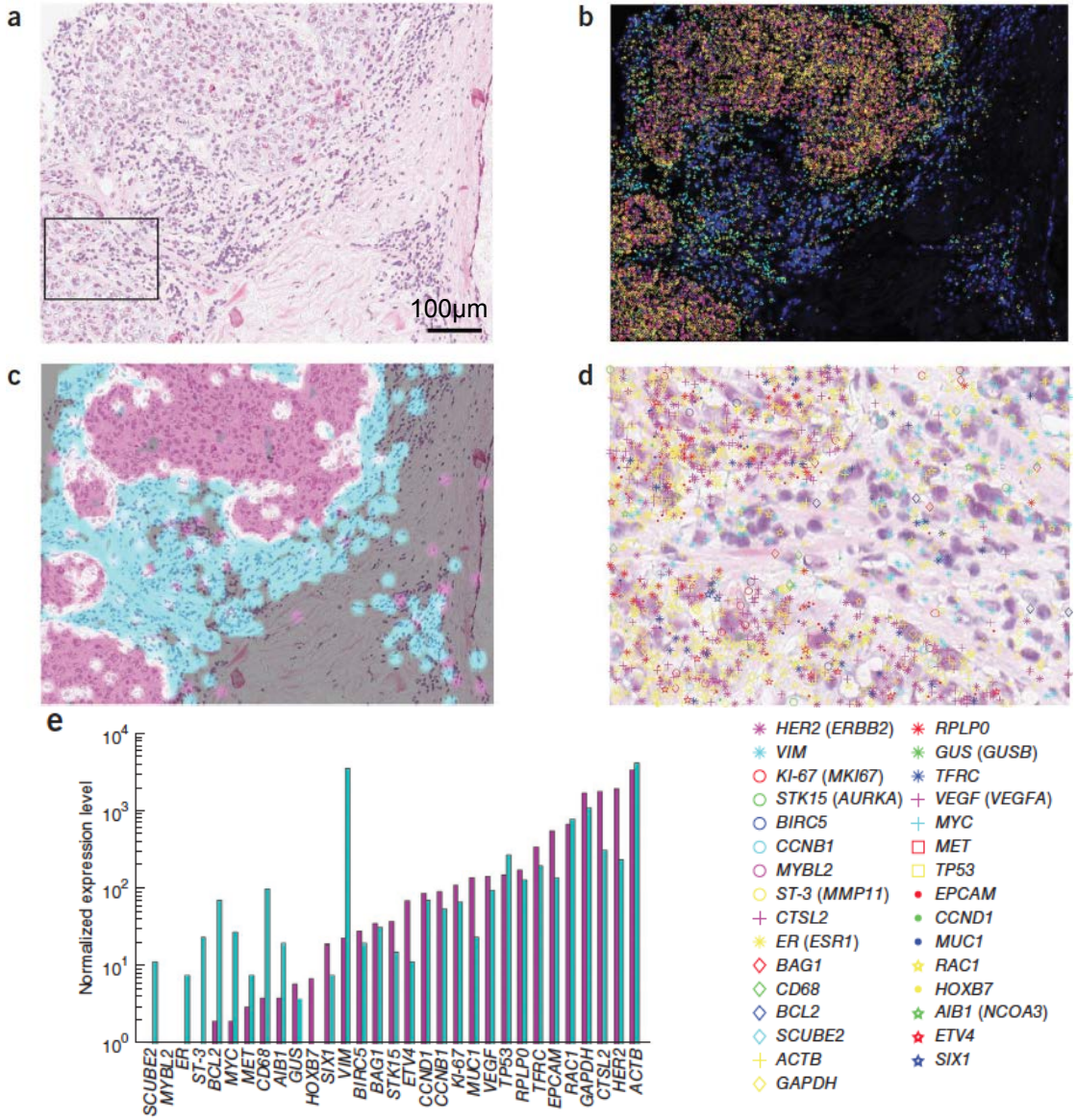
Rongqin et al., 2013

Her2 positive fresh frozen breast cancer



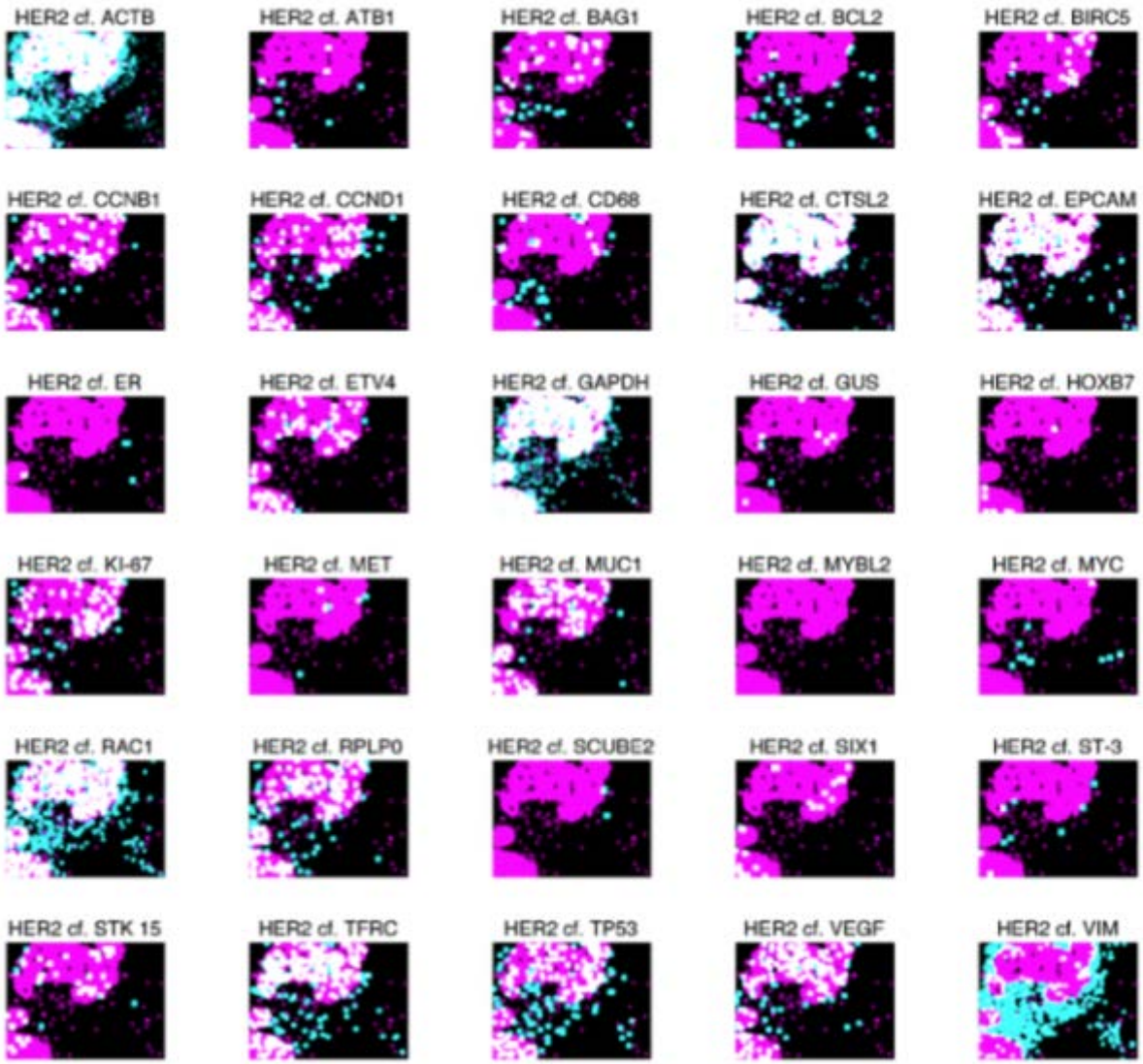
Rongqin et al., 2013

Gene expression profiling by barcode padlock probe



Rongqin et al., 2013

Expression patterns



Rongqin et al., 2013

Maximum number of reads

- 450 cells covering area of 0.16mm^2: 11,423 reads (average of 25 reads/cell)
- Covering 5.5% of the area

- Increase of 20% before loss of signal due to overlap of signals
- 270,000 reads per mm^2

- Comparison of in situ sequencing data with qPCR and published RNA seq data

Summary

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- In situ detection of up to 31 short RNA fragments
- Visualizing multiplexed gene expression in human breast cancer tissue sections
- Four-base-pair fragments detectable (4^4)

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- Depending on the method, target sequences still have to be determined
- Critical step: detecting every single nucleotide by using different probes
- Efficiency 30%
- Interactions between LNA primers
- Dependent on tissue quality, thickness

Outlook

- Detection of mutations in the microenvironment of tumors and other lesions
- Longer transcripts detectable
- Characterization and identification of tissue without a priori information
- Can be combined with in situ proximity ligation assay to detect interactions or post-translational modifications of proteins

Questions?

Thank you for your attention!