Section in situ hybridization

Kirsi Sainio

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Section / cellular ISH types

- Radioactive ISH for cells and tissue sections – radiolabeling of probes and detection by autoradiography
- Non-radioactive ISH – probes labeled with haptens or fluorochromes – cellular, chromosomal or tissue section ISH

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Sections

- Paraffin/resin embeddeds sections
- Frozen sections
- Vibratome sections
- Electron microscopy samples

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Optimizing ISH

- Optimized ISH for section (as well as whole mount) protocols share several common goals:
  - retention of tissue morphology
  - rendering tissue permeable to probe
  - retaining target mRNA within the tissue
  - effective penetration and binding of probes
  - reduction of nonspecific background

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

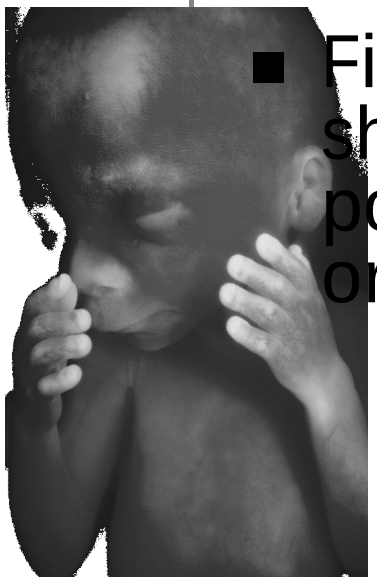
Optimizing ISH

- The critical parameters that result in successful ISH are type of fixative and length of tissue fixation, method for embedding fixed tissue, agents used for sample permeabilization, choice of hybridization conditions, and post-hybridization treatment

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Fixation

- Perfusion is much better at preserving tissue quality and RNA integrity because of the rapid spread of fixative through the cells
- In addition, perfusion results in ISH data with low background due to clearance of blood cells from the tissue
- Fixation by immersion, on the other hand, should be used when perfusion is not possible - for example with clinical samples or embryonic tissues



Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

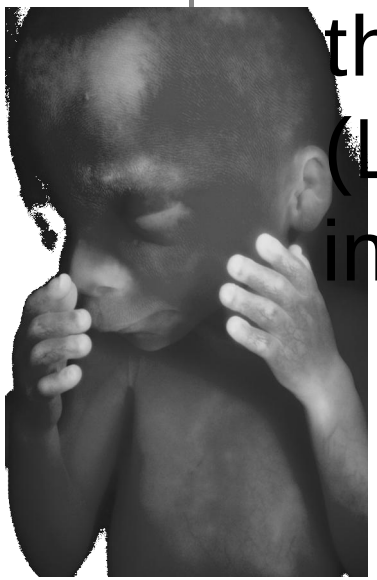
Fixatives

- Fixation should ideally prevent the loss of cellular RNAs during hybridization while preserving accessibility of the target RNA to the probe
- Precipitating fixatives (such as ethanol/acetic acid or Carnoy's Solution) function by precipitating proteins to trap the RNA inside cells

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Fixatives

- They provide the best probe penetration
- Tissues fixed by precipitating fixatives are subject to loss of target mRNA and the cell's morphological structure (Lawrence and Singer, 1985), resulting in poor ISH data quality



Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Fixatives

- The primary fixative of choice of most investigators is 4% neutral buffered formalin or 4 % paraformaldehyde
- Aldehyde fixatives are not always the best alternative although it seems that they tend to be the ONLY alternative

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Fixatives

- Tissue fixation by formaldehyde works by crosslinking amino groups, thereby preventing loss of the mRNA target
- During hybridization, high temperature and formamide remove some of these crosslinks

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Fixatives

- This promotes penetration of the probe, but may also lead to unwanted loss of the target RNA
- Thus, the ratio between the temperature of hybridization and the strength of fixation is very important to obtain an optimal signal

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Fixatives

- When using RNA probes the hybridization temperature should be high enough to ensure specific binding of the probe
- Fixation of the tissue under alkaline pH sometimes dramatically improves the signal when using RNA probes

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Embedding

- Cryostat sections of frozen tissue and paraffin embedded tissue sections have both been effectively used for ISH
- In general, paraffin-embedded tissues show better morphology than frozen tissue
- Paraffin embedding requires more tissue processing and can result in RNA loss and low ISH signal (Pintar and Lugo, 1985)

Embedding

- Paraffin sections should be used with caution for ISH experiments on mammalian tissues where sensitivity is critical
- paraffin sections still have particular value in preparation of clinic, pathological and research samples for long-term protection of tissue morphology

Permeabilization

- The most critical step in successfull ISH both in sections and in whole mounts
- Usually enzymatic (proteinase K) or chemical (HCl) permeabilization
- Different samples require different treatments!!
- For instance brain tissues fixed in 4% paraformaldehyde overnight: deproteination by PK is either unnecessary or detrimental to RNA retention

Permeabilization

- PK digestion of the cell may result in loss of mRNAs or a loss of morphology
- addition of HCl diluted in triethanolamine increases detection sensitivity in paraformaldehyde fixed samples, possibly due to its ability to denature ribosomes, thus exposing additional target mRNAs to probe

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Specificity

- In sections background signal arises primarily from nonspecific retention of probe in tissue sections (due to electrostatic interactions between probe and tissue macromolecules)
- Several chemical functional groups in proteins (such as amine and carboxylate groups) are believed to induce this nonspecific binding

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

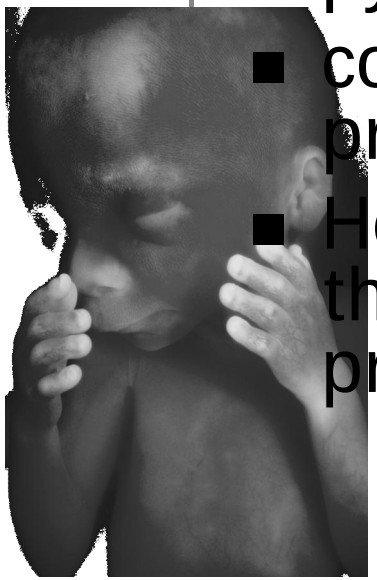
Specificity

- Minimize this source of background by treating tissue slides with acetic anhydride and triethanolamine (Hayashi et al., 1978)
- Acetylation of amine groups by acetic anhydride, routinely used in ISH protocols, maybe important in reducing backgrounds (for probes larger than 2.0 kb) (Lawrence and Singer, 1985)

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Specificity

- Another way to decrease nonspecific probe binding is to saturate the binding sites on proteins by incubating tissue with prehybridization solution
- ficoll, bovine serum albumin, polyvinyl pyrrolidone, and nucleic acids
- compete with the nonspecific binding of probes to tissue
- However, addition of the above reagents to the hybridization buffer does not completely prevent background signal



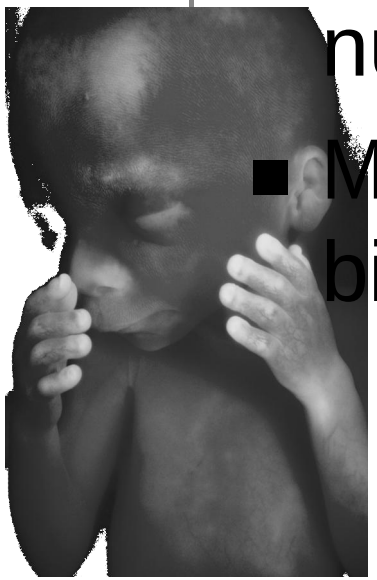
Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Specificity

- Nuclease treatment after hybridization is still necessary for reducing this nonspecific signal (nuclease treatment degrades unhybridized, single stranded probe)
- Without RNase treatment, the background with [^{33}P]-labeled RNA probes may be so high that specific hybridization signal is not discernable
- RNA probes tend to exhibit high levels of nonspecific binding, so RNase treatment could help if this is a problem

Specificity

- High stingency washing conditions after cRNA-mRNA ISH decrease the background
- Mostly washes away the unbound nucleotides and off-target hybrids
- May also affect somewhat the specific binding

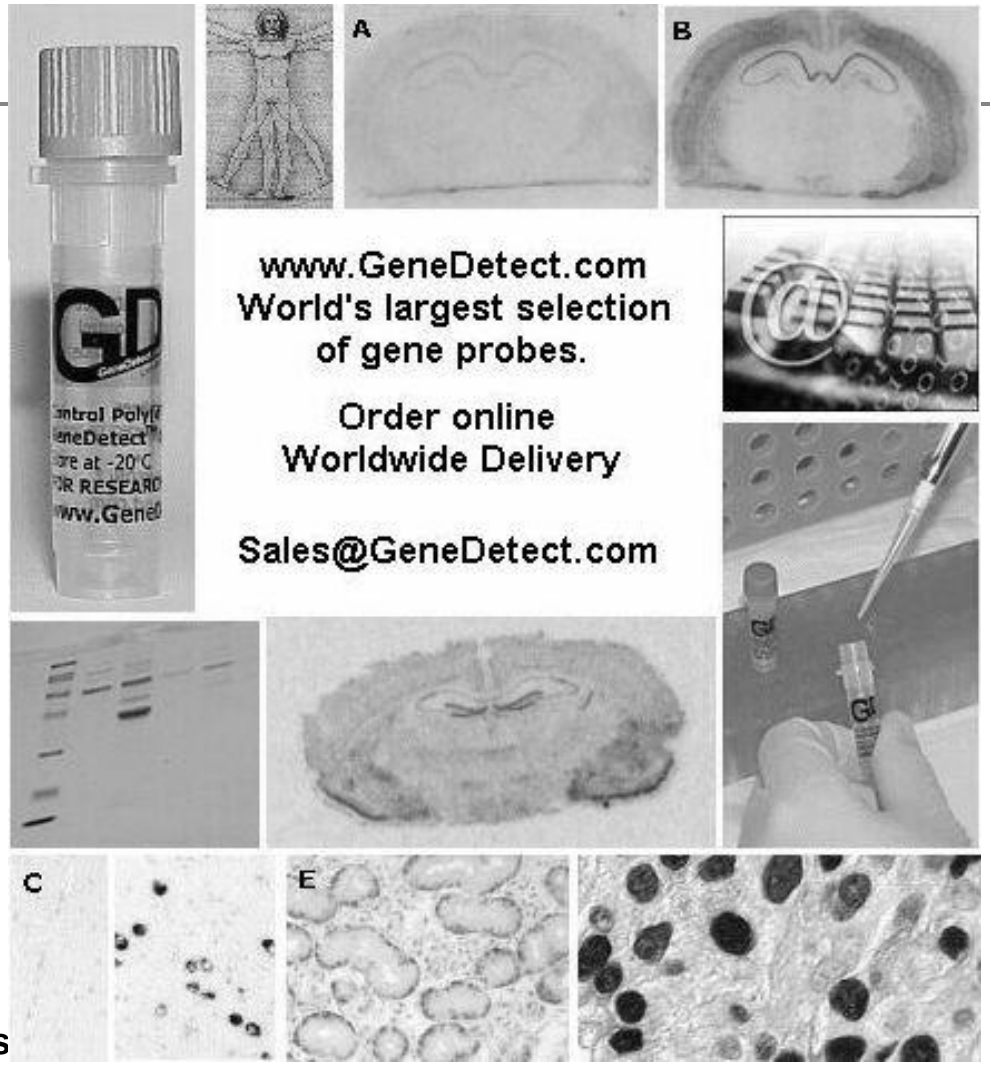


Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Specificity

- While there are different recipes for making hybridization buffers, the inclusion of dextran sulphate in the hybridization solution increases probe binding to target mRNA
- including 10% dextran sulphate enhances ISH signal several fold
- too much dextran sulphate in the hybridization buffer will induce high background, which is difficult to remove in post hybridization washes

Probes



Kirs
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

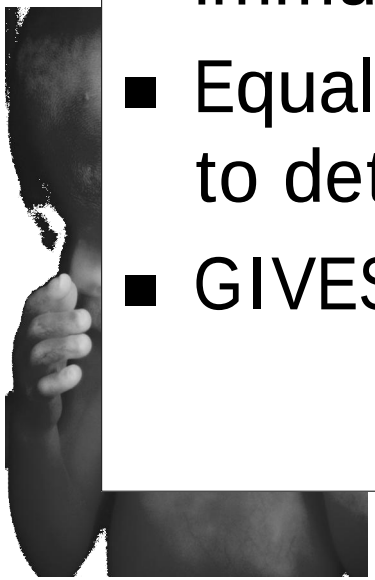
Labels

- Radioactive methods are sensitive, but require radionucleotides, are time-consuming and give poor detection in cellular level (autoradiography detection)
- Demanding method, but once set-up works fairly constantly and gives good results

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

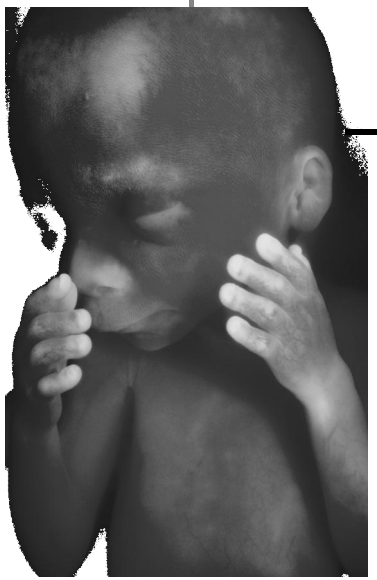
Labels

- Non-radioactive methods are also sensitive in section level, give more possibilities in the choice of label, are quick, give good resolution in single cell level, give a possibility to double-labelling or even combination of ISH and immunohistochemistry
- Equally demanding method, sometimes difficult to detect small amount of target
- GIVES THE DETECTION IN SINGLE CELL LEVEL



Labels

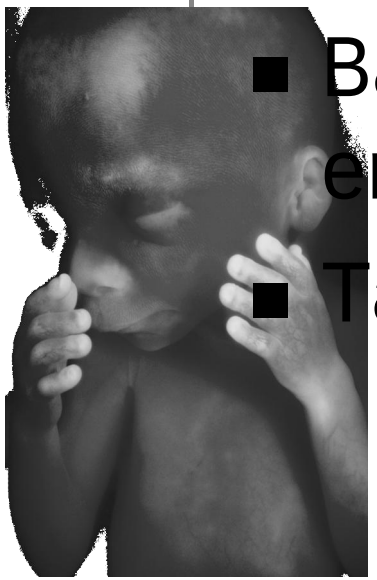
- Radiolabels:
  - For RNA ISH S^{35}-labeled UTP most often used, also P^{33} can be used
  - S^{35} labelled RNA probes usually give higher backgrounds
  - dithiothreitol (DTT) should be added to all solutions used in prehybridization, hybridization, and posthybridization washes



Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

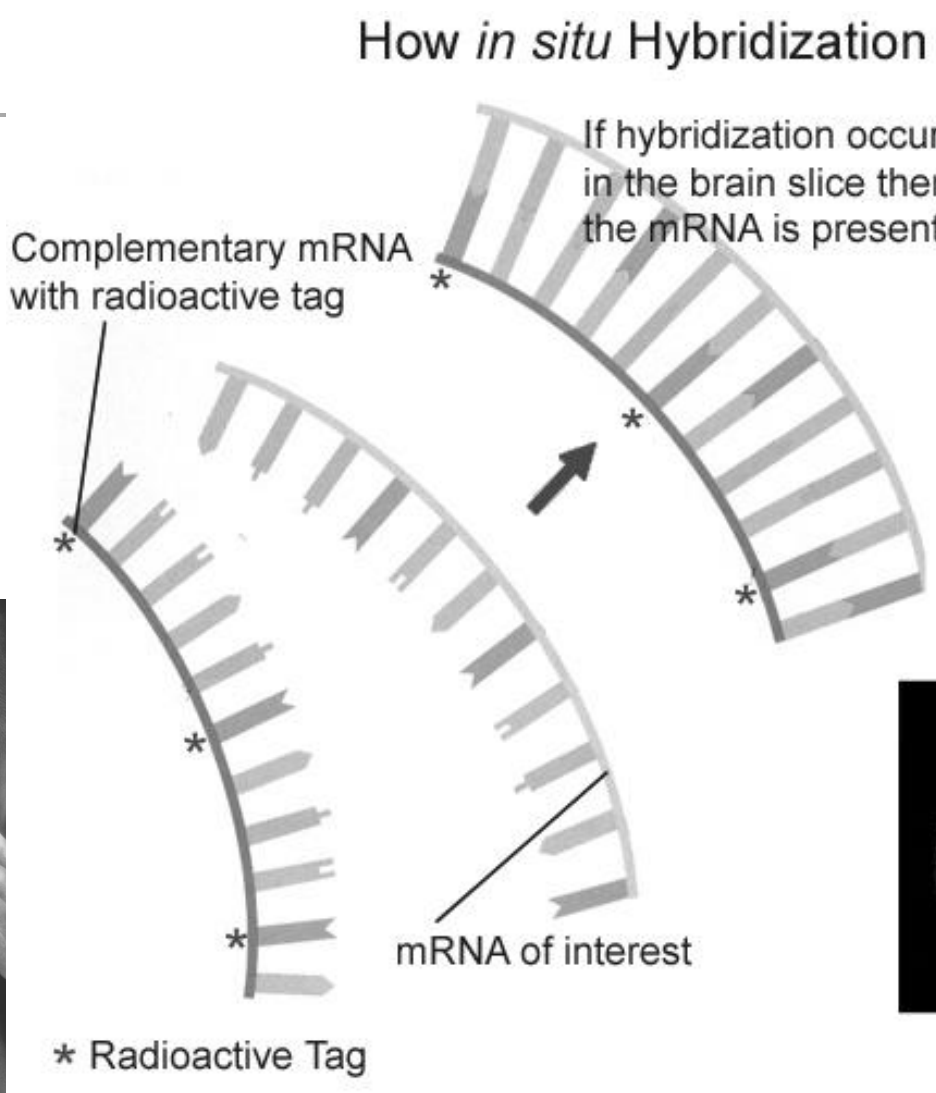
Radioactive ISH

- Detection possible only by autoradiography
- If this is not done properly, it can spil the whole ISH!
- Based on ”standard” photography emulsion/development process
- Takes several days/weeks

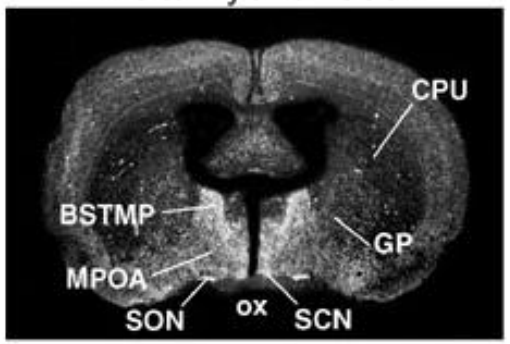


Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

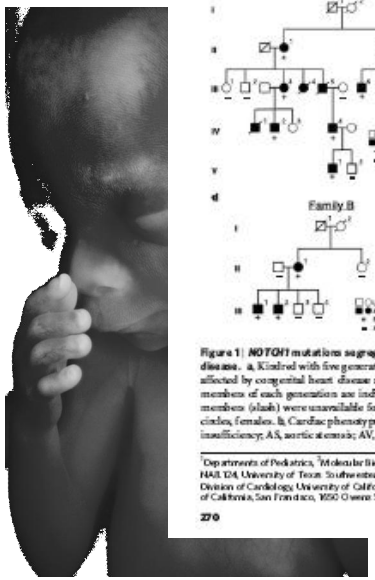
Radiolabeled probes



Localization of the OFQ receptor in the hypothalamus using *in situ* hybridization



Radioactive ISH



nature Vol 437 | 8 September 2005 | doi:10.1038/nature03940

LETTERS

Mutations in *NOTCH1* cause aortic valve disease

Vidu Garg[1,5], Alecia N. Muth[1]†, Joshua F. Ransom[1]†, Marie K. Schluterman[1], Robert Barnes[3,4], Isabelle N. King[1,5]†, Paul D. Grossfeld[6] & Deepak Srivastava[1,2,4,5]†

Calcification of the aortic valve is the third leading cause of heart disease in adults[1]. The incidence increases with age, and it is often associated with a bicuspid aortic valve present in 1–2% of the population[2]. Despite the frequency, neither the mechanisms of valve calcification nor the developmental origin of a two, rather than three, leaflet aortic valve is known. Here, we show that mutations in the signalling and transcriptional regulator *NOTCH1* cause a spectrum of developmental aortic valve anomalies and severe valve calcification in non-syndromic autosomal-dominant human pedigrees. Consistent with the valve calcification phenotype, *Notch1* transcripts were most abundant in the developing aortic valve of mice, and Notch1 repressed the activity of Runx2, a central transcriptional regulator of osteoblast cell fate. The hairy-related family of transcriptional repressors (Hrt), which are activated by Notch1 signalling, physically interacted with Runx2 and repressed Runx2 transcriptional activity independent of histone deacetylase activity. These results suggest that *NOTCH1* mutations cause an early developmental defect in the aortic valve and a later de-repression of calcium deposition that causes progressive aortic valve disease.

Abundant evidence suggests a major inherited component to the aetiology of aortic valve disease in children and adults[3,4]. The most severe type of aortic valve obstruction in children results in failure of the fetal left ventricle to grow, a condition known as hypoplastic left heart syndrome. About 10% of relatives of hypoplastic left heart syndrome patients have bicuspid aortic valve, often undiagnosed, suggesting a common genetic aetiology with phenotypic heterogeneity[4]. The valve calcification often observed in bicuspid aortic valve is a result of inappropriate activation of osteoblast-specific gene expression[4], but the mechanism is unknown.

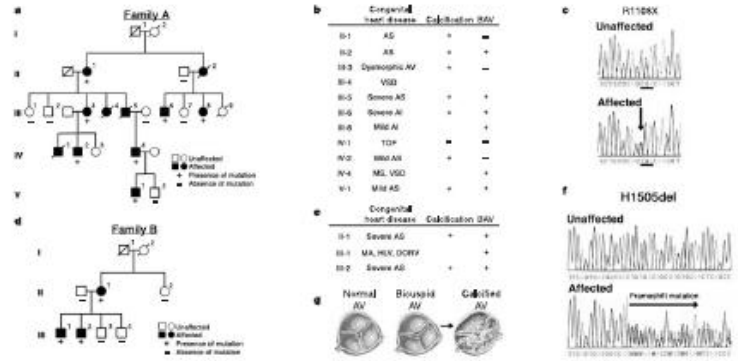
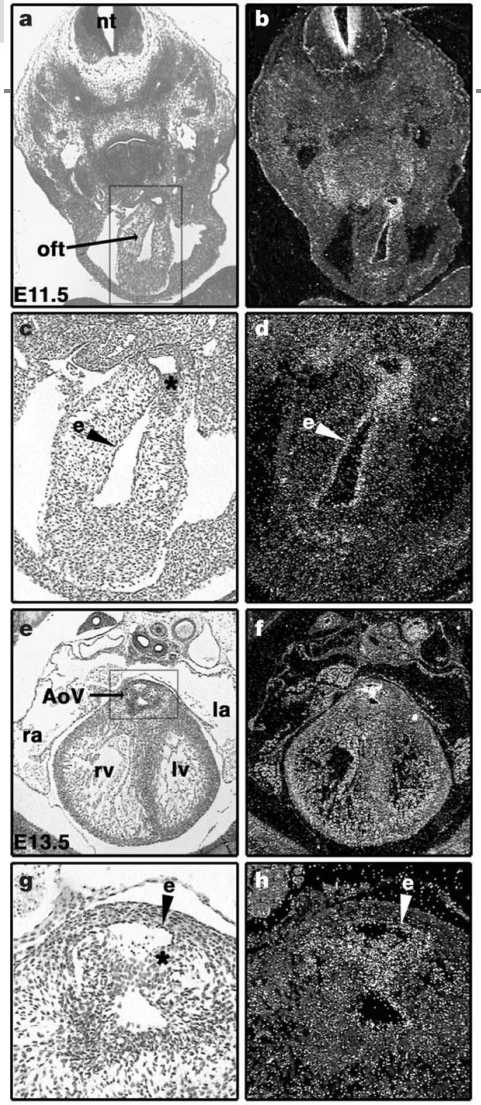
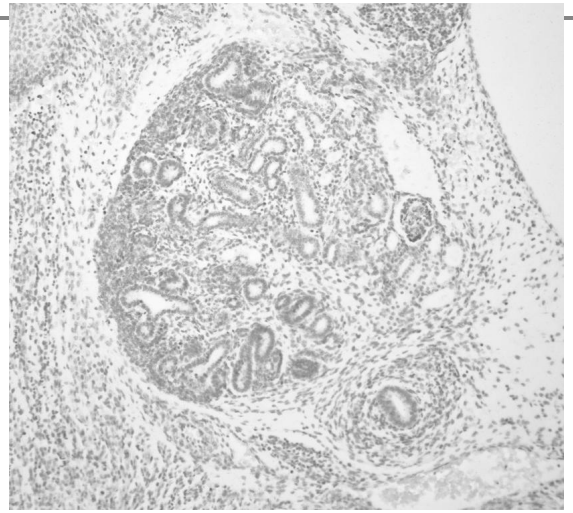


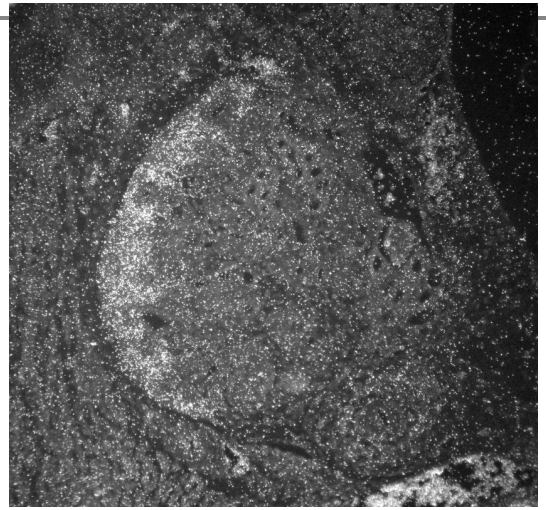
Figure 1 | *NOTCH1* mutations segregate with familial aortic valve disease. a, Kindred with five generations (indicated with Roman numerals) affected by congenital heart disease and valve calcification. Participating members of each generation are indicated numerically. Deceased family members (slash) were unavailable for mutation analysis. Squares, males; circles, females. b, Cardiac phenotype in affected family members. AI, aortic insufficiency; AS, aortic stenosis; AV, aortic valve; BAV, bicuspid aortic valve; TOF, tetralogy of Fallot; VSD, ventricular septal defect. c, Sequence chromatogram of affected family members. d, Kindred with three members affected by congenital heart disease. e, Cardiac phenotype of family B. DO RV, double-outlet right ventricle; HLV, hypoplastic left ventricle; MA, mitral atresia; MS, mitral stenosis. f, Sequence chromatogram of affected members in family B. g, Schematic of normal trileaflet aortic valve, bicuspid aortic valve and calcified aortic valve.

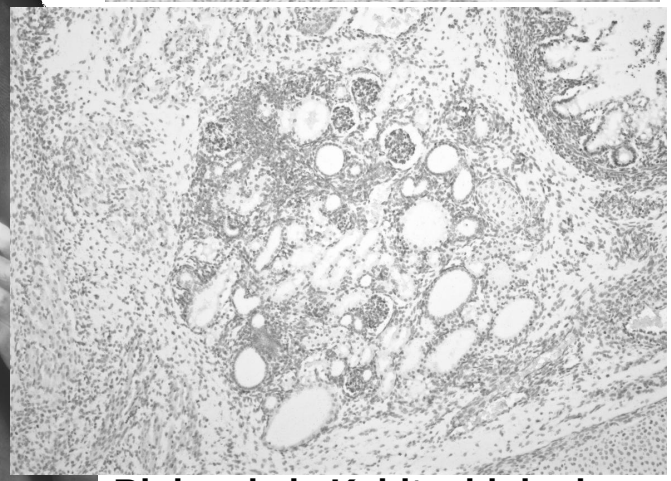
[1]Departments of Pediatrics, [2]Molecular Biology and [3]Internal Medicine, and [4]the McDermott Center for Human Growth and Development, 6000 Harry Hines Boulevard, Room NA8.124, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9148, USA. [5]Children's Medical Center, Dallas, Texas 75235, USA. [6]Department of Pediatrics, Division of Cardiology, University of California, San Diego 92123, USA. †Present address: Gladstone Institute of Cardiovascular Disease and Department of Pediatrics, University of California, San Francisco, 1650 Owens Street, San Francisco, California 94158, USA.

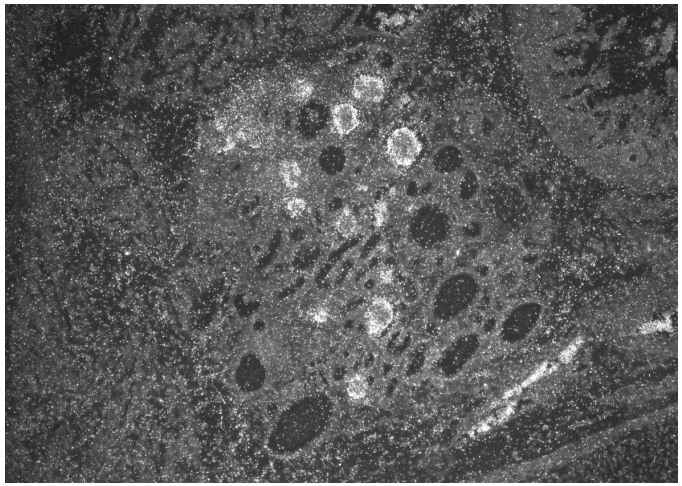


Analysis of the results





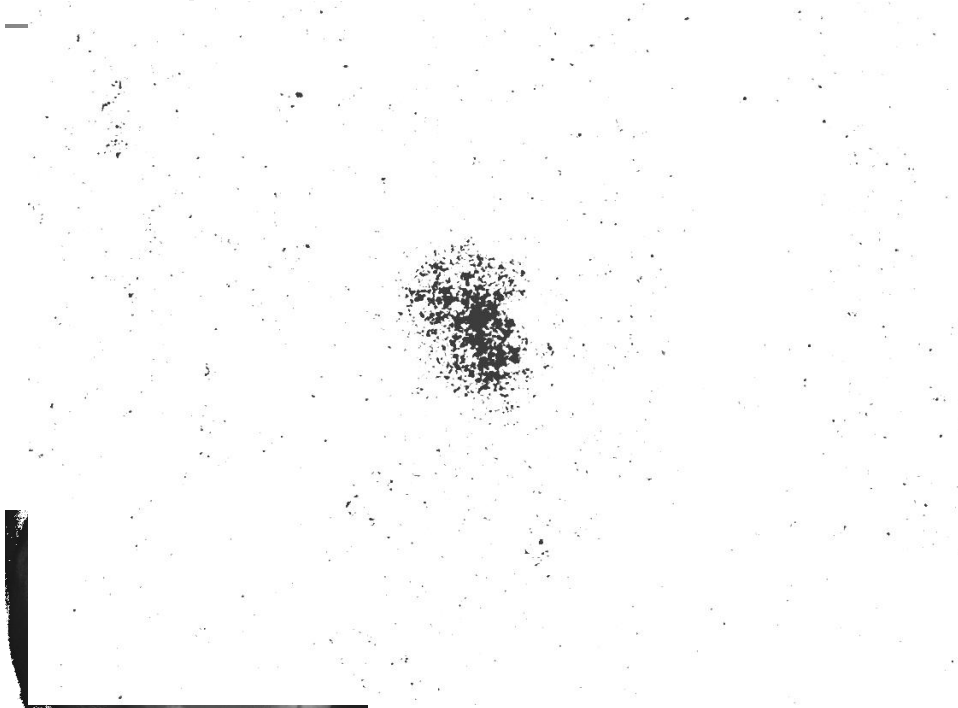


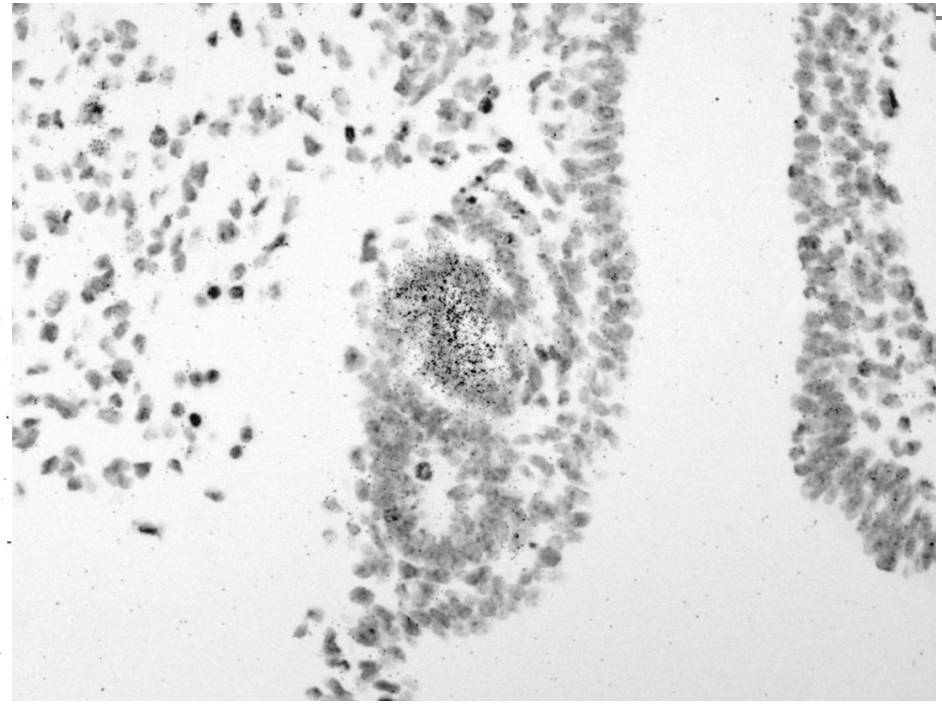


How to visualize autoradiography ?

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

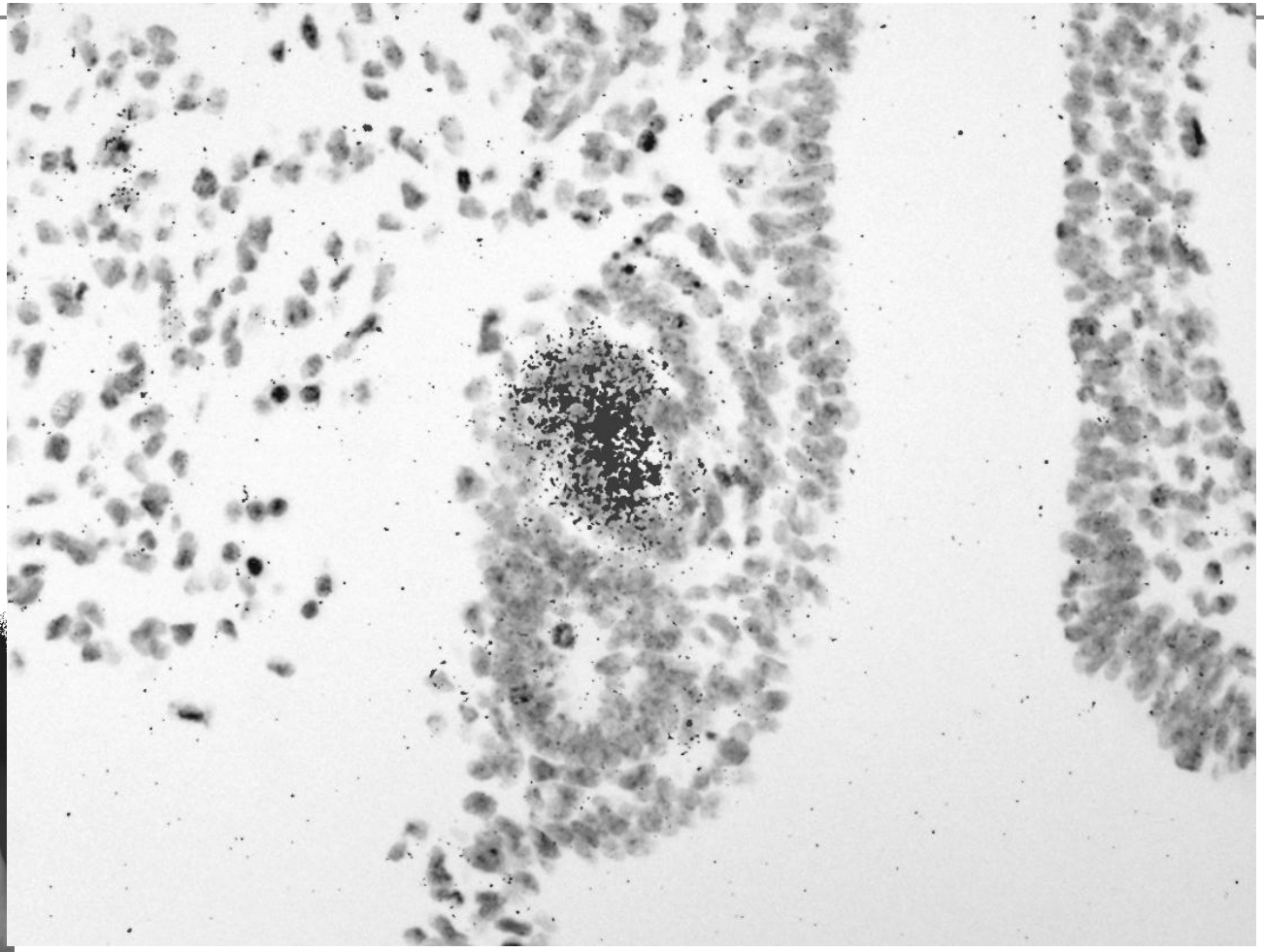
Photoshop helps ...



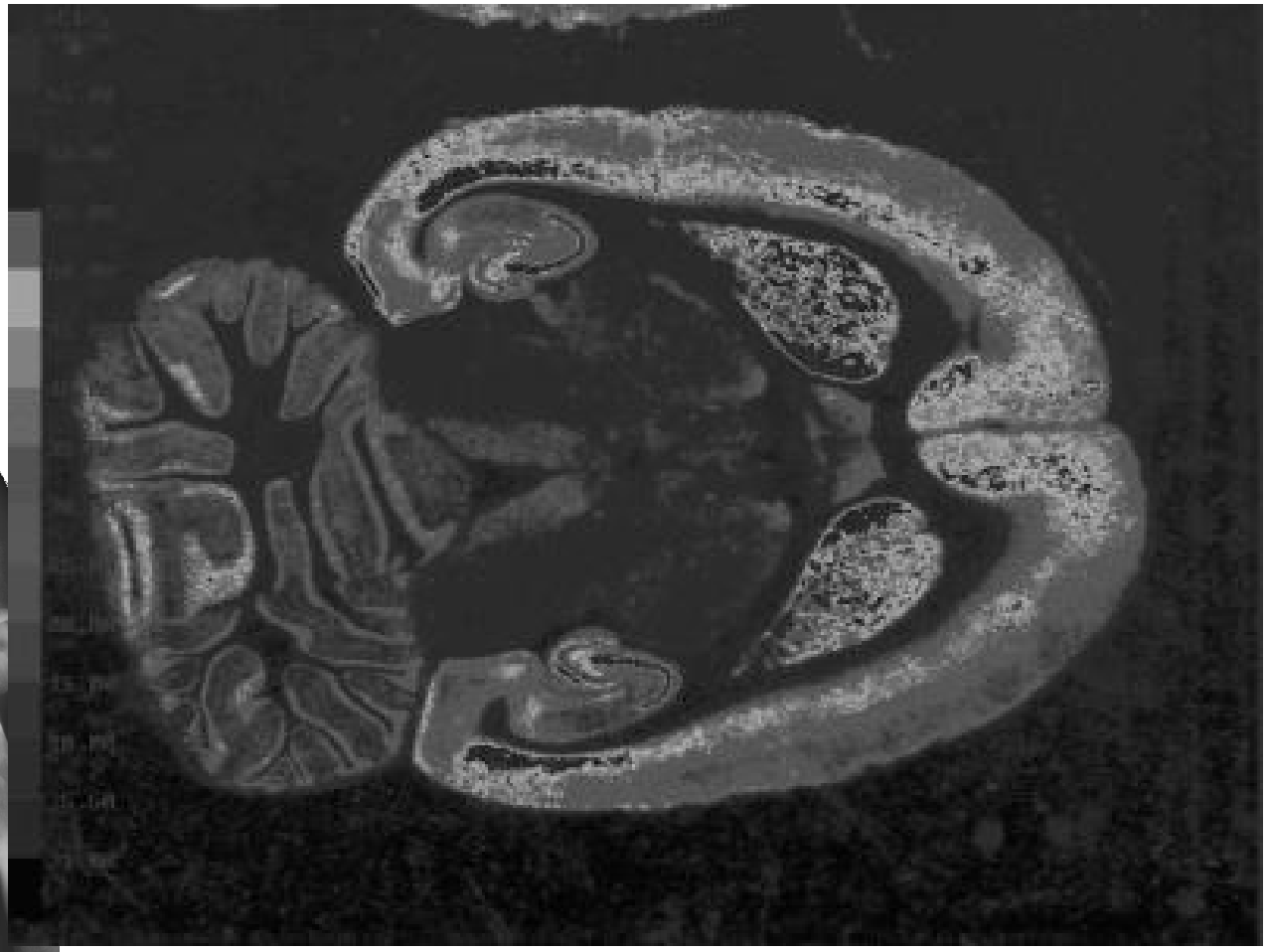


Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

... to make it look like a real thing



Artificial colors to visualize autoradiography



Biokemia ja Kehitysbiologia
Biolääketieteen laitos

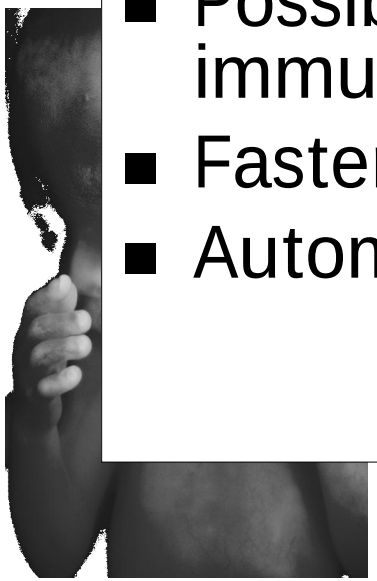
Radioactive ISH

- When sensitive method is needed
- Time is sometimes money!
- Not suitable for high-throughput studies
- More hazardous waiste products
- Autoradiography is difficult and can spoil the whole thing...

Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

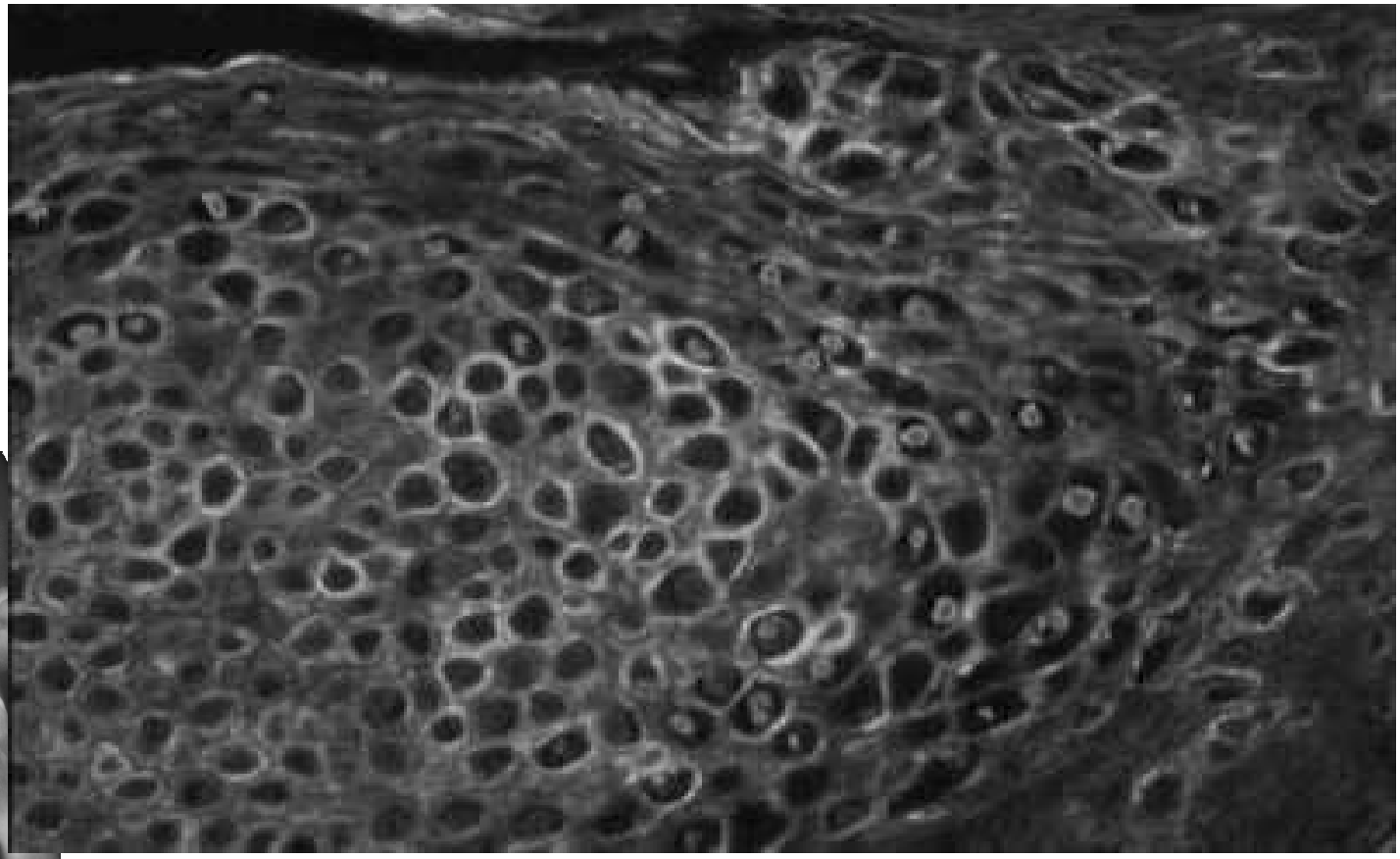
Non-radioactive ISH

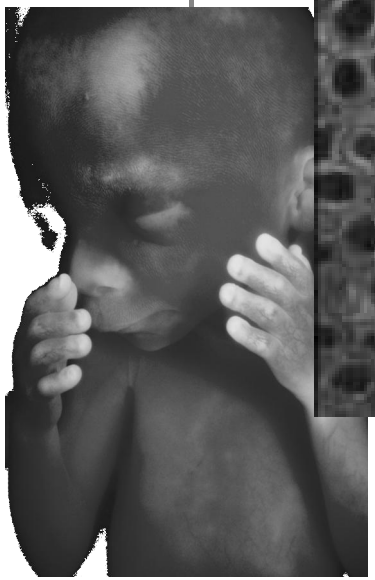
- Nonisotopic labeling systems (such as digoxigenin and biotin) are also frequently used for section ISH studies
- Same labels and detection methods than in whole mount ISH
- Possibility to multiple labelings and modifications
- Possibility to include protein immunohistochemisty
- Faster, high throughput studies are possible
- Automated systems possible



Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

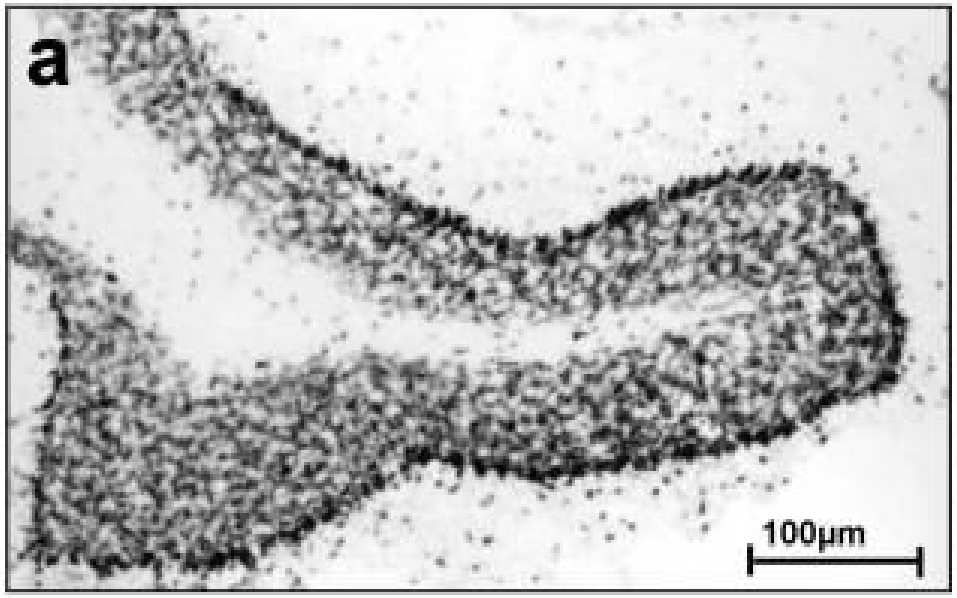
MULTI ISH/immunohistochemistry

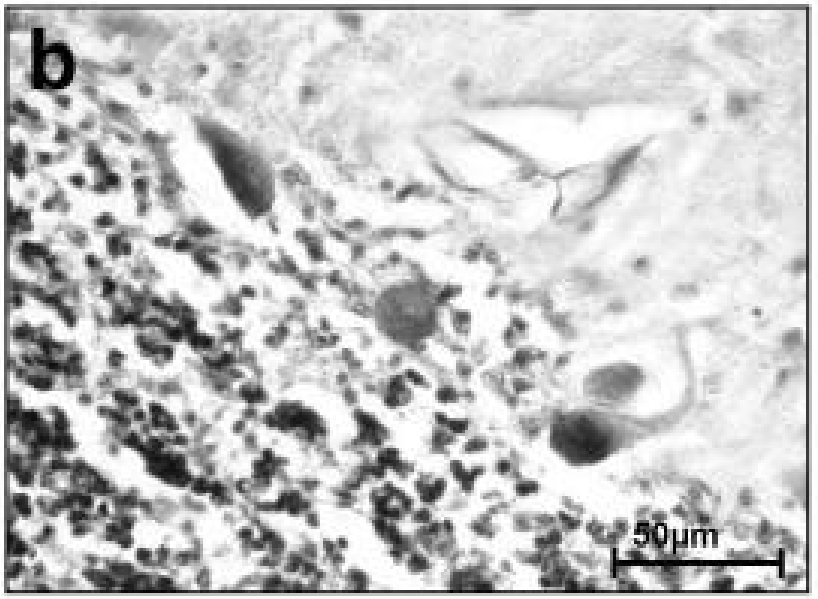


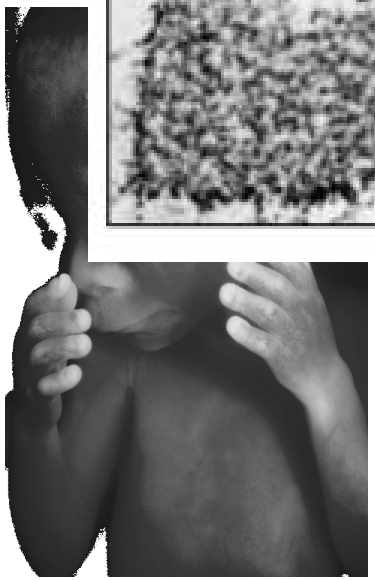


Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Non-radioactive section ISH

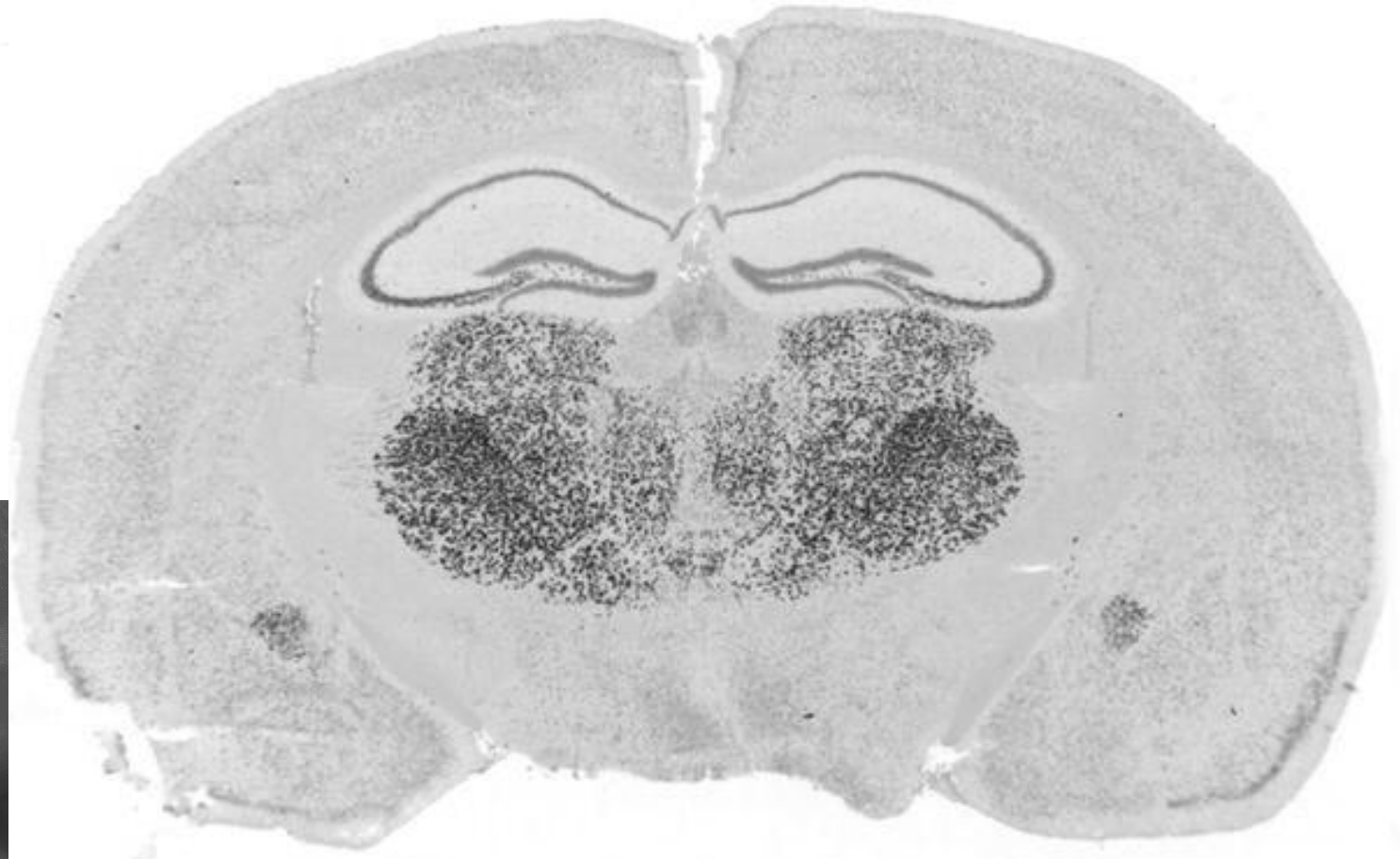




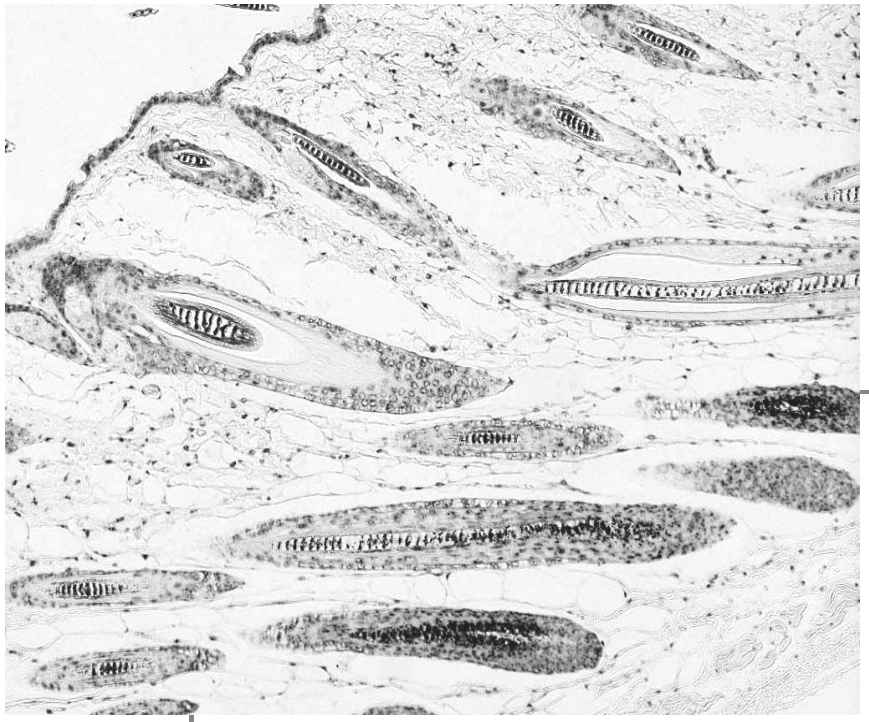


Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

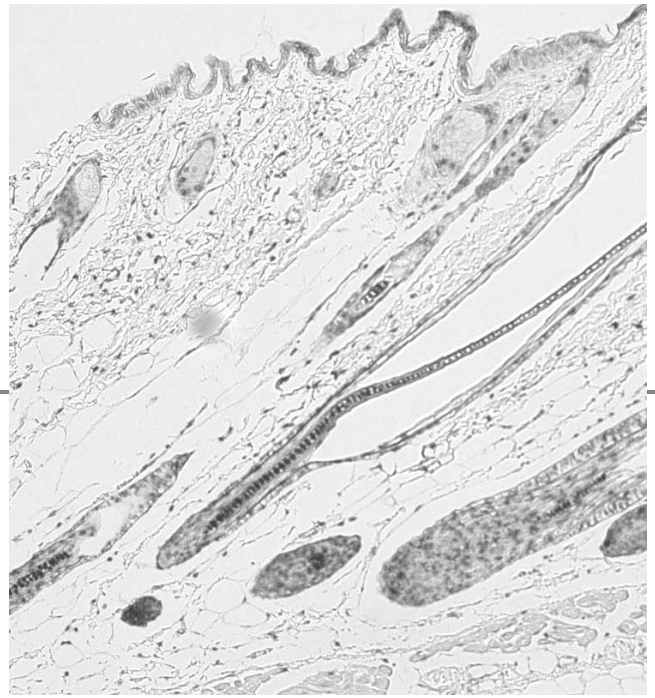
When it is nice, it is nice...



Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

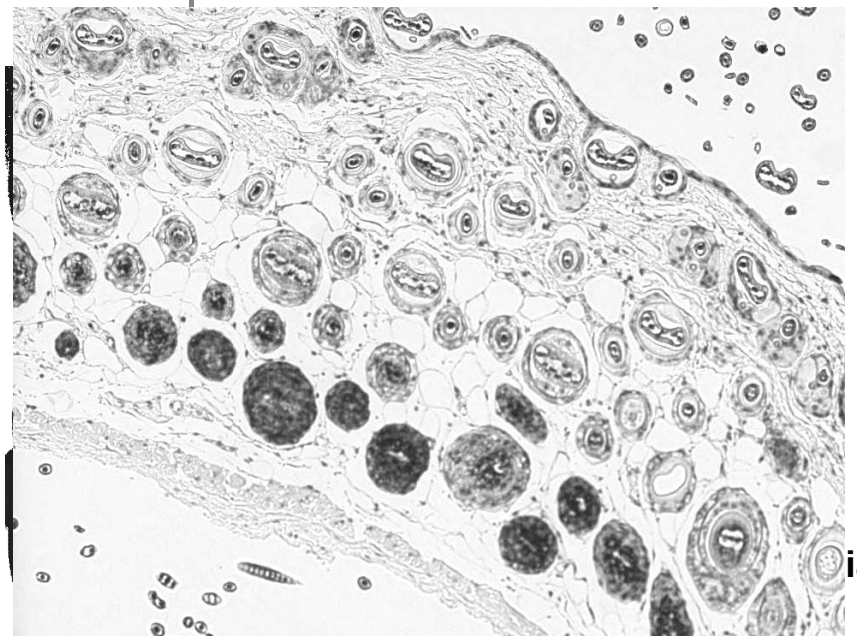


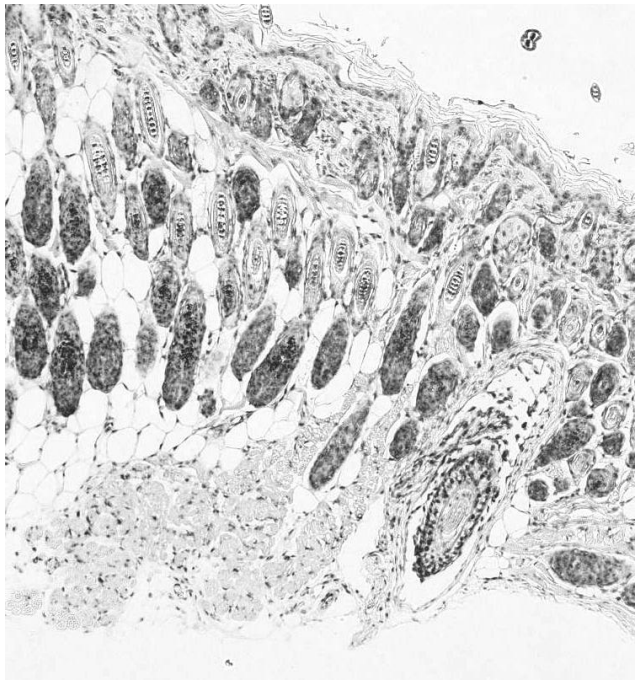
Rmpr



5 days

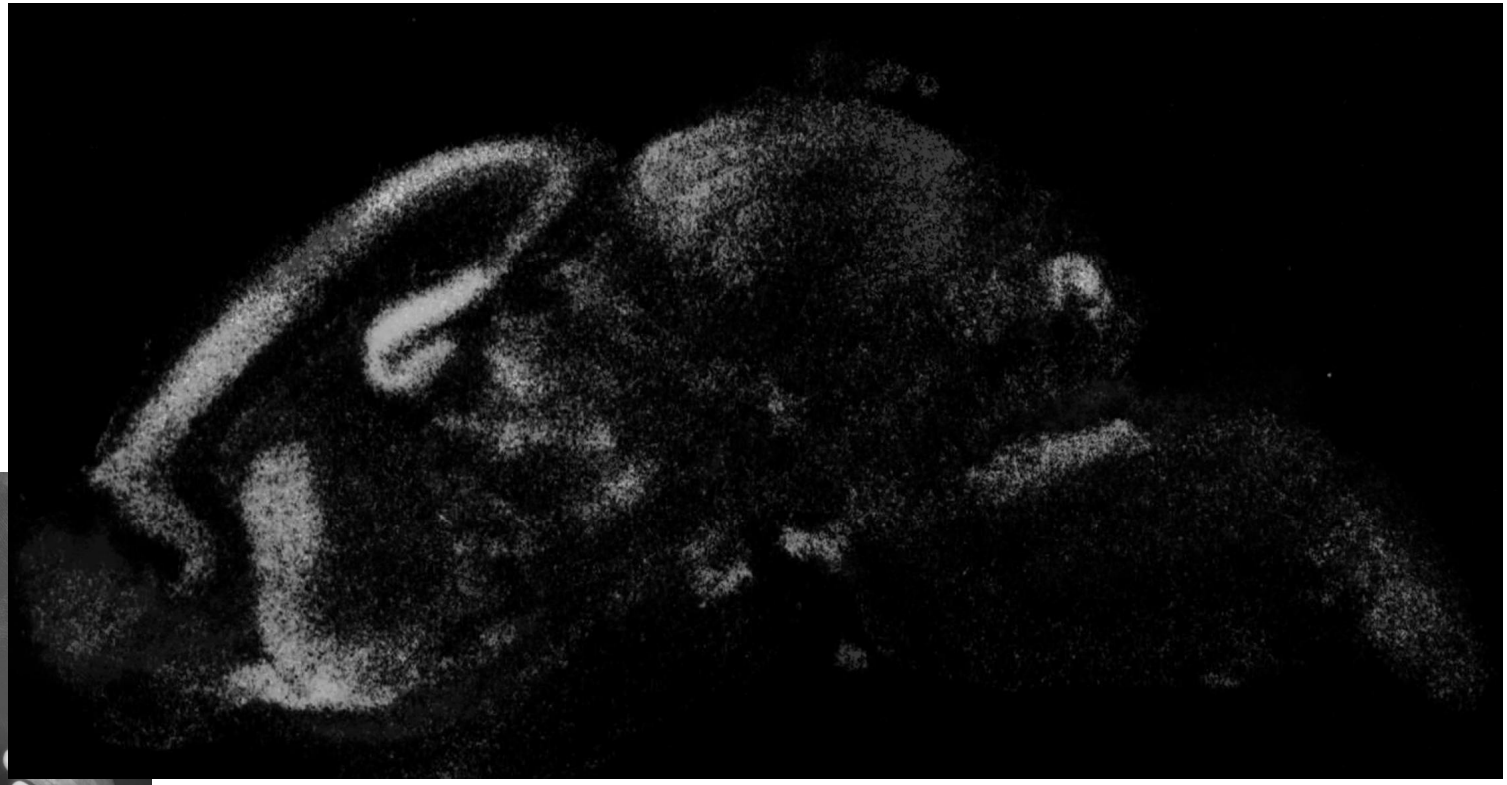
1 month

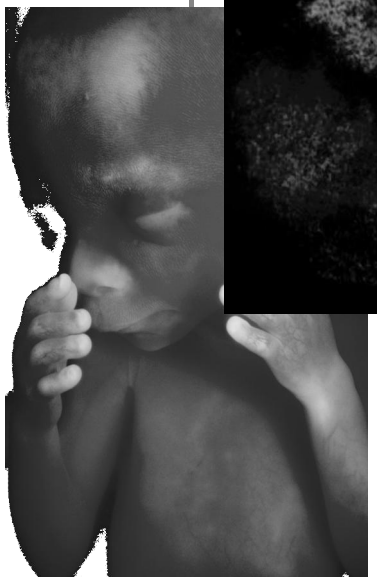




ia

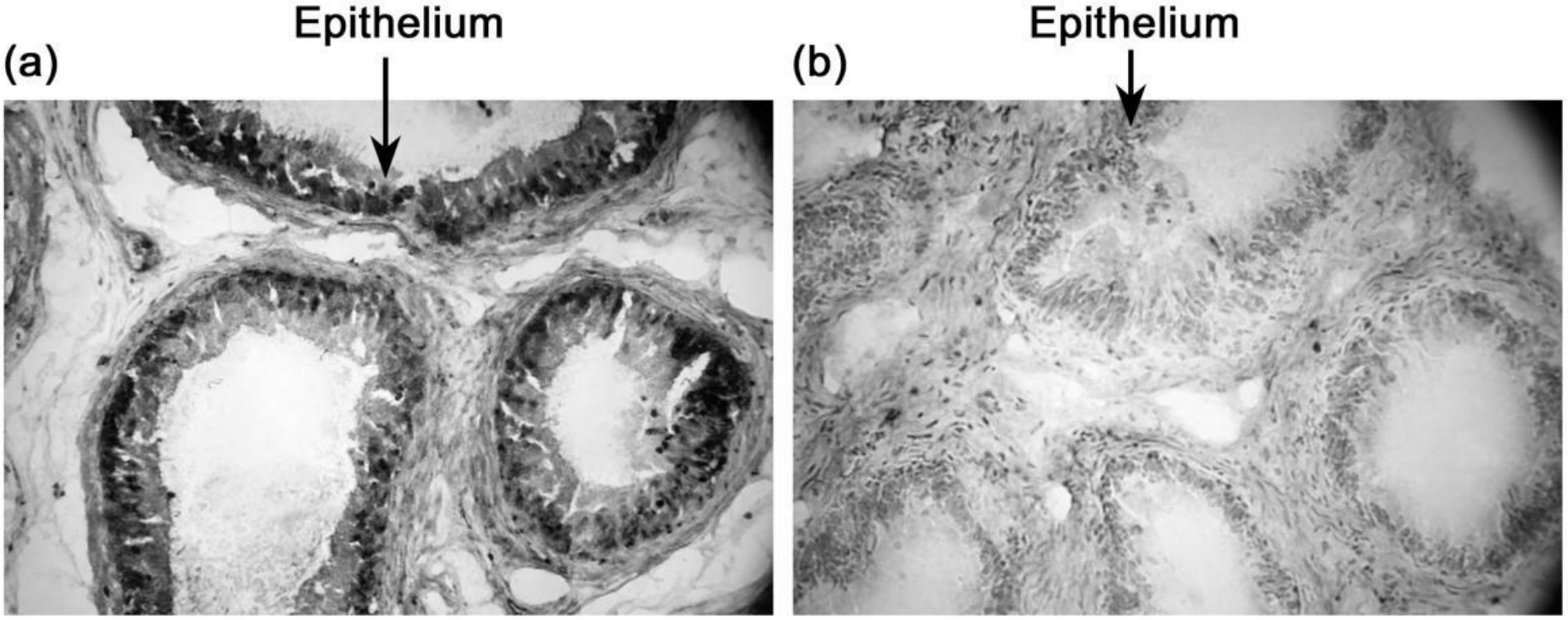
Artificial coloring can be applied also here

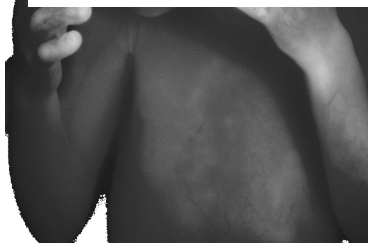




Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

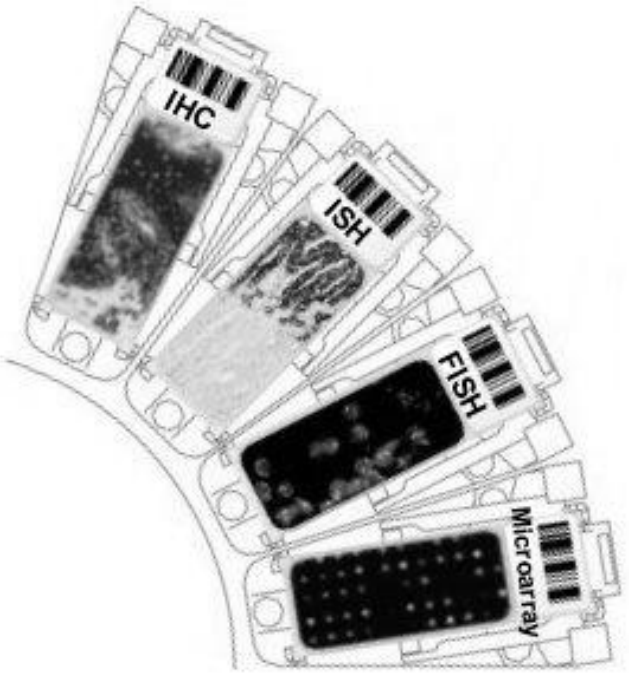
Different haptens and detection methods can be used

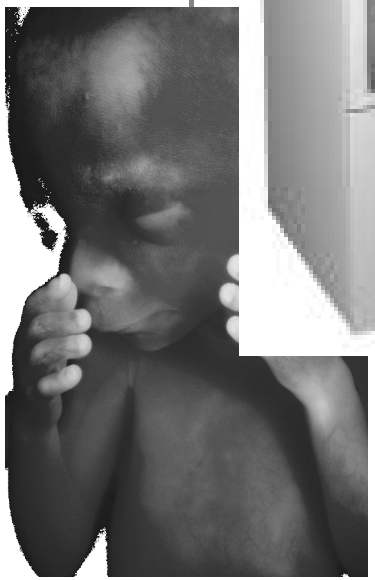




Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

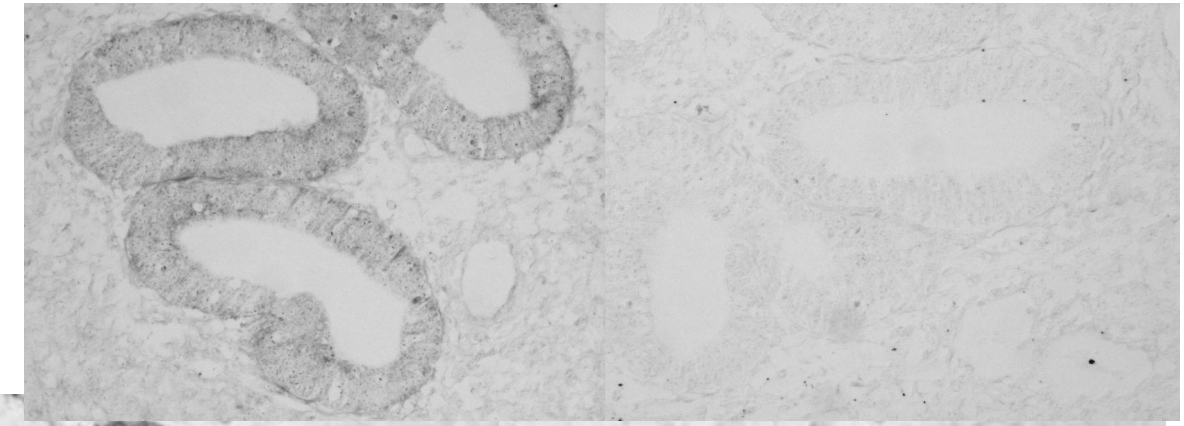
Automated section ISH

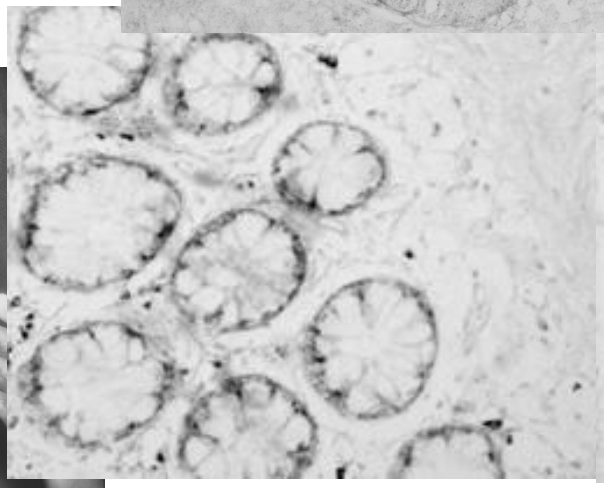


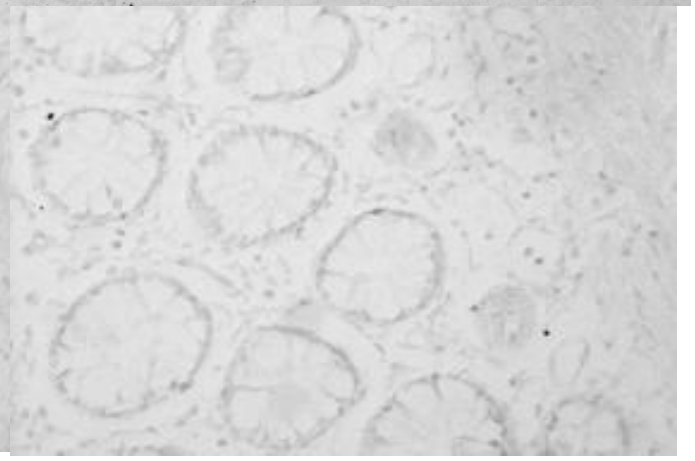


Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Automated section ISH

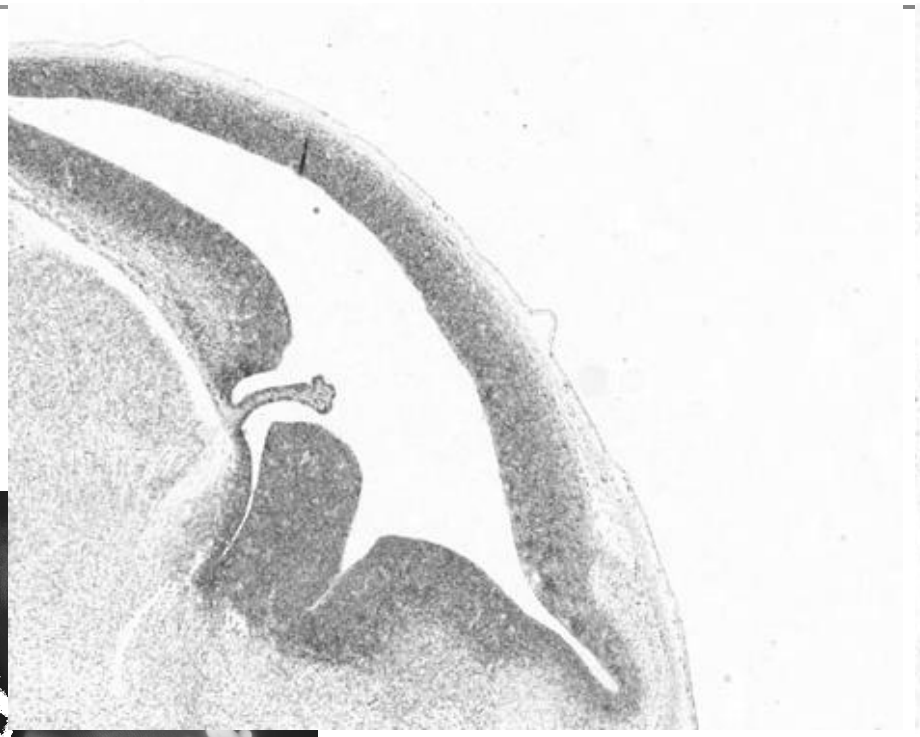


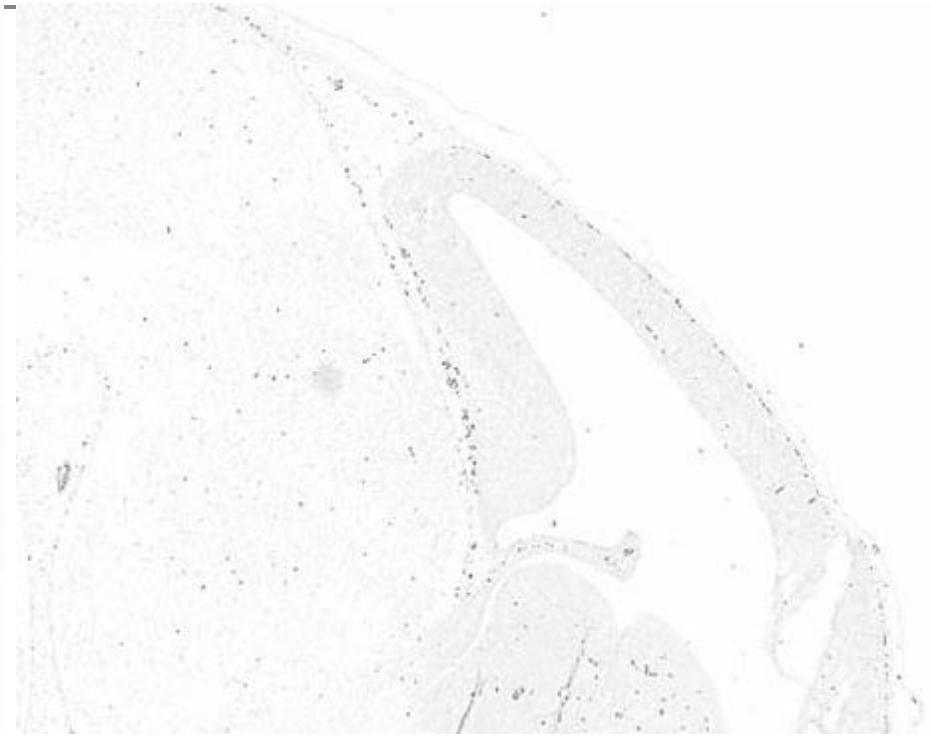


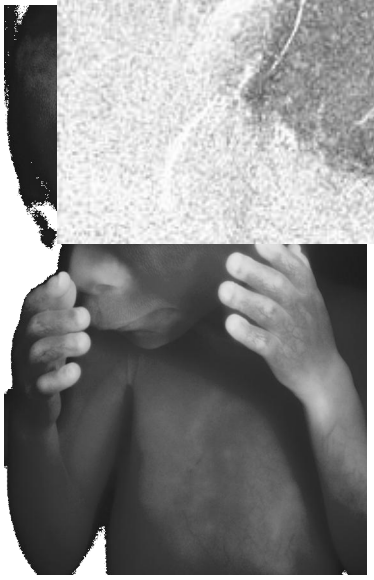


Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Reliable results

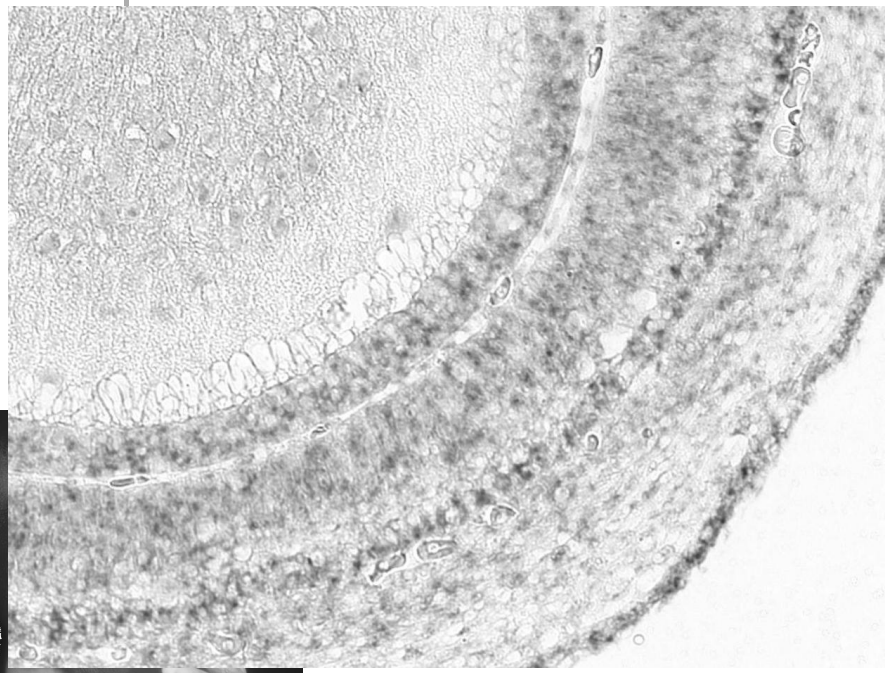


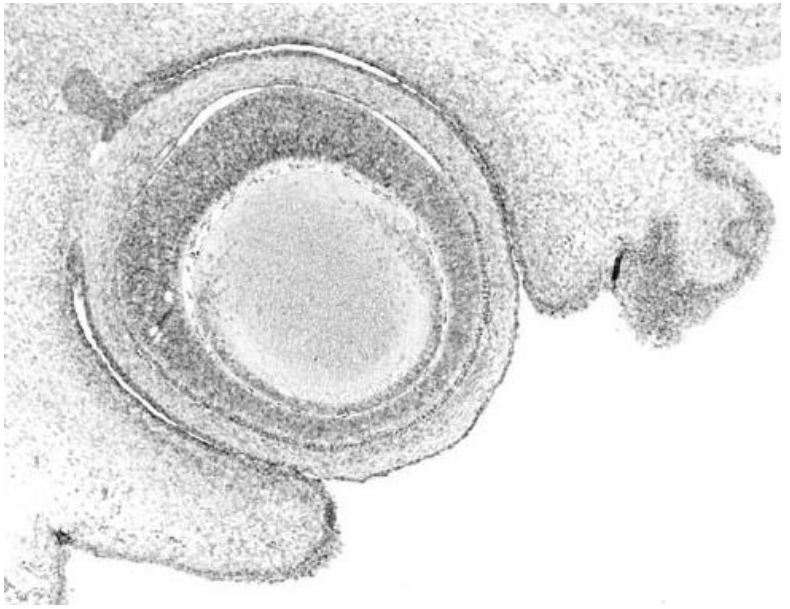




Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

Cellular level detection

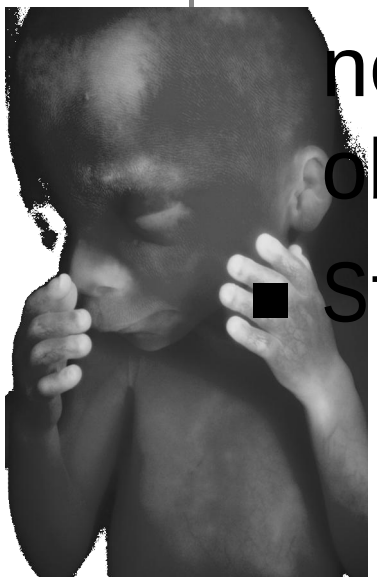




Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

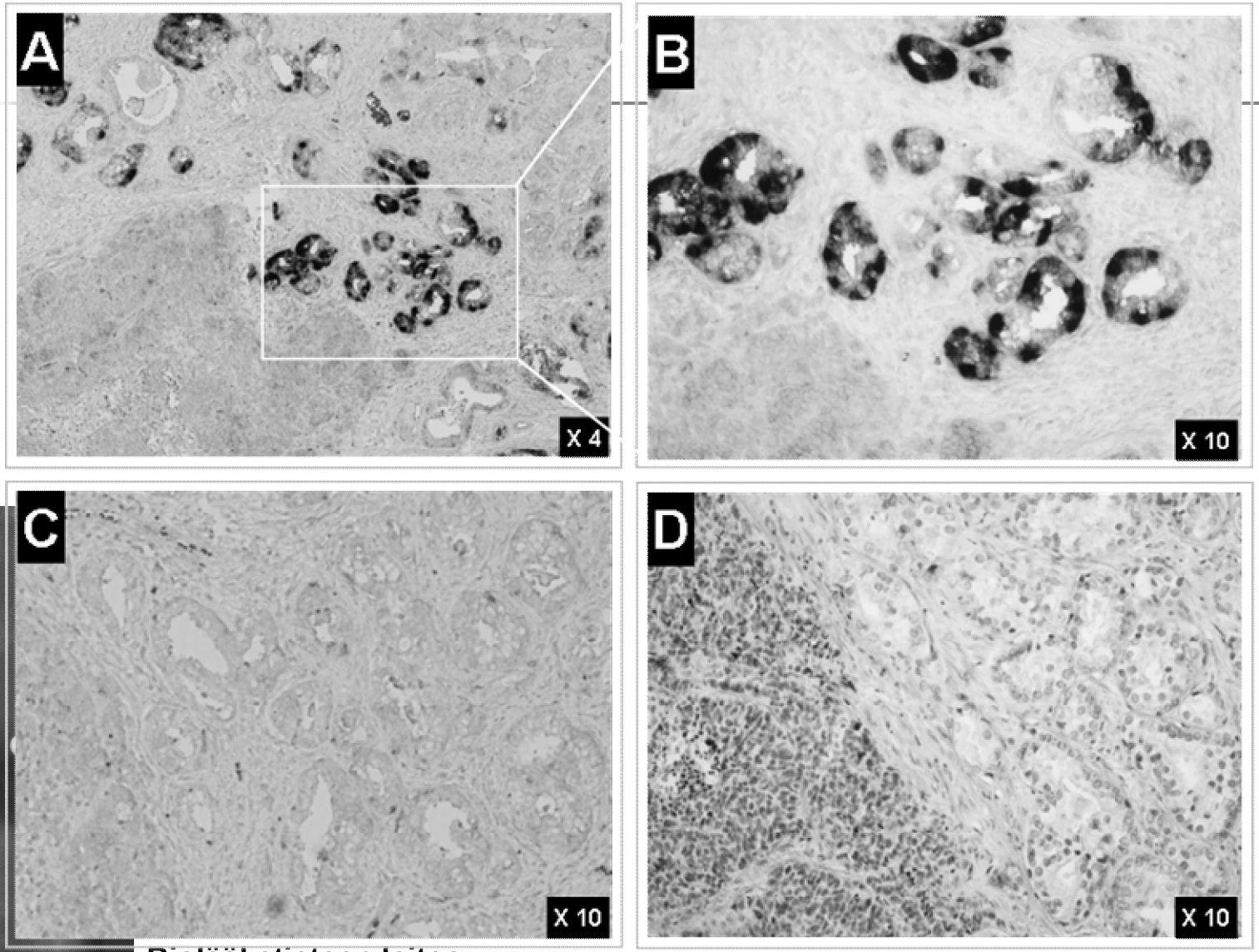
Power and pitfalls

- Reliable, fast, easy to use, gives good results
- Optimization possible and easy
- Relatively expensive, sometimes does not give any detection without any obvious reason
- Still worth trying!

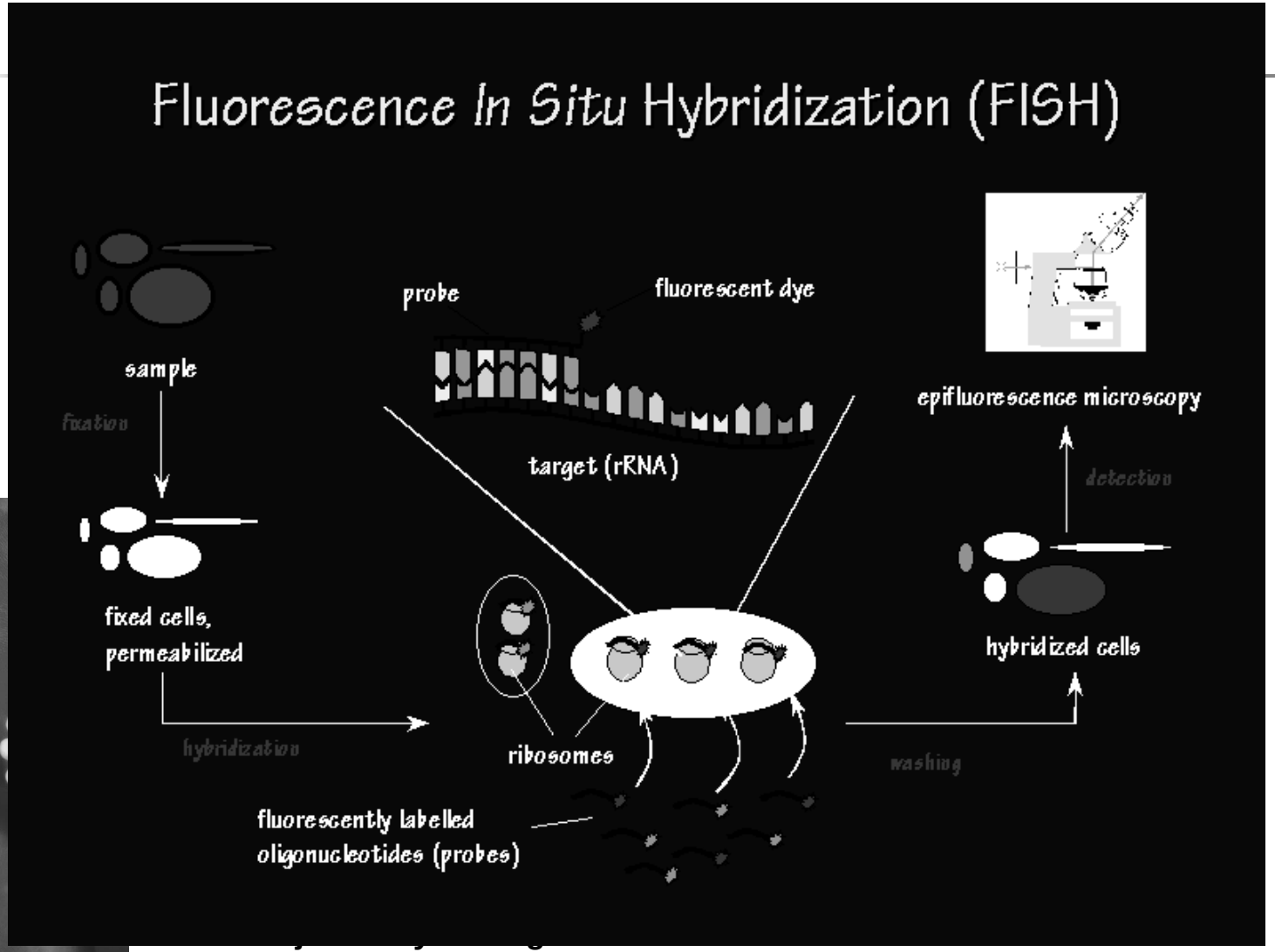


Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

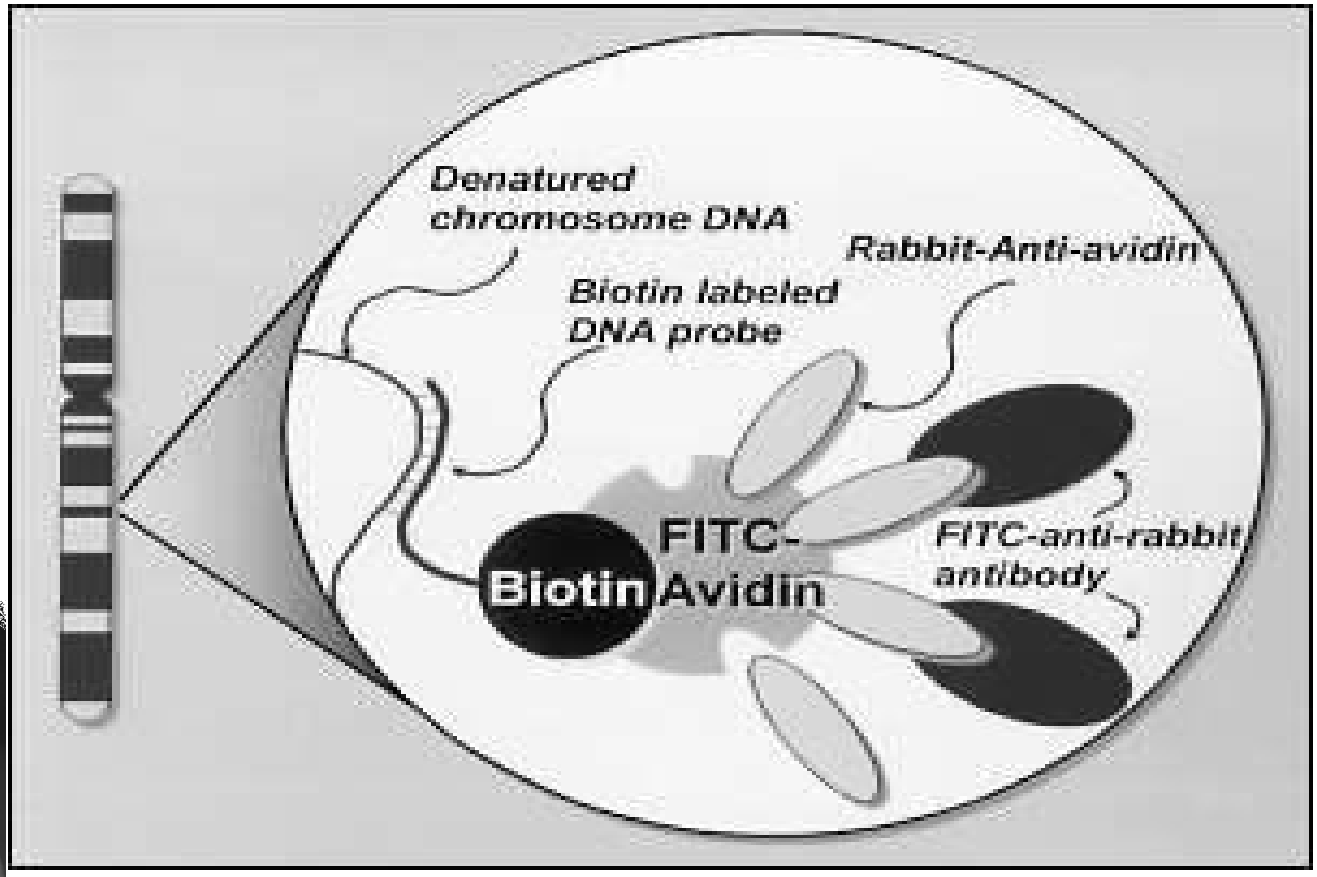
Clinical applications



FISHybridization

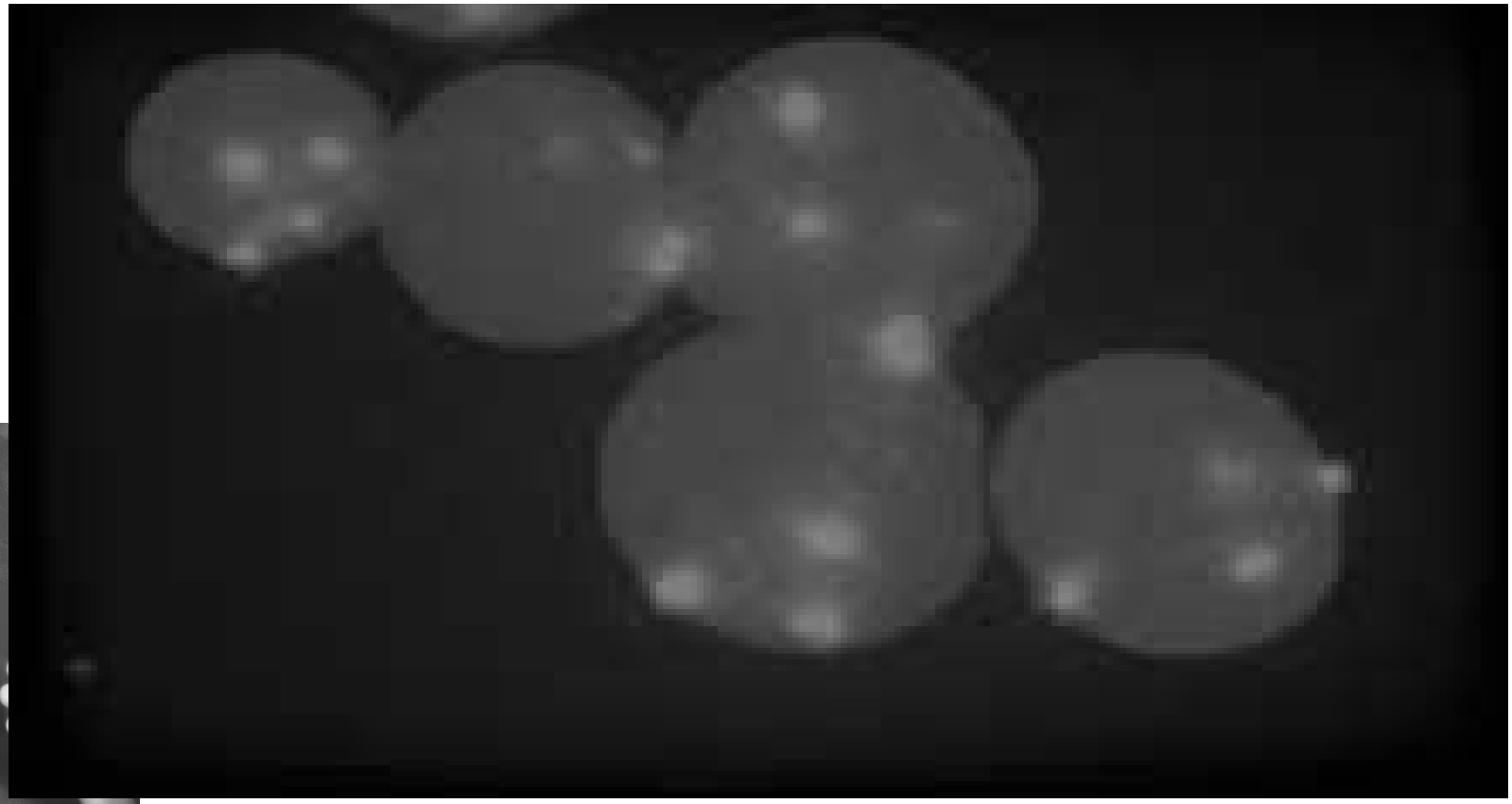


FISH



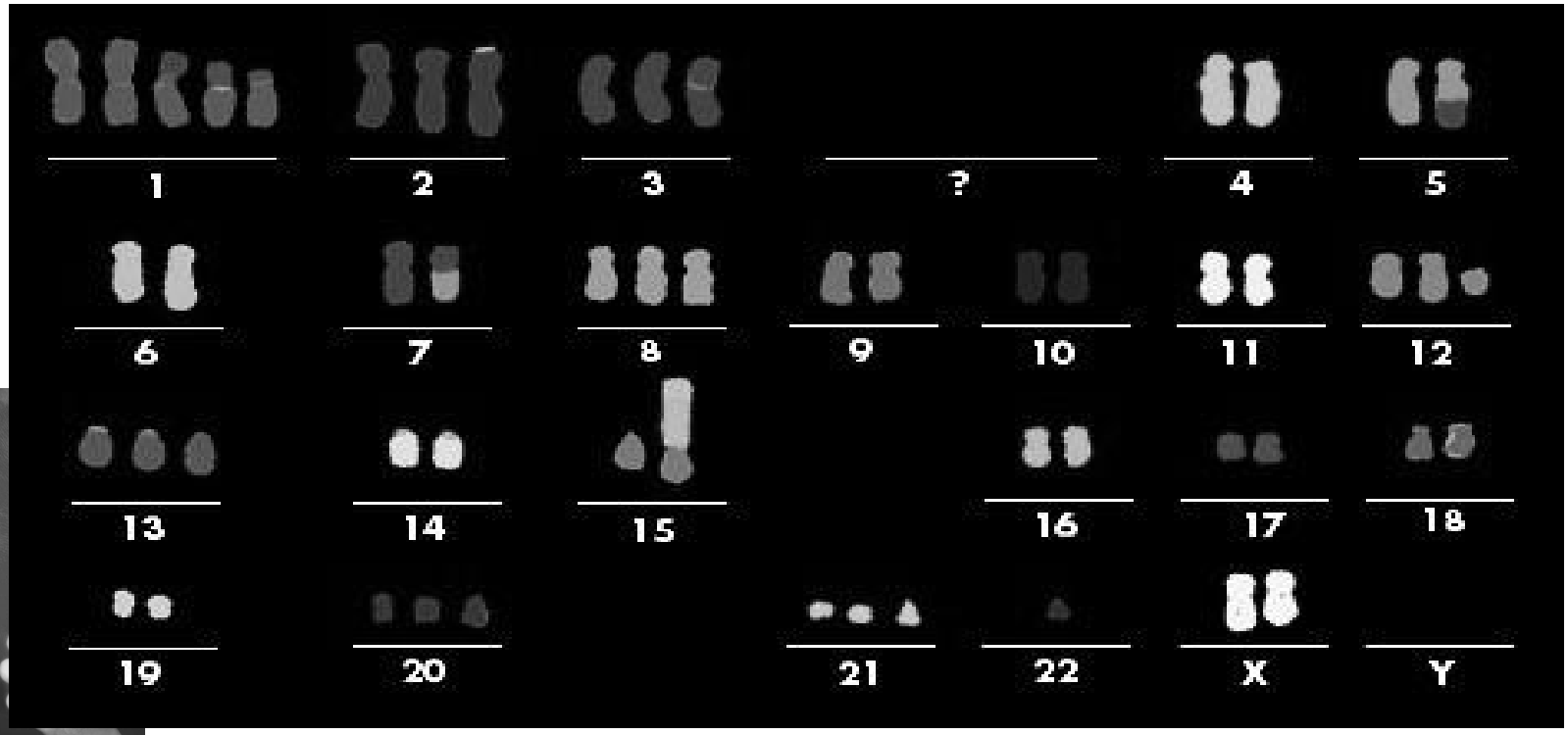
Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

FISH in cells



Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos

FISH in isolated chromosomes



Kirsi Sainio
Biokemia ja Kehitysbiologia
Biolääketieteen laitos