## In situ hybridization

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## ISH

- Detection of DNA or RNA
- Single or double stranded
- Chromosomal or cellular nucleic acids

## ISH

- Type of a hybrid?
- DNA-DNA
  - In situ re-naturation of target DNA in ISH cannot be prevented since the probe and the target have the same thermal stability!

## ISH

- DNA-RNA
  - More thermally stable hybrids
  - Choice of hybridization conditions that favour DNA-RNA hybridization instead of DNA-DNA hybridization

## ISH

- RNA-RNA
  - Choice of the complementary probe sequence for detection of tissue mRNA – method of choice when gene activity needs to be monitored
  - Choice of the hybridization conditions: thermally the most stable form of hybridization

## ISH

- For tissue/whole mount -ISH, single stranded probes are recommended:
  - The probe is not self-annealing in the solution
  - Large concatenates that would penetrate sections or whole chromosomes poorly, do not occur

## History

- 1969
  First *in situ* hybridisation experiments published
  RNA from [3H] labelled HeLa cells hybridised to unlabelled HeLa cells
- First *in situ* hybridisation experiments published [3H] labelled rRNA from cultured *Xenopus* cells hybridised to unlabelled *Xenopus* ovary

## History

- 1970
- Tritium-labelled cRNA probe against satellite DNA (from a CsCl gradient)used to label *Drosophila* salivary gland, and mouse chromosomes *in situ*
- 1975
- [3H] labelled probes to detect genes for 18S & 28S rRNA in mouse chromosome spreads

## History

- 1977
- radio-labelled chick myosin heavy chain (MHC) cDNA probes (synthesized from MHC mRNA extracted from chick muscle) used to detect MHC mRNA in cultured cells
- 1979
- [3H]-poly (U) probe to detect maternal mRNA on tissue sections

## History

- 1982 1st edition of 'Molecular Cloning - a
- Laboratory Manual' by Maniatis, Sambrook and Fritsch published
- 1983
- [35S]-antisense gene specific probes to specific transcripts on tissue sections
- 1986
- First fluorescent probe (Y specific) used to identify regions on chromosomes

## History

- 1987-88
- Hapten labelling of nucleic acid probes becomes available (DIG -Boehringer Mannheim)
- 1989
- *in situ* hybridisation to used to detect mRNA in whole Drosophila embryos
- 1994
- more hapten labels become available

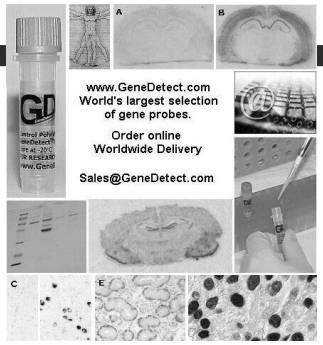
## History

- 1994
- non-radioactive *in situ* hybridisation to used to detect two different mRNAs
- ~1999
- Automated *in situ* hybridisation machines become available…

## Probes

- Today it is possible to order short nucleic acid probes, clone probes, use PCR for probe preparation or use genomic DNA
- The method of choice depends on WHAT NEEDS TO BE DETECTED and WHAT ARE THE POSSIBILITIES IN YOUR LAB!

## Probes




## Labels

- Non-radioactive methods are sensitive, 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, BUT YOU HAVE TO KNOW HOW TO DO IT!

## Labels

- **Non-radioactive labels:**
  - Direct or indirect labelling
  - In direct label the reporter is directly bound to the nucleic acid label and can be monitored immediately after the hybridization
  - In indirect labels the reporter is not directly subject to harsh hybridization- and washing conditions
  - The indirect reporter does not interfere with the hybridization !

## Labels

- **Digoxigenin**
  - From the plant *Digitalis purpurea* or *Digitalis lantana*
  - Does not occur in animals
    - Easy to raise detection methods (antibodies) that do not give background
    - Can be incorporated relatively easily into uridine via random priming, nick translation, PCR, 3'-end labeling or in vitro transcription

## Labels

- **Biotin**
  - First enzymatic labeling of biotin-dUTP
  - now also other biotinylated nucleotides available
  - Direct detection with biotin antibodies or with biotin-streptavidin methods

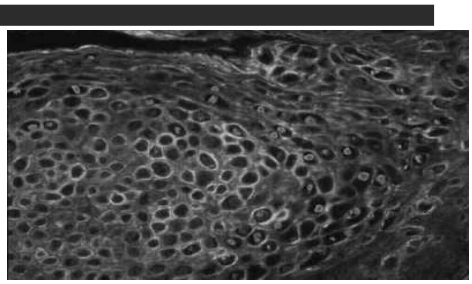
## Labels

- **Fluorochromes**
  - Fluorescein coupled to UTP
  - Indirect method, no additional visualization needed
  - More specificity with non-direct detection via antibodies

## Labels

- **Multiple labeling and detection**
  - Combinations of DIG, biotin and fluorochrome-labled probes makes it possible to do multiple ISH or ISH combined with immunohisto-chemistry
  - Utilizes different fluorochromes: FITC –TRITC-AMCA

**MULTI ISH/immunohistochemistry**



## Kinetics of hybridization

- **In principal:**
  - Basic knowledge of the kinetics of nucleic acid re-annealing is required when choosing the method and to ideally use the method that was chosen!!!

## Nucleic acid hybridization

- Hybridization depends on the ability of denatured DNA or RNA to re-anneal with complementary strand in an environment just below their melting point ($T_m$)

## Kinetics of hybridization

- $T_m$ is the temperature at which half of the DNA is present in a denatured form
- Different in genomic DNA isolated from different organisms!
- Depends on GC content in the sequence

## Kinetics of hybridization

- **Temperature**
  - Theoretically maximal rate for DNA hybridization is at +25$^0$C
  - The rate and temperature relationship is however quite broad and hybridization can be done in temperatures 16$^0$C – 32$^0$C BELOW the $T_m$

## Kinetics of hybridization

- **pH**
  - Not critical, hybridization rate is maximal in pH from 5-9 at 25$^0$C
  - Neutral pH buffers are used
  - More stringent hybridization conditions are obtained in higher pH

## Kinetics of hybridization

- **Monovalent cations**
  - Sodium ions (salt) interact electrostatically with nucleic acids
  - In practice higher salt conditions increase the stability of the hybrid
  - Low salt concentrations make more stringent conditions

## Kinetics of hybridization

- **Divalent cations**
  - Free divalent cations strongly stabilize duplex nucleic acid
  - For denaturation they have to be removed from the mixture
  - For stringency they have to be removed or complexed by citrate or EDTA

## Kinetics of hybridization

- **Formamide**
  - Allows hybridization in lower temperatures than the melting point as it reduces the thermal stability of double-stranded polynucleotides
  - DNA-DNA /DNA-RNA/ RNA-RNA hybridization can be done in 30$^0$C-45$^0$C in 50% of formamide
  - If higher temperatures are needed for stringency, formamide concentration can be increased

## Kinetics of hybridization

- **Probe length**
  - Maximal hybridization rates are obtained with long probes
  - However, in whole-mount ISH, probe penetration may be a limiting factor
  - Probe length affects the thermal stability:
    - Change in $T_m$ x n = 500 (n=nucleotides)
    - this gives you the value which relates the shortest fragment length in a duplex molecule to change in $T_m$

## Kinetics of hybridization

- **Probe length:**
  - In practice: the longer the probe, the higher the hybridization temperature can be used
  - If oligonucleotide probes are used, the hybridization temperature is low, the formamide concentration low, the salt concentration high
  - If long probes (DNA or RNA) are used, the higher the temperature, the higher the formamide concentration and the lower the salt concentration

## Kinetics of hybridization

- **Probe concentration**
  - There has to be enough probe for the nucleation reaction
  - This is the reaction at which the first few base pairs are hybridized – probe concentration affects the rate and efficiency of the nucleation reaction = rate limiting step in hybridization

## Kinetics of hybridization

- **Probe concentration**
  - The higher the probe concentration, the higher the re-annealing rate
  - However, high probe concentrations require also high stringency conditions and good washing conditions and does not usually give better end results

## Kinetics of hybridization

- **Dextran sulphate**
  - Affects the probe concentration and gives higher hybridization rates in aqueous solutions
  - In such solutions dextran sulphate is strongly hydrated and prevents the macromolecules to be solved in water

## Kinetics of hybridization

**Blocking agents:**

- Denhardt's solution
  - Prevents the non-specific attachment of the probe to slide or any surface
  - Used in combination with salmon sperm DNA/yeast DNA and detergents

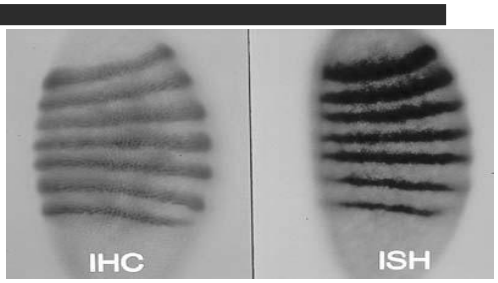
## Kinetics of hybridization

- **Powdered non-fat milk**
  - Easier and cheaper than Denhardt's – but for RNA probes should be RNAase-free!!

## Kinetics of hybridization

- **Heparin**
  - Used as a blocking agent
  - If dextran sulphate is used in hybridization mix, used at a concentration of 500μg/ml, if no Dextran is added, 50 μg/ml is enough

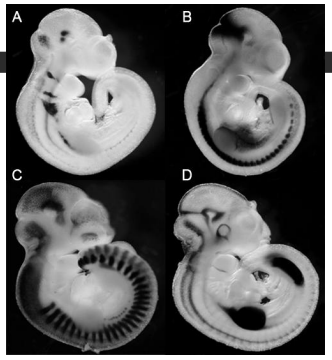
## Whole-mount ISH




## The protocol

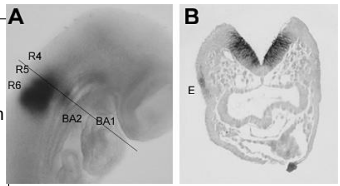
- Whole mount *in situ* hybridization, based on *Wilkinson* protocol, modified by Murray Hargrave (m.hargrave@cmcb.uq.edu.au), Koopman lab, and Sariola lab (Satu Kuure, Kirsi Sainio)

## The optimal result

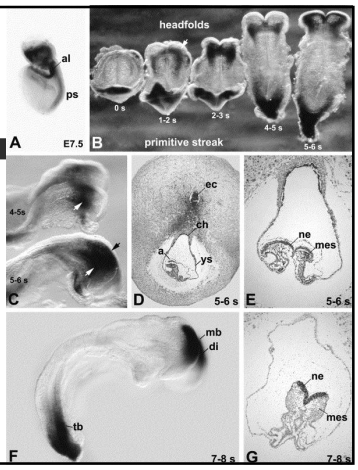



## Analysis

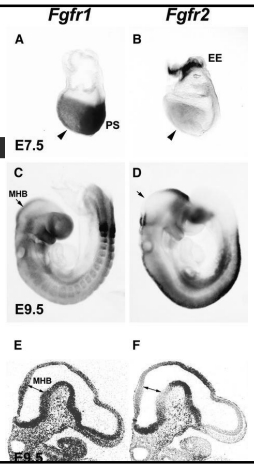
- *Fgf3* expression in the developing pharyngeal region. Whole-mount in situ hybridization of a 8 somite stage embryo. Note expression in the ectoderm covering the future 2nd branchial arch. BA1 and 2; branchial arch 1 and 2; R4, 5 and 6, rhombomeres 4, 5 and 6 (courtesy J. Partanen)



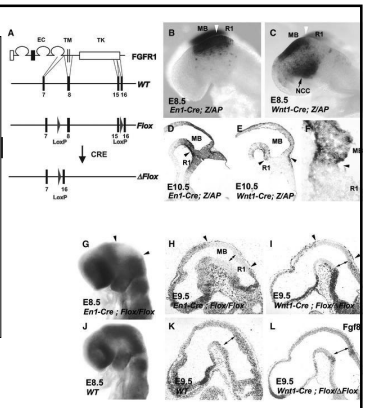

- *Drapc1* expression from E7.5 to E8.5. Whole-mount in situ hybridization (A–C,F), in situ hybridization on sections (D,E,G). (A)



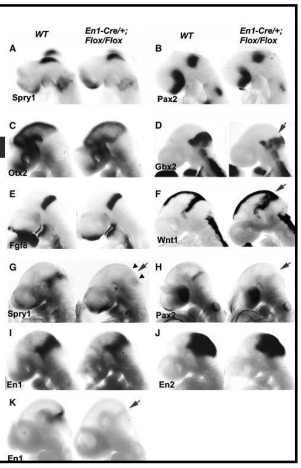
- Expression of *Fgfr1* and *Fgfr2*. Whole-mount and radioactive *in situ* hybridization analysis of the expression (courtesy J. Partanen)

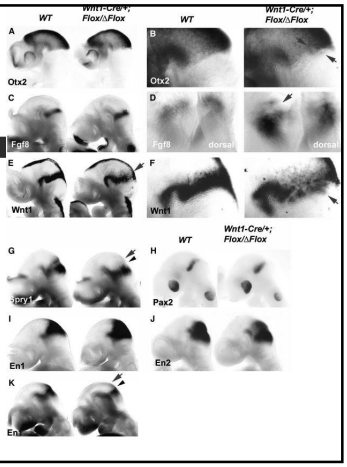



- Schematic presentation of the conditional *Fgfr1*flox allele and its inactivation by the Cre-recombinase
- Analysis of mutant embryos by ISH (courtesy J. Partanen)

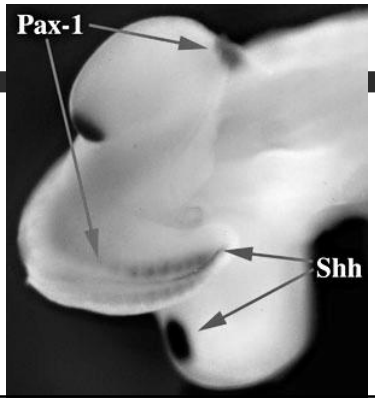



- Analysis of mutant embryos by whole mount ISH (courtesy J. Partanen)

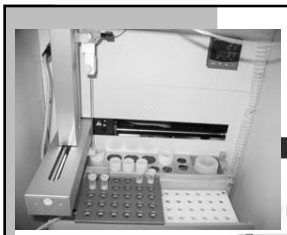


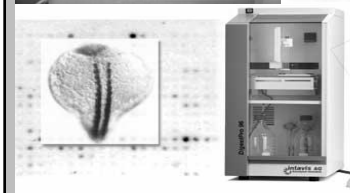

## Double labeling




## Intavis InSituPro



manual ...

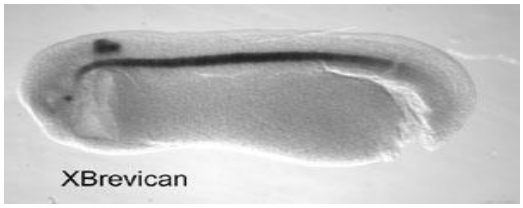


... in situ detection

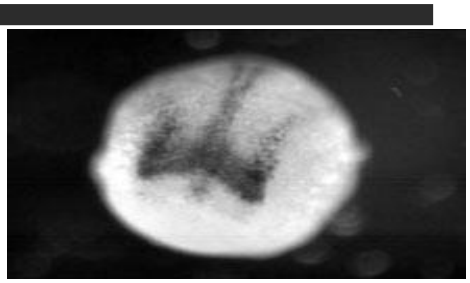
... peptide synthesis

... protein digestion

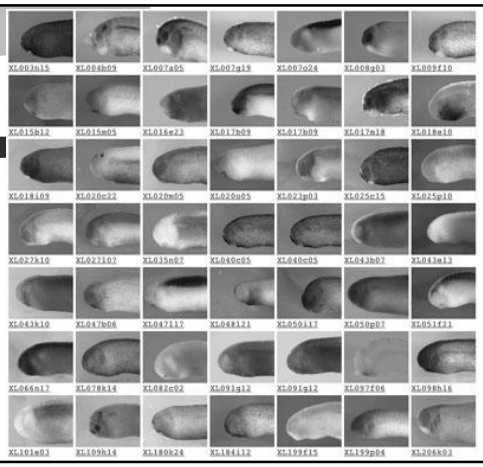
## InSituPro




## InSituPro



**What is the real benefit of automated ISH?**



## Section *in situ* hybridization

## Section / cellular ISH types

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

## Sections

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

## 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

## 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

## 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

## 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

## 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

## 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

## Fixatives

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

## 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

## 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

## 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

## 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

## 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)

## 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

## 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

## 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

## 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

## 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

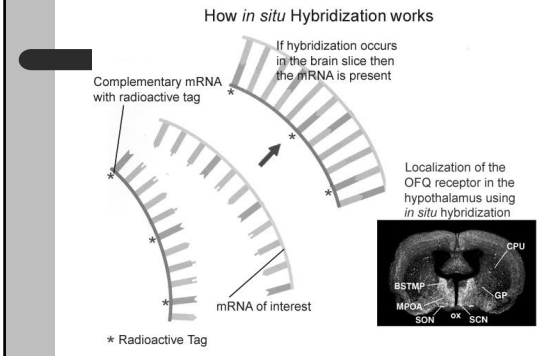
## Labels

- **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

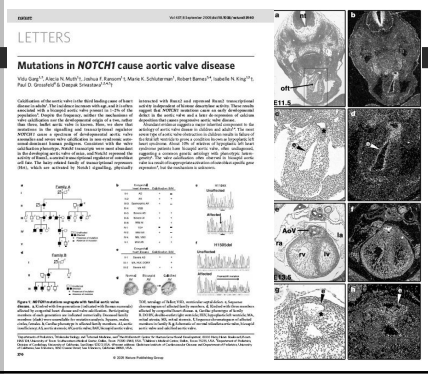
## Radioactive ISH

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

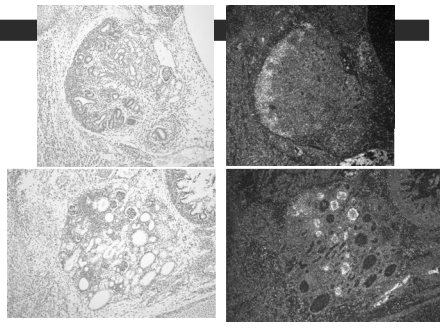
## Radiolabeled probes



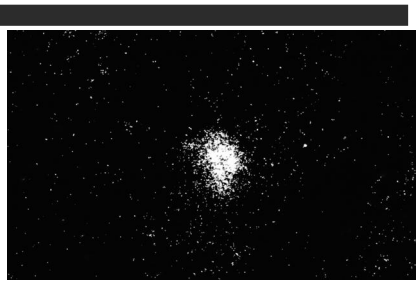

## Radioactive ISH



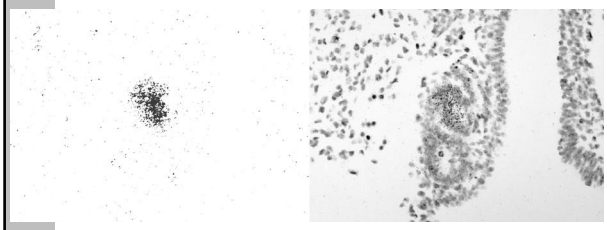

## Analysis of the results



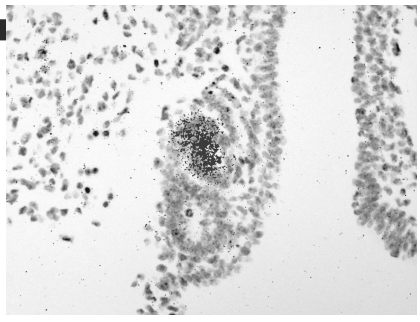
## How to visualize autoradiography ?



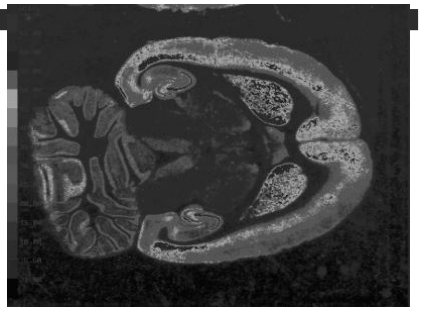
## Photoshop helps …



## … to make it look like a real thing



**Artificial colors to visualize autoradiography**



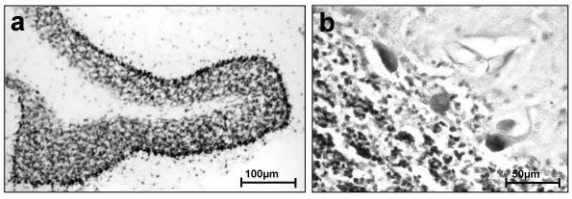
## Radioactive ISH

- 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…

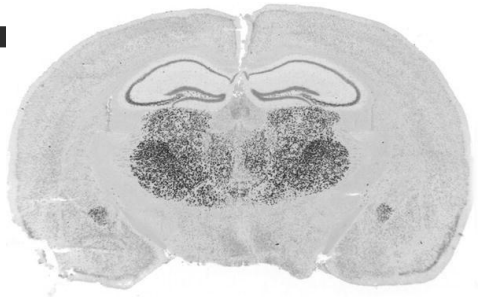
## Non-radioactive ISH

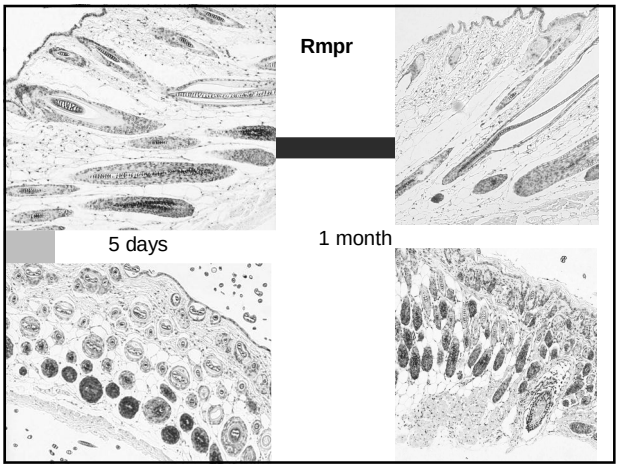
- Nonisotopic labelling systems (such as digoxigenin and biotin) are also frequently used for section ISH studies
- Same labels and detection methods than in whole mount ISH
- Possibility to multiple labelling and modifications
- Possibility to include protein immunohistochemisty
- Faster, high throughput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

## Non-radioactive section ISH

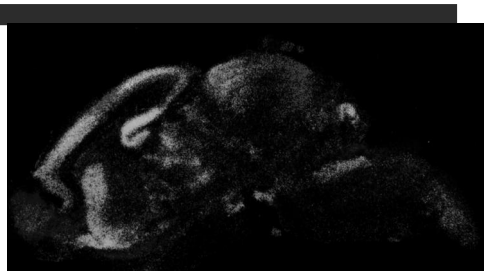



## When it is nice, it is nice…

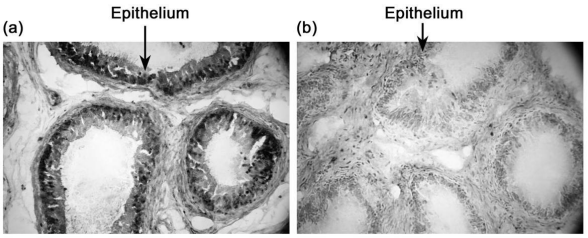




## Artificial coloring can be applied also here

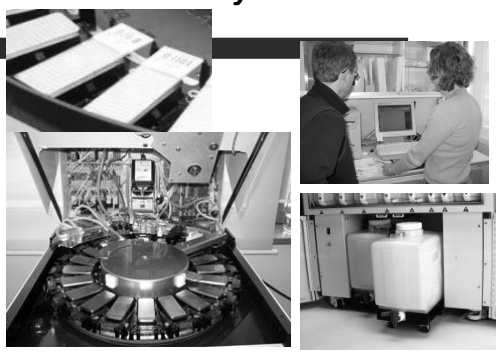


## Different haptens and detection methods can be used

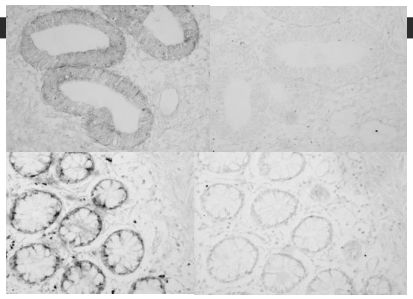



## Automated section ISH

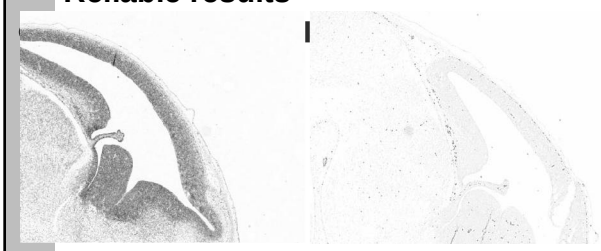

## Ventana Discovery



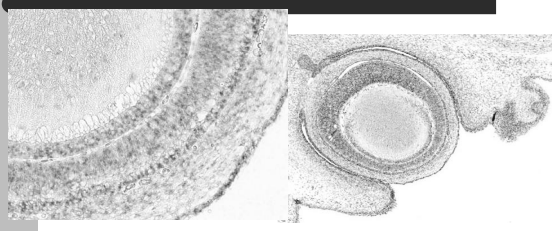
## Automated section ISH



## Reliable results



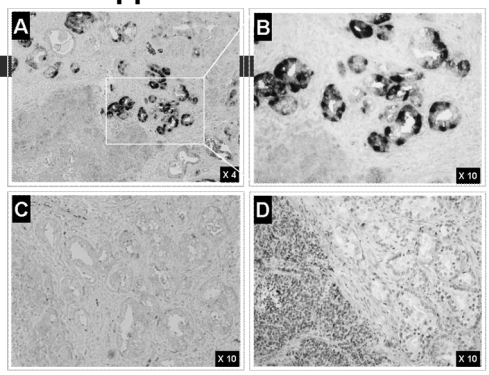
## Cellular level detection



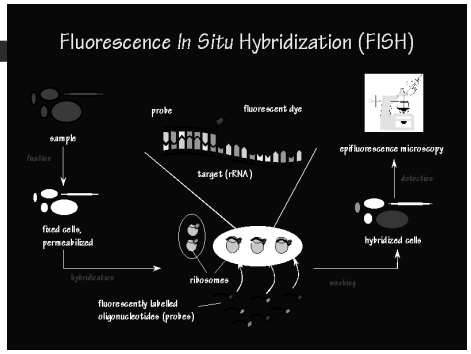
## Power and pitfalls

- 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!

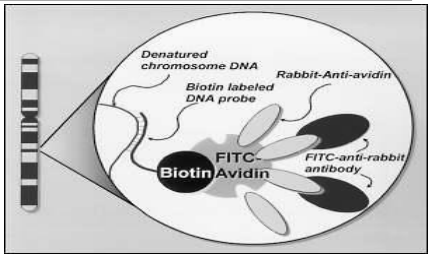
## Clinical applications



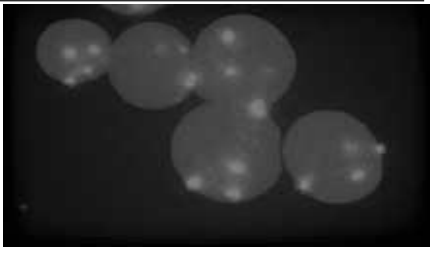

## FISHybridization



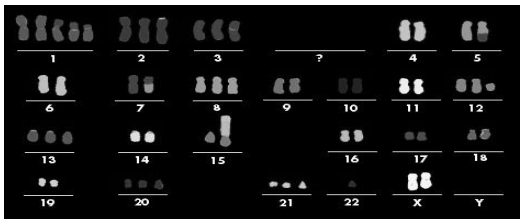

## FISH




## FISH in cells



## FISH in isolated chromosomes




## ISH in ES-cells

- Easier to use EBs
- Non-radioactive probes
- For instance transfected or genetically manipulated ES-cells to check the expression