In situ hybridization

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Biolääketieteen laitos

ISH

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

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ISH

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

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ISH

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

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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

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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

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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!

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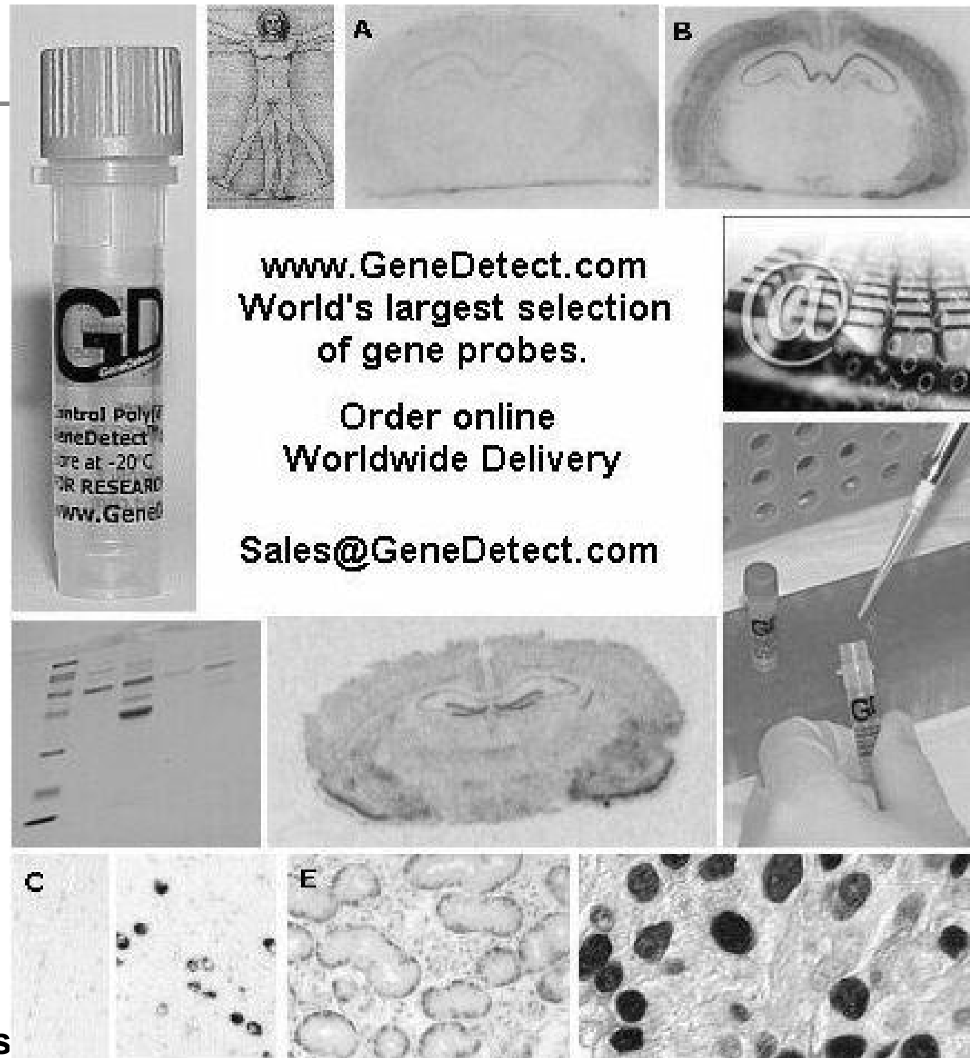
Probes



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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!

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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 !

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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

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Labels

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

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Labels

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

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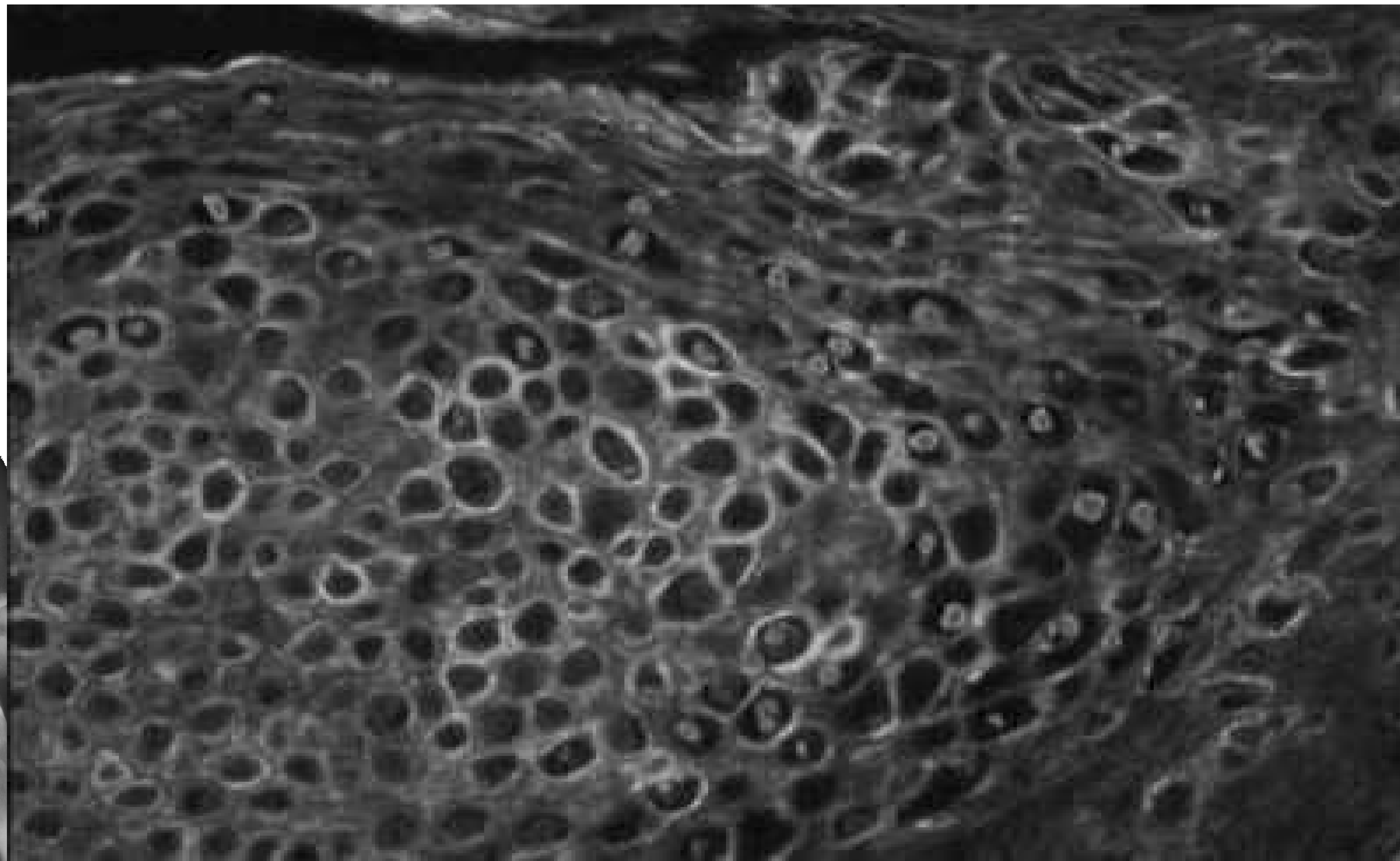
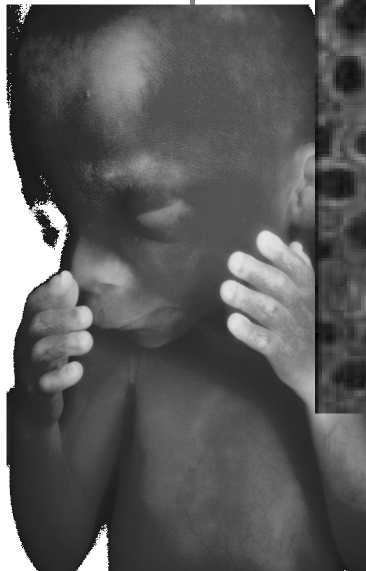
MULTI ISH/immunohistochemistry





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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!!!

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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)

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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

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Kinetics of hybridization

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

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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

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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

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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

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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 30^0C-45^0C in 50% of formamide
  - If higher temperatures are needed for stingency, formamide concentration can be increased

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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

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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

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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

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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

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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

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Kinetics of hybridization

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

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Kinetics of hybridization

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

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Whole-mount ISH

IHC ISH

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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)

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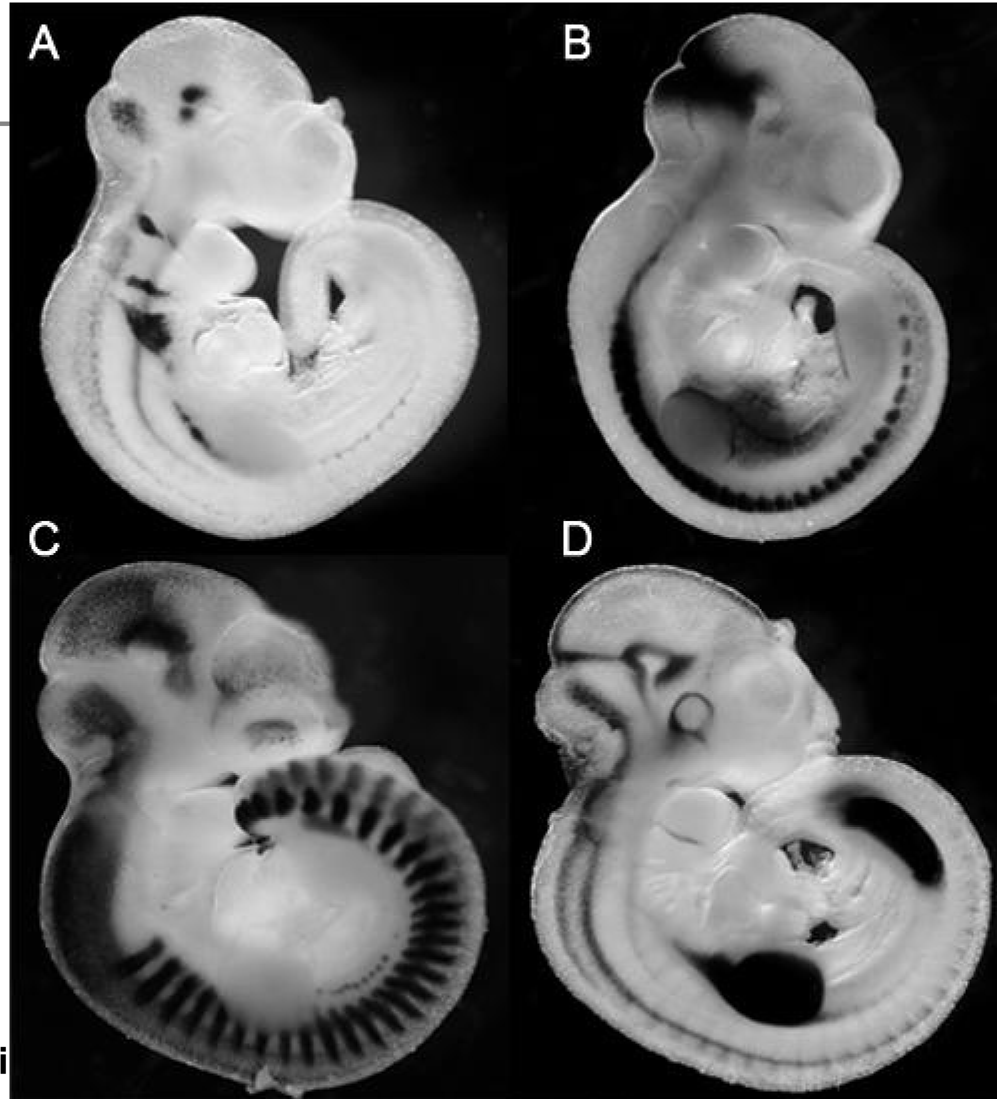
The result



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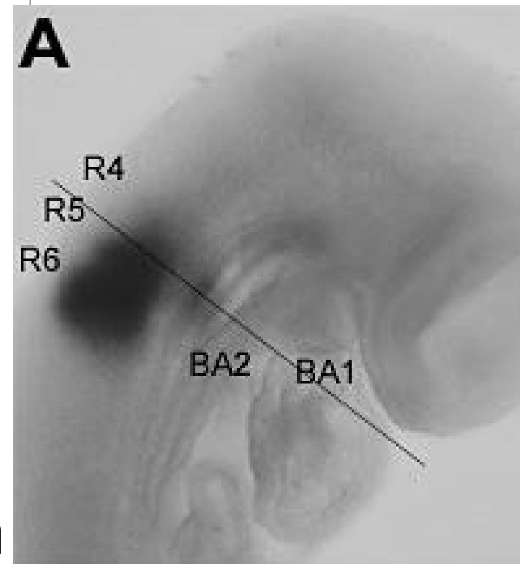
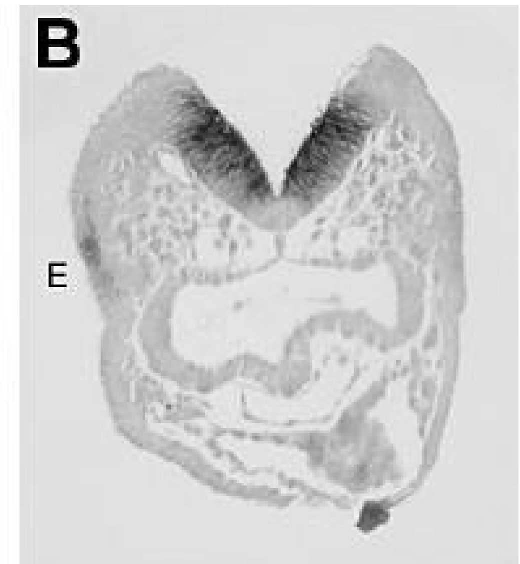
Analysis

- 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.





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- *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)

headfolds
al
ps
A E7.5
0 s 1-2 s 2-3 s 4-5 s 5-6 s
B primitive streak
4-5s
5-6 s
C
ec ch a ys
D 5-6 s
ne mes
E 5-6 s
mb di tb
F 7-8 s
ne mes
G 7-8 s

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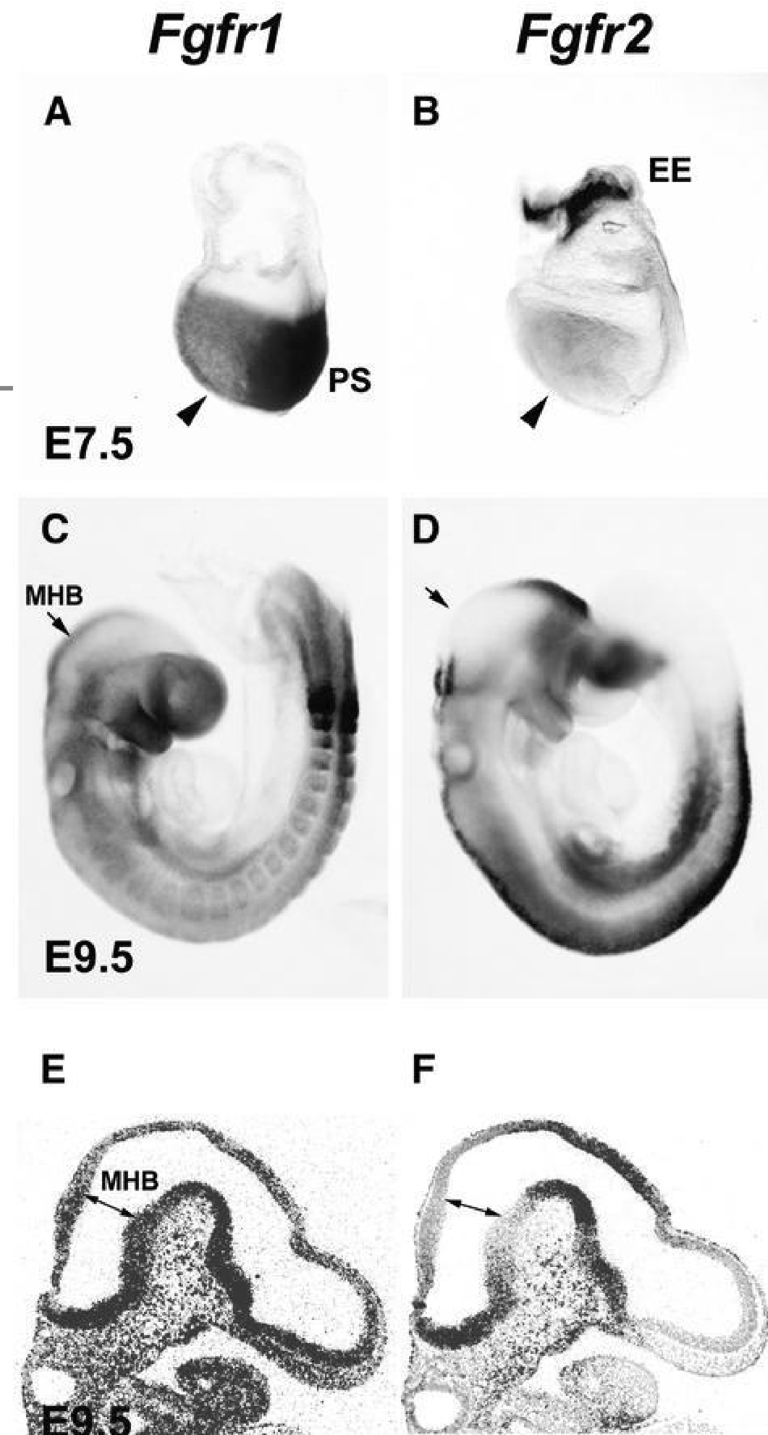
- Expression of *Fgfr1* and *Fgfr2*. Whole-mount and radioactive *in situ* hybridization analysis of the expression



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- The conditional *Fgfr1* allele, *Fgfr1*flox, and its inactivation by *En1-Cre* and *Wnt1-Cre*. (A) Schematic presentation of the *Fgfr1*flox allele and its inactivation by the Cre-recombinase. The structures of the FGFR1 protein

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WT | En1-Cre/+; Flox/Flox | WT | En1-Cre/+; Flox/Flox

A Spry1
B Pax2
C Otx2
D Gbx2
E Fgf8
F Wnt1
G Spry1
H Pax2
I En1
J En2
K En1

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WT

Wnt1-Cre/+; Flox/ΔFlox

A Otx2

B Otx2

C Fgf8

D Fgf8 dorsal dorsal

E Wnt1

F Wnt1

WT

Wnt1-Cre/+; Flox/ΔFlox

G Spry1

H Pax2

I En1

J En2

K En1

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Double labeling

Pax-1

Shh

K
B
B

Intavis InSituPro

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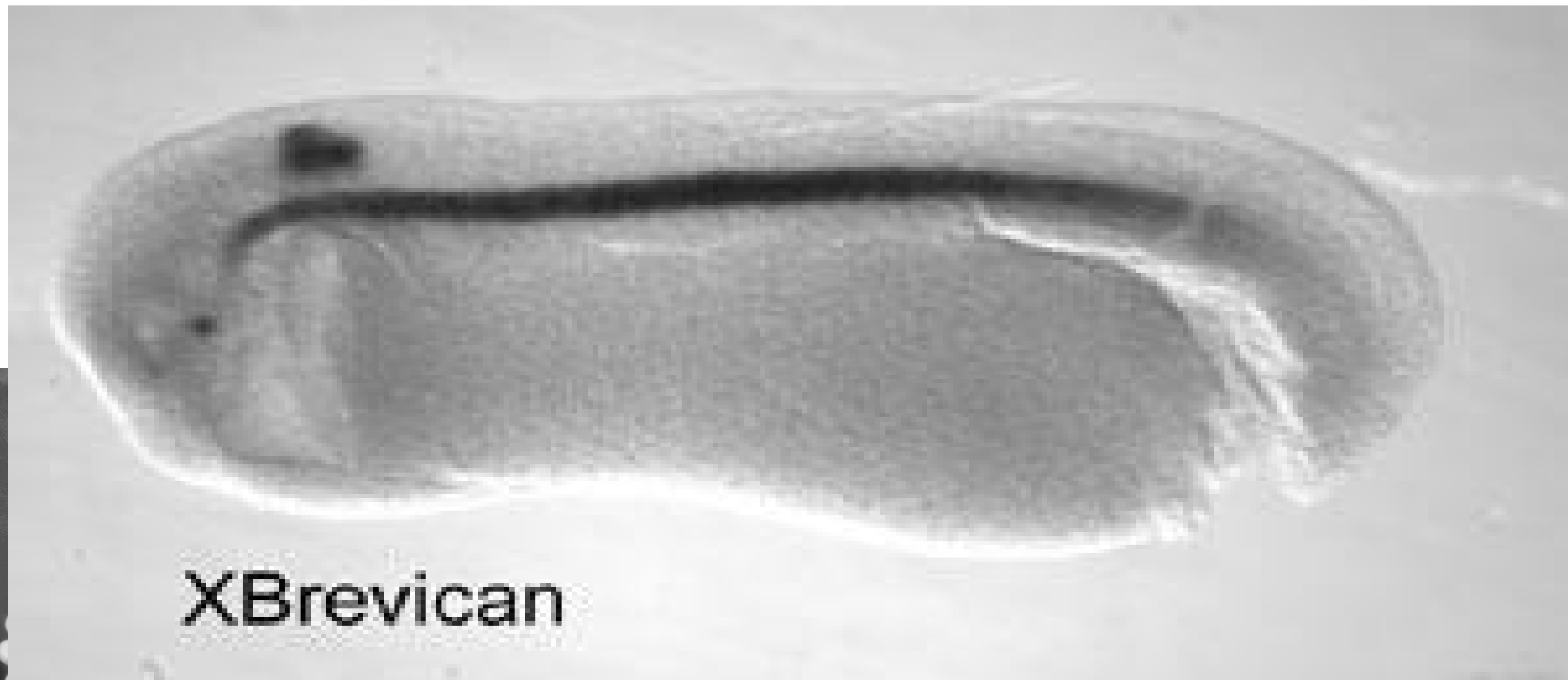
InSituPro



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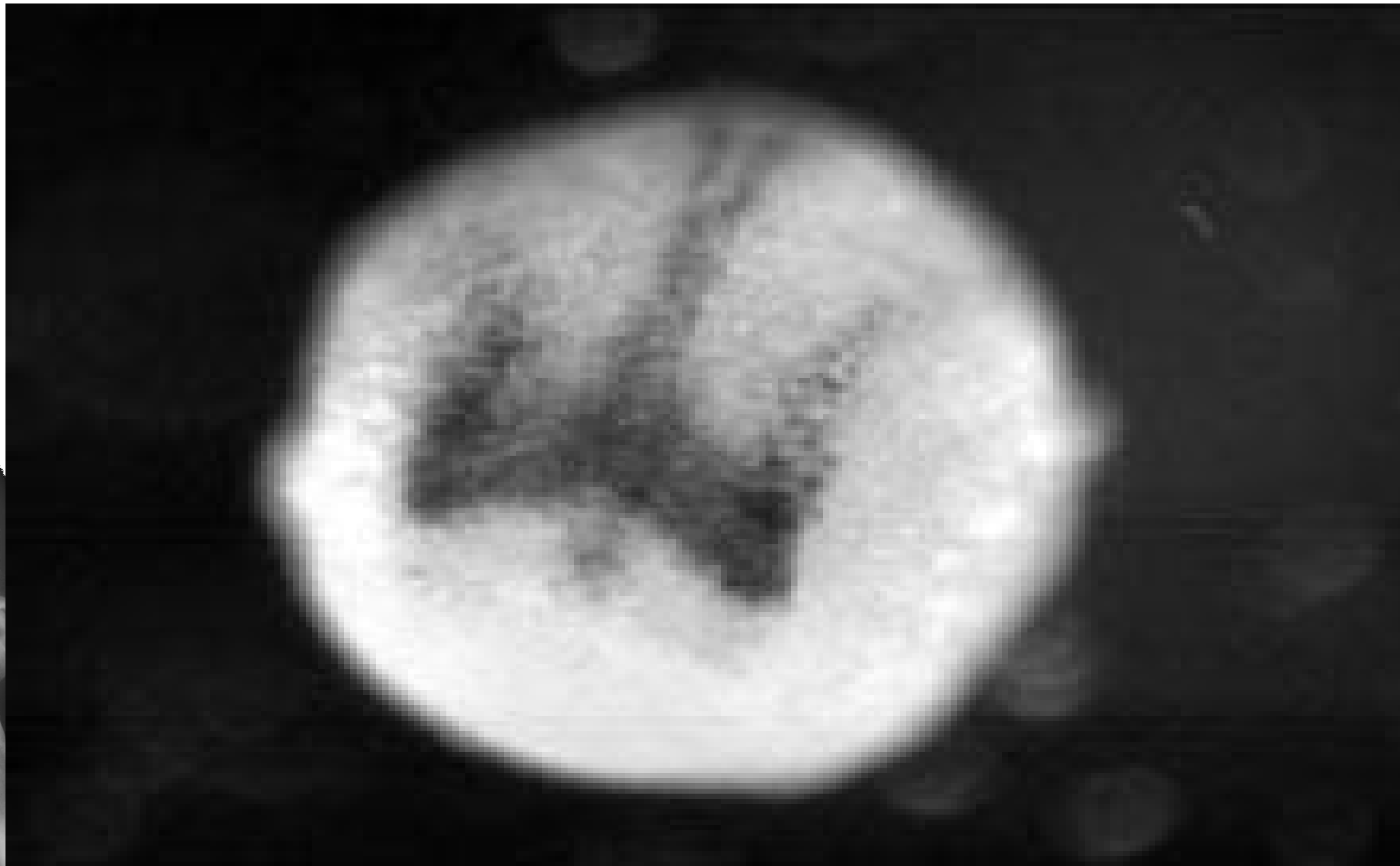
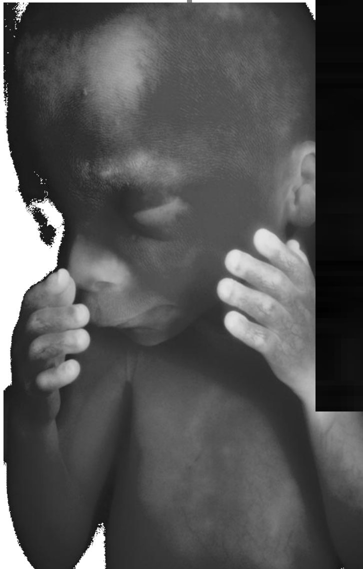
InSituPro





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What is the real benefit of automated ISH?

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XL003n15	XL004b09	XL007a05	XL007g19	XL007o24	XL008g03	XL009f10
XL015b12	XL015m05	XL016e23	XL017b09	XL017b09	XL017m18	XL018e10
XL018i09	XL020c22	XL020m05	XL020o05	XL023p03	XL025c15	XL025p10
XL027k10	XL027l07	XL035n07	XL040c05	XL040c05	XL043b07	XL043e13
XL043k10	XL047b06	XL047l17	XL048l21	XL050i17	XL050p07	XL051f21
XL066n17	XL078k14	XL082c02	XL091g12	XL091g12	XL097f06	XL098h16
XL101e03	XL109h14	XL180k24	XL184i12	XL199f15	XL199p04	XL206k03