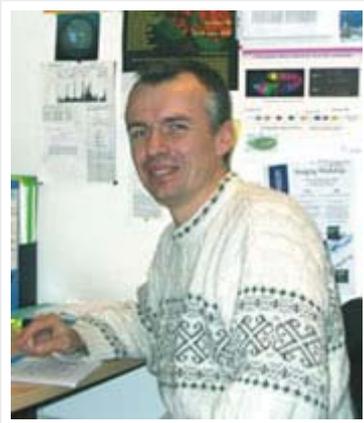




## Two-colour fluorescent in situ DNA hybridization on whole mount *Drosophila* embryos and larval imaginal discs (PROT07)



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### Introduction

Increasing evidence in literature reveals that the organization of chromosomal domains in the cell nucleus plays an important role in gene expression during cellular differentiation and development (Spector, 2003, Taddei *et al*, 2004). Fluorescent *in situ* DNA hybridization (FISH) allows to study the positioning of single copy genes into the nucleus, and is therefore widely applied to address the question of how is gene positioning regulated.

We describe here a two-colour FISH method for interphase nuclei of whole mount drosophila embryos. This procedure has been adapted from the protocol described by Gemkow *et al*, 1998, and allows us to study the relative position of two distinct single copy genes in the nucleus. This FISH protocol can be applied to other drosophila tissues, such as larval imaginal discs (Bantignies *et al*, 2003). For simplicity, we will use "tissues" throughout the text to design embryos and larval discs in the steps common to all tissues, whereas we will specify "embryos" or "larval imaginal discs" in those steps that apply to only one type of tissue.

### Procedure

This procedure is a two-day protocol (without counting the time for preparation of fixed embryos and the time of image acquisition and analysis). DNA hybridization is usually performed overnight between the two days. The method consists of 5 parts: 1) Fixation of whole embryos, 2) Labeling of DNA probes, 3) *In situ* DNA hybridization to fixed embryos, 4) Immunodetection of both FISH signals, 5) Image acquisition.

### Fixation of embryos or larval discs

#### Whole embryos

This procedure is a standard protocol that leads to embryo dechoriation, followed by a simultaneous fixation and devitellinization of the embryos.

1. Collect eggs on an apple juice-agar plate with fresh yeast and rinse them into a sieve (pore size less than 0.5 mm);
2. Dechorionate embryos with bleach (3 to 5 minutes), and rinse them extensively with dH<sub>2</sub>O;
3. Fix with 5ml of 4% paraformaldehyde (make it fresh from powder) in Buffer A (dissolve the p-formaldehyde

by putting the solution in boiling water bath for few minutes). Use this solution to transfer the embryos to a glass flask, or transfer the embryos with a paintbrush into a vial that already contains the fixing solution and the heptane;

4. Add 5ml of heptane (1 volume of heptane for 1 volume of [Buffer A](#)), and mix by vigorous rotation for 25 minutes at room temperature (RT) on a mini-shaker;
5. Remove the aqueous phase (lower phase), and keep the heptane phase (top phase) and the embryos (that are located in between both phases);
6. Devitellinize embryos by adding 5ml of 100% MeOH to the heptane phase. Mix by hand for 1 minute, or mix 15 seconds with Vortex at medium speed. Good embryos will fall down to the bottom MeOH phase;
7. Collect embryos at the bottom of the flask by pipetting with a Gilson blue tip (cut the last ~0.5cm at the end of the tip to prevent embryo damage), and transfer them into an eppendorf tube;
8. Wash twice in 100% MeOH, and store embryos in 100% MeOH (see [note 1](#));
9. Proceed with *in situ* hybridization (part 3).

## Larval imaginal discs

1. Dissect the discs in PBS 1X, and transfer them into an 1.5ml eppendorf tube on ice;
2. Fix as soon as possible in PBT (PBS 1X; 0.1% Tween20), 4% p-formaldehyde (dissolve the p-formaldehyde by putting the solution in a boiling water bath for few minutes) for 20 minutes on a rotating wheel at RT (see [note 2](#));
3. Proceed with *in situ* hybridization (part 3) .

See [comment 1](#).

## Probe labeling

This step is a critical step to obtain good FISH signals. Two different kits are used to produce Biotin-labeled and Digoxigenin-labeled probes. Suitable DNA probes are obtained from fragments of digested plasmids, from digested or sonicated BACs, or from genomic PCR reactions. Generally, single copy genes are detected with a probe made of 15-25 kb of DNA complementary to the locus of interest. (see [note 5](#))

## Probe Concentration for Hybridization

For single-colour FISH: use 150 to 200ng in about 30µl of FISH hybridization buffer ([FHB](#))/per hybridization. Perform hybridization in a total volume of 30µl. Typically, 1µg of labelled DNA resuspended in 100-200µl of [FHB](#), are sufficient for 3 to 6 single-colour FISH experiments.

For two-colour FISH: use 200 to 400ng per probe in 20µl of [FHB](#) (per probe) for each hybridization. Perform hybridization in a total volume of 40µl (2x 20µl).

## Probe labeling and purification

Probe labeling with the BioNick™ Labeling System (biotinylated probe):

- DNA - 1µg
- dNTPs 10X Mix - 5µl
- Enzyme 10X Mix - 5µl
- ddH<sub>2</sub>O (from the kit) - to 50µl final

1. Mix well and centrifuge briefly;
2. Incubate 1-2 hours at 16°C. Before stopping the reaction, load 1/10 (5µl) of the reaction in a 1% agarose gel. A smear of biotinylated fragments ranging from 500 to 200 nt should be obtained;
3. Stop the reaction with 5µl of stop buffer;
4. Add 500µl of PB buffer from the PCR Purification Qiagen Kit;
5. Purify through the purple Qiagen column;
6. Elute with 50µl of ddH<sub>2</sub>O (Sigma);
7. Concentrate the probe by drying, using a speed-vaac until about 2-5µl are left (do not overdry);
8. Resuspend the labeled probe in the FISH Hybridization Buffer ([FHB](#)). The volume of [FHB](#) depends on the type of FISH experiments that must be performed (see above for the concentration of probes required for single or two-colour FISH).

Probe labeling with the DIG-Nick translation Mix (Digoxigenin-labeled probe):

- DNA - 1µg
- DIG 5X Mix - 4µl
- ddH<sub>2</sub>O (Sigma) - to 20µl final

1. Mix well and centrifuge briefly;
2. Incubate 1-2 hours at 15°C. Before stopping the reaction, check 1/10 (2µl) of the reaction on a 1% agarose gel. As for biotin labeling, a smear of Digoxigenin-labeled fragments ranging from 500 to 200 nt should be obtained;
3. Stop the reaction by adding 1µl of 0.5 M EDTA, pH 8.0 and heating for 10 minutes at 65°C;
4. Proceed as for the biotinylated probe (steps 4 to 8).

## Probe Denaturation

Denature the probes in [FHB](#) by incubating for 10 minutes at 95°C, then immediately add the probes to the tissues (without prior cooling).

## In situ DNA Hybridization to fixed embryos or discs

All the following steps are performed in 1.5ml eppendorf tubes, on a rotating wheel at RT. The embryos or discs should sediment at the bottom of the tube between each change of buffer. If necessary, you can help sedimentation by very gentle centrifugation, 1 minute at 500 rpm.

## Pre-Hybridization

1. Re-hydrate fixed embryos into PBT (PBS 1X; 0.1% Tween 20) by passing through the following solutions (1ml each, 3-5 min each step):
  - 90% MeOH; 10% PBT
  - 70% MeOH; 30% PBT
  - 50% MeOH; 50% PBT
  - 30% MeOH; 70% PBT
  - 100% PBT
2. For larval imaginal discs, skip the re-hydration step and start directly at the RNaseA step below, after fixation;
3. Incubate tissues in 1ml of 100-200µg/ml RNaseA in PBT for at least 2 hours at RT on a rotating wheel (or overnight at 4°C);
4. Incubate tissues for at least 1 hour at RT in 1ml of PBS-Tr (PBS 1X; 0.3% Triton X-100) (see [comment 2](#));
5. Transfer tissues into a pre-Hybridization Mixture ([pHM](#)) by passing through the following solutions (1ml each, 20 minutes each step):
  - 80% PBS-Tr; 20% [pHM](#)
  - 50% PBS-Tr; 50% [pHM](#)
  - 20% PBS-Tr; 80% [pHM](#)
  - 100% [pHM](#).

## Hybridization

1. Denature embryonic or larval DNA by incubation for 15 minutes at 80°C in 100% [pHM](#). While the tissues are still at 80°C, remove as much buffer as possible and add the denatured probes in [FHB](#) to the tissues (without prior cooling) (see the probe labeling section and also see [comment 3](#));
2. Cover the tissue suspensions with a drop of mineral oil, and incubate in a thermomixer at 37°C with a gentle agitation (about 450 rpm) for 14-17 hours (i.e. overnight).

## Post-hybridization washes

1. Add 500µl of solution 1 (described in Solutions for FISH hybridization) and remove supernatant (remove as much liquid as possible). This helps to immediately remove the mineral oil.
2. Wash the tissues by subsequent passages through post-hybridization wash solutions 1 to 7 (1ml each, about 20 minutes each step):
  - Perform washes with post-hybridization wash solutions 1 (2 washes), 2, 3, 4 in the thermomixer at 37°

- C with a gentle agitation (about 800 rpm);
- Perform washes with post-hybridization wash solutions 5, 6, 7 on a rotating wheel at RT (see [note 3](#) and [comment 4](#)).

## Immunodetection of the FISH signals

1. Rinse rapidly the tissues in blocking solution;
2. Block for at least 2 hours at RT (or 4°C overnight), in 0.5ml of blocking solution;
3. Incubate in 0.5ml of blocking solution containing the fluorescent labeled anti-DIG or anti-Biotin antibodies for 1 hour at RT (described in Solutions and antibodies for detection of the FISH signals). For two-colour FISH, incubate first with the anti-DIG alone for 1 hour at RT, then add the anti-Biotin and incubate an additional hour at RT with both antibodies;
4. Rinse tissues in PBS-Tr (3x 5-10 minutes each wash);
5. Wash three more times in PBS-Tr, 20 minutes each wash;
6. Counterstain DNA with DAPI (1:1000 of the stock solution) in PBT for 10 minutes at RT;
7. Wash once in PBT;
8. Rinse quickly in PBS (in order to remove all traces of detergent) and leave about 50µl of PBS to put the tissues on the slide with a cut yellow tip;
9. On the slide, remove as much PBS as possible around the tissues with a Gilson p200 pipette;
10. Mount tissues with about 40µl of freshly prepared ProLong antifade and put the slides at least one night at 4°C before acquisitions. Mounted tissues in ProLong antifade can be stored for up to 3 weeks at 4°C.

See [note 6](#)

See [comment 5](#) and [comment 6](#).

## Acquisition

Analyze the slides under a fluorescent microscope equipped with a CCD camera, a 63X or a 100X oil objective and appropriate filters for the different fluorochromes (i.e. Dapi, FITC and Rhodamine). An example of two-colour FISH on whole mount drosophila embryos is shown in [Figure 1](#). This figure represents the different imaging steps, from image acquisition to display of two-colour FISH signals of individual representative nuclei.

## Materials & Reagents

**Buffer A** Prepare as follows for 10 ml of buffer (make it fresh):

- 8.3ml - ddH<sub>2</sub>O
- 0.6ml - KCl (1 M stock solution) (60mM)
- 30µl - NaCl (5 M stock solution) (15mM)
- 10µl - spermidine (0.5 M; 1000X) (0.5 mM)
- 10µl - spermine (0.15 M; 1000X) (0.15 mM)
- 40µl - EDTA (0.5 M; 250X), pH 8.0 (2mM)
- 10µl - EGTA (0.5 M; 1000X), pH 8.0 (0.5 mM)
- 1.0ml - PIPES (150 mM; 10X), pH 7.4 (15 mM)

**pHM** pre-Hybridization Mixture (make it fresh):

- 50% formamide ((Fluka)
- 4X SSC
- 100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0
- 0.1% Tween 20

**FHB** FISH Hybridization Buffer (can be stored at -20°C for long time storage, and pre-warmed at 37°C before use):

- 2X SSC
- 10% dextransulfat (Pharmacia)
- 50% deionized formamide (Sigma)
- Salmon Sperm DNA at 0.5 mg/ml (0.05% final, i.e. 2% of a 2.5% sonicated and autoclaved stock solution)

## General reagents

- Bidistilled H<sub>2</sub>O (ddH<sub>2</sub>O)
- 10X PBS, pH 7.4 (Gibco)
- PIPES (Sigma)
- NaH<sub>2</sub>PO<sub>4</sub> (Prolabo)
- 20X SSC (Euromedex)
- Triton X-100 (USB)
- Tween 20 (USB)
- CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) (Sigma)

## Solutions for preparation of fixed whole mount embryos

- Bleach (Sodium hypochlorite with 2.6% of active chlore). Important: after opening, the bottle of bleach is conserved for no longer than one month
- [Buffer A](#) (see above table)
- Paraformaldehyde or p-formaldehyde (in powder, Prolabo, Cat. No. 28 794-295)
- Heptane
- 100% MeOH

## Kits and reagents for probe labelling

- Biotinylated probe: BioNick<sup>TM</sup> Labeling System (Invitrogen LT) (contains Biotin-14-dATP, Cat. No. 18247-015, for 50 reactions)
- Digoxigenin labeled probe: DIG-Nick translation Mix (Roche Diagnostics) (contains DIG-11-dUTP, Cat. No. 1 745 816, for 40 reactions)
- EDTA: 0.5M, pH 8.0
- PCR Purification Qiagen Kit
- ddH<sub>2</sub>O (Sigma)

## Solutions for FISH hybridization

- PBT (PBS 1X; 0.1% Tween20)
- PBS-Tr (PBS 1X; 0.3% Triton X-100)
- RNase A (Sigma R-6513); stock solution 10mg/ml; 100X)
- Dextranulfat Mw 500,000 (Pharmacia 17-0340-01): to make a 50% solution, we use the recipe of Pharmacia: weigh 5g of Dextranulfat in a 50ml Falcon tube, add 7ml of pre-warmed ddH<sub>2</sub>O at 60°C, and agitate for at least 30 minutes using a magnetic stirrer
- Formamide (Fluka) and deionized formamide (Sigma F-9037)
- Mineral oil (Sigma)
- pre-Hybridization Mixture ([pHM](#)) (see above table)
- ISH Hybridization Buffer ([FHB](#)) (see above table)
- Post-hybridization wash solutions (make them fresh)
  1. 50% formamide (Fluka) ; 2X SSC ; 0.3% CHAPS
  2. 40% formamide ; 2X SSC ; 0.3% CHAPS
  3. 30% formamide ; 70% PBT
  4. 20% formamide ; 80% PBT
  5. 10% formamide ; 90% PBT
  6. 100% PBT
  7. 100% PBS-Tr

## Solutions and antibodies for detection of the FISH signals

- BSA (Sigma, 98% electrophoresis purity)
- Normal donkey or goat serum (Jackson laboratories)
- Blocking solution: PBS 1X; 0.3% Triton X-100; 1% BSA; 10% normal donkey or goat serum
- FITC-anti-Biotin antibody made in goat (Vector Labs): 1:550 (i.e. 0.9µl in 500µl blocking solution)
- Rhodamine-anti-Digoxigenin antibody made in sheep (Roche): 1:45 (i.e. 11µl in 500µl blocking solution)
- DAPI (4'6' Diamidino-2-phenylindole) stock solution at 0.1mg/ml in 180 mM Tris-HCl, pH 7.5.

## Mounting Media

- ProLong antifade (Molecular Probes)

## Author Notes

1. At this stage, embryos can be stored at -20°C for several weeks if not immediately used.
2. Unlike for embryos, there is no way to store discs for a long period of time at this stage. For a short period (1 to 3 days), the discs can be stored at 4°C in PBT (see comment #8).
3. At this stage, tissues can be stored at 4°C for up to 1 week if not immediately used for the detection reactions.
4. The protocol described above is not long (two days) but then, if the FISH signals are good (as should be the case if you follow all the stages of this procedure), image acquisition and analysis usually takes much longer. The detailed procedure of image acquisition and analysis falls beyond the scope of this protocol, but you will find below the essentials on our way to acquire and analyse FISH signals.

**About image acquisition:** All our images are acquired with a cooled Micromax YHS 1300 CCD camera (Roper Scientific) driven by the Metamorph software (Roper Scientific), and mounted on a DMRXA Leica microscope. Images are acquired with a 100X Plan/Apo objective (NA 1.4) mounted on a piezo electric (Roper Scientific). Optical sections are collected at 0.5µm intervals along the z-axis. For each color channel, we usually take z-stacks of 6-7µm (i.e. 13 to 15 images if collection at 0.5µm intervals) in order to have entire embryonic or larval nuclei in the z-axis. This step can be performed by using the Acquire Multiple Wavelengths mode coupled with the Acquire Z Series mode of the Metamorph software. In this way 3D stacks, one per channel, can be reconstituted (if the background of the images is high, apply the Nearest Neighbors 2D Deconvolution mode of Metamorph on the stack) and color combined to give a multichannel 3D stack.

**About image analysis:** This can be the trickiest part... To determine the relative position of two genes in the nucleus is not as trivial as it seems. Be prepared to count the relative position of your two favorite genes in many nuclei of different specimens to end up with statistical significant values. On multichannel 3D stacks, the distance between the visually determined center of mass of dots corresponding to DNA hybridization signals can be determined using the x,y,z-measured distance module of the Metamorph software. The pixel size in your image can be calibrated with Metamorph (for example, the pixel size is 0.065 µm when using a 100X objective and a camera binning of 1), and distances between signals can then be expressed in micrometers for many nuclei of different tissues. Then, the 3D-distance distribution between two loci into the nucleus can be plotted by using the Microsoft Excel software (see Bantignies *et al*, 2003 for examples).

Please note that these imaging procedure require a real imaging expertise. Users that are new to this field are strongly recommended, during initial experiments, to ask for advice and support from the nearest advanced imaging lab or facility. See [comment 7](#).

5. Concerning the probe labeling, we have recently been successfully using the FISH Tag DNA kit from Invitrogen. With this labeling technology, the fluorescent dyes are directly incorporated into the DNA probes. This system has two main advantages:
  - First, it is more sensitive and more reliable. The consequence is that you can easily decrease the length of your genomic probes, i.e. you can make a good probe of a single copy locus with as less as 5 PCR fragments of 1 to 1.5 kb covering approximately 10 kb of genomic DNA.
  - Second, the in situ DNA Hybridization steps are exactly the same, but after you do not need secondary antibodies to detect the probes. Therefore, escape steps 1 to 5 of the "Immunodetection of the FISH signals" paragraph, and directly go to step 6 (DAPI staining) until step 10 (mounting).

All the procedures for the direct labeling of your probes are well described in the FISH Tag DNA Kit manual that you can download from the Invitrogen website. For the Two colour FISH, we recommend to use the Alexa Fluor 488 dye (FISH Tag™ DNA Green Kit, Cat. No. F32947) and the Alexa Fluor 555 dye (FISH Tag™ DNA Orange Kit, Cat. No. F32948). Note that for the Nick Translation reaction, we are generally using less of the DNase I amount recommended (1/2 to 1/3) in order to give the appropriate length for the probes (ranging from 250 to 500 bp), and we are performing the reaction for at least 1h30 and no more than 2 h at 15°C. This is really important for a good incorporation of the dyes. The trick for a good probe is to have at least 2 dyes incorporated per 100 bp, best if 4 to 6 dyes per 100 bp. The calculation of this parameter is well described in the Invitrogen manual.

6. The described FISH protocol can be coupled with an immunostaining reaction (FISH-I). This allows us to study the relative position of a gene with specific regulatory factors, or specific nuclear compartments (nuclear bodies, nucleolus, lamin...).

Basically, first perform the FISH protocol as described here, and second perform immunostaining with the

antibody of interest.

- After the post-hybridization washes, embryos or wing imaginal discs are blocked in PBS, 0.3% Triton (PBSTr), 10% Normal Goat Serum (NGS) for 2 h at RT and incubated overnight at 4°C in PBSTr, 10% NGS with the primary antibody. After this incubation, tissues are washed several times in PBSTr (3 short washes of 5-10 min, 3 longer washes of 20-30 min).
- If you use the undirect labeling system, block again in PBSTr, 1% BSA, 10% NGS for 2 h at RT, and incubated sequentially in blocking buffer with the secondary antibodies: first, with anti-digoxigenin-Rhodamine (Roche Diagnosis) at a dilution of 1:50 for 1h at RT, second, with anti-biotin-FITC (Vector laboratories) diluted at 1:550 (without removing the anti-DIG) for an additional hour. Remove the solution containing the anti-DIG and anti-biotin antibodies. Third, incubate with an anti-mouse or rabbit -Cy5 (Jackson Laboratories) in PBSTr, 10% NGS, at a dilution of 1:200 for 1 h at RT. After these incubations, tissues are washed several times in PBSTr (3 short washes of 5-10 min, 3 longer washes of 20-30 min).
- If you use the direct labeling system recommended in [note 5](#), then block again in PBSTr, 10% NGS for 1 h at RT (facultative), and incubate with a secondary anti-mouse or rabbit -Cy5 (Jackson Laboratories) in PBSTr, 10% NGS, at a dilution of 1:200 for 1 h at RT. Wash several times in PBSTr.
- For both, then directly go to step 6 of the “Immunodetection of the FISH signals” paragraph (DAPI staining) until step 10 (mounting).

We have been successfully using this procedure with antibodies against proteins, which bind strongly to chromatin (for example, the Polycomb group proteins), or lamin. However, some other nuclear proteins might have a more labile nuclear localization and might not resist to that procedure (for example, components of the RNAi machinery). Importantly, you will find some examples of this FISH-I procedure in references 6-8.

So good luck everybody... FB, CG and GC

## Reviewer Comments

Reviewed by: [Cornelia Fritsch](#) and [Donna Arndt-Jovin](#), MPI for Biophysical Chemistry, Göttingen

1. If fixed in [Buffer A](#) and p-formaldehyde, the IDs may be stored in methanol at -20°C for months without changes in hybridization efficiency. Exchange the buffer stepwise to methanol after washing discs out of the fixative with PBT.
2. For the alternative protocol listed below do a post-RNase fixation step for 20 minutes in 4% p-formaldehyde/PBST.
3. Although 80°C has been found to be adequate for embryos some probes show improved hybridization if IDs are denatured at 83°C.
4. We generally perform the first wash for 10 minutes at 45°C followed by solution 1 again at 37°C to improve stringency.
5. For delineation of the nuclei in order to measure distances of the hybridization locus to the membrane or center of the nucleus it is recommended to stain the nuclear membrane in both embryos and discs with a monoclonal antibody specific for *Drosophila* lamin and a secondary goat anti-mouse Cy5 labelled secondary antibody (see alternative protocol).
6. Alternative method for fixation and hybridization of imaginal discs with lamin staining of the nuclear envelope.

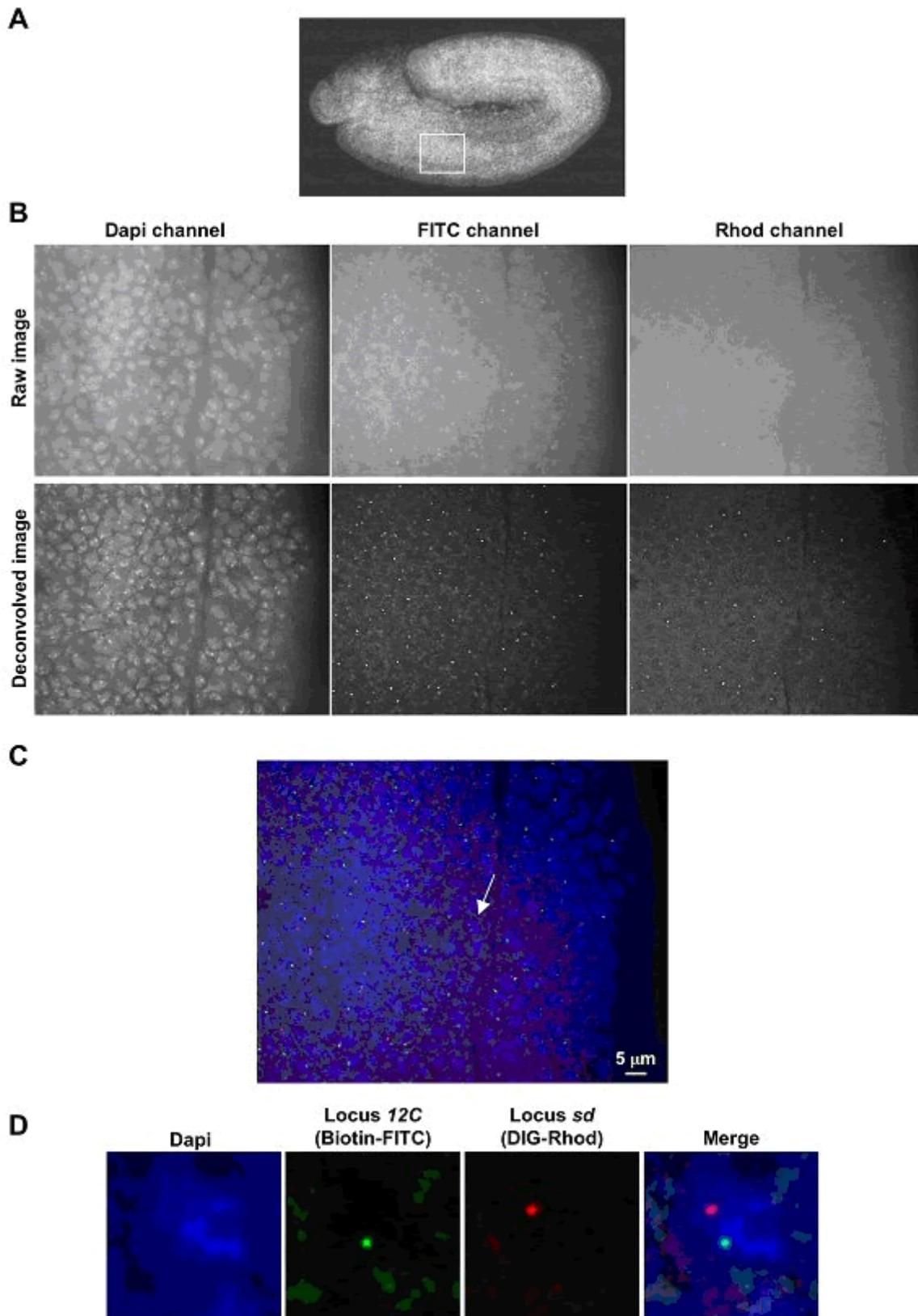
### Fluorescence in situ hybridization in larval imaginal discs

Imaginal discs (ID's) were dissected from third instar larvae, fixed for 20 minutes in 4% paraformaldehyde, 0.2% Triton X-100 in [Buffer A](#) (60mM KCl, 15mM NaCl, 0.5mM spermidine, 0.15mM spermine, 2mM EDTA, 0.5mM EGTA, 15mM PIPES pH 7.4), followed by washing 5 minutes in 5% BSA/PBS, two times in PBS and then two times in PBT (PBS + 0.1% Tween 20). RNA was digested with 300µg/ml RNaseA in PBT for 3 hours. The discs were washed twice in PBT for 10 minutes, post-fixed for 20 minutes in 4% paraformaldehyde/PBS and reequilibrated by washing 2 times for 15 minutes in 5% BSA/PBS and finally PBS. From PBS discs were stepwise equilibrated into hybridization mixture (50% formamide, 2xSSC, 50mM NaPO<sub>4</sub> pH 7.0, 0.1% Tween 20) over a period of 1 hour. The larval DNA was then denatured at 83°C for 15 minutes after which as much of the buffer as possible was removed. The denatured probe (~1µg DNA) in the presence of a 20-fold excess of carrier yeast tRNA and a 10-fold excess of cot 1 human DNA in hybridization buffer was then immediately applied to the discs and incubated over night at 37°C with gentle agitation. After hybridization, the discs were washed for 10 minutes each with the following buffers: 50% formamide, 2xSSC (30mM sodium citrate dihydrate, 0.3M sodium chlorine), 0.1% Triton X-100, 0.3% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) (wash buffer) first at 45°C, then at 37°C; 40% formamide, 0.1xSSC, 0.1% Triton X-100, 0.3% CHAPS at 37°C; 20% formamide/PBS and twice with PBS, all three steps at room temperature. Blocking was performed with 1% BSA/PBT for 30 minutes after which the discs were incubated for 2 hours with sheep anti-DIG Fab fragments directly labelled with Cy3 and

monoclonal mouse anti-lamin (Risau, W. *et al*, 1981) in 1% BSA/PBT. The imaginal discs were then washed three times for 20 minutes in PBT after which they were incubated for 1 hour with Cy5 labelled goat anti-mouse (Fab)2 in BSA/PBT and then washed three times for 15 minutes with PBT. Counterstaining was performed with 5 $\mu$ M DAPI/PBT for 5 minutes and after 15 minutes washing with PBT, the discs were mounted in mowiol.

7. Data can be collected using a confocal scanning microscope for multiple channels. It is preferable to delineate the nuclei by means of lamin staining since most CLSMs do not have UV excitation sources. In addition, the actual distance to the periphery is much more accurate than in trying to determine the boundary with a DNA stain. There are usually fewer problems with registration of images from a CLSM but you should know your instrument, the software and the limitations in all cases as cautioned by the Cavalli lab.

## Figures



**Figure 1: Example of two-color FISH in *Drosophila* embryos**

A. A *Drosophila* embryo stained with Dapi and acquired with a 20X objective. The white rectangle corresponds to the the area of the embryo imaged when the magnification is increased by switching to a 100X oil objective.

B. Upper panels: raw 16-bit images (top) acquired with a 100X oil objective and the corresponding dichroic filters for the different fluorochromes (Dapi / Leica Filter A4, FITC / Leica Filter L5, Rhodamine / Leica Filter Cy3). Lower

panels: the deconvolved images corresponding to the upper panels. Each raw image was deconvolved using the Huygens deconvolution software with the Huygens MLE single tif procedure (Scientific Volume Imaging). For figure display, all images were converted to 8 bits, mounted and cropped with Adobe photoshop.

C. Merged image of Dapi labeling (blue), the 12C locus (green), and the sd locus (red). Only one slice along the z-axis is shown, containing approximately 150 nuclei. One or two FISH signals are seen in most of the nuclei. The two gene signals are not necessary in the same z-axis slice, and that is why some nuclei have only one FISH signal, or no signal at all. The arrow indicates a nucleus chosen for enlargement.

D. An enlarged individual nucleus. Dapi staining is shown in blue, the 12C locus (labeled by biotin and revealed by the FITC-anti-Biotin antibody) is green, the sd locus (labeled by Digoxigenin and revealed by the Rhodamine-anti-DIG antibody) is red, and the merge of the three channels is shown in the right panel.

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