Multicolour 3D-FISH in vertebrate cells (PROT23)

Marion Cremer, Claudia Weierich & Irina Solovei
LMU Biozentrum
Department Biology II
Grosshadernerstr. 2
82152 Martinsried-Planegg, Germany

Email feedback to:
Marion.Cremer@lrz.uni-muenchen.de

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Introduction

Multicolour 3D-FISH in combination with confocal microscopy, 3D image reconstruction and quantitative image analysis is an efficient tool for the analysis of the 3D genome structure and of the spatial relationship of defined nuclear targets comprising entire chromosome territories down to the level of single gene loci. Until a few years ago the drawback of confocal microscopy was its limitation to three or at maximum to four different fluorochromes that could be visualized simultaneously. Recent developments of a "new generation" of confocal microscopes allow the simultaneous excitation and distinct visualization of five different fluorochromes (the number can be increased if colour unmixing software is used) within one experiment, opening the way for a simultaneous delineation of numerous differently labeled intranuclear targets.

Here we provide protocols for the preparation of complex DNA-probe sets suitable for 3D-FISH with up to six different fluorochromes, protocols for 3D-FISH on cultured mammalian cells (growing in suspension or adherently growing), and protocols for an efficient 3D-FISH on tissue sections, that have all been used successfully by our group. We restrict to protocols describing the labeling of a given DNA probe (such as chromosome specific probes, BACs or plasmids etc.) with a single hapten or fluorochrome. We should mention here that the term M-FISH (which is originally the abbreviation for multiplex (!) FISH and not for multicolour FISH) is often related to the combinatorial labeling of a probe with different, usually two or three fluorochromes/haptens in order to increase the number of distinguishable targets. While this approach has been widely used as a tool for the complex analysis of metaphase chromosomes and interphase cytogenetics, its successful application for 3D-FISH on 3D preserved nuclei in combination with confocal microscopy has been shown only in a few studies (see e.g. Bolzer *et al.* (2005)). This is mainly due to the fact that analysis of confocal image stacks containing combinatorial labeled probes is highly demanding and requires specialized skills. For the special aspects with regard to the generation of DNA probes by combinatorial labeling we kindly ask you to refer to the papers of Bolzer *et al.*, (2005) and Fauth *et al.*, (2001).

Finally we want to emphasize that multicolour FISH on 3D preserved nuclei is a somewhat delicate method where minor deviations or experimental mistakes can easily change the quality of an experiment. For readers that are interested to concern this technique in more detail we refer to our previous and recent publications (Solovei *et al.* 2002a; Solovei *et al.* 2002b; Walter *et al.* 2006)

Procedure

Probe preparation and labeling procedures

High quality of probe labeling is crucial for efficient 3D-FISH experiments. Most DNA-probes are efficiently labeled by the PCR amplification techniques described below, which saves time and material. Even complex probe pools, containing e.g. large sets of BAC pools can be labeled by this method. Direct labeling of probes by incorporating fluorochrome-conjugated nucleotides (such as FITC-dUTP, TAMRA-dUTP, TexasRed-dUTP and others) has been quite successful following our protocols and can be considered equally efficient in comparison to hapten-labeled probes (Biotin, Digoxigenin and DNP).

Probe preparation

Although a large number of labeled DNA probes are commercially available, we strongly encourage to generate own probes. This saves money and insures higher flexibility in designing experiments. An official source to obtain chromosome specific DNA probes is provided by M. Rocchi, University of Bari (http://www.biologia.uniba.it/rmc/).

In the following section we present protocols for the generation of DNA probes frequently used in 3D-FISH experiments

Chromosome painting probes

Chromosome painting probes are usually generated from flow sorted chromosomes. The genomic DNA is initially amplified by DOP PCR using the universal primer 6MW (Telenius *et al*., 1992). The amount of this primary amplification product can be increased by one or a few further rounds of DOP amplification (see note 1). The amplified DNA is used for the subsequent labeling DOP-PCR (see figure 1).

Standard reaction for (re-)amplification for a single DOP amplification reaction

48.5µ	MM
30-200ng	DOP amplified DNA (normally corresponds to 1µl)
0.5µl	Taq-Polymerase

Mastermix for the amplification of DNA by DOP-PCR using the 6MW-primer (see comment 1)

MM for 20 reactions		**concentration in reaction**
200µl	PCR Buffer D 5x (Invitrogen Cat K1220-02D)	1X
100µl	6 MW-primer (20µM) (sequence CCGACTCGAGNNNNNNATGTGG)	2µM
100µl	Polyoxyethylene ether W1 (1%) (SIGMA Cat P7516)	0.1%
80µl	dNTP-mix (2.5mM each)	200µM
490µl	H_2O	

For a single DOP-PCR reaction we use 48.5µl MM + 1µl of the DOP amplified DNA + 0.5µl Taq-Polymerase (5U/µl).

Features of DOP amplification cycles (see note 2)

		Primary	**Secondary**
	Initial denaturation	96°C 3'00"	96°C 3'00"
	Denaturation	94°C 1'00"	
Low stringency (x8)	Annealing	30°C 1'30"	
	Extension	time ramp 14°C/min 72°C 2'00"	
	Denaturation	94°C 1'00"	94°C 1'00"
High stringency (x35)	Annealing	56°C 1'00"	56°C 1'00"
	Extension	72°C 2'00"	72°C 2'00"
	Final extension	72°C 5'00"	72°C 5'00"
	Approximate time	4hr 15'	3hr

Standard reaction label-PCR for a single DOP labeling reaction

48.5µl	MM
30-200ng	DOP amplified DNA (normally corresponds to 1µl)
0.5µl	Taq-Polymerase

Labeling DOP-PCR e.g.Bio-dUTP (see note 3 and note 4 and comment 1)

MM for 20 reactions		**Final concentration**
100µl	GeneAmp PCR Buffer 10x (Applied Biosystems, N808-0130)	1x (50mM KCl, 10mM Tris, pH8.3)
80µl	$MgCl_2$ (25mM)	2mM
100µl	6 MW-primer (20µM)	2µM
50µl	ACG-mix (each 2mM)	100µM

80µl	dTTP (1mM)	80µM
20µl	Bio-dUTP (1mM)	20µM
530µl	H_2O (bi-distilled)	

We use 48.5µl MM + 1µl of the DOP amplified DNA + 0.5µl Taq-Polymerase (5U/µl) for a single DOP labeling reaction.

Features of DOP-labeling cycles

	Initial denaturation	94°C 3'00"
	Denaturation	94°C 1'00"
x20	Annealing	56°C 1'00"
	Extension	72°C 0'30"
	Final extension	72°C 5'00"
	Approximate time	1hr15'

Locus specific probes from BAC clones

We usually order BAC probes from the C.H.O.R.I. BACPAC Resources Center.

Genomic DNA from BAC clones can be obtained by any conventional DNA purification method. Prior to amplification of the BAC-DNA RNAse-treatment and concentration measurements (by gel control or spectrophotometer) should be performed (50-100ng/ µl). For a primary amplification and subsequent labeling of BAC-DNA for 3D-FISH we use a modified DOP-PCR employing two different primers in separate amplification reactions described as DOP2 and DOP3 by Fiegler *et al*. (2003) (see note 5).

We perform the amplification and labeling reactions in separate PCR-setups for each DOP2 and DOP3 primer.

Standard reaction for a single DOP 2 or DOP3 amplification reaction

33µl	MM
2µl	DNA (50-200ng)
15µl	H_2O(Bi-distilled)
0.5µl	Taq-Polymerase

DOP2 and DOP3-PCR (see note 6)

MM for 20 reactions	**Final concentration in final reaction set up**
200µl PCR Buffer D 5x (Invitrogen Cat K1220-02D)	1x
100µl DOP2* or DOP3**-primer (20µM)	2µM
100µl Polyoxyethylene ether W1 (1%) (SIGMA Cat P7516)	0.1%
80µl dNTP-mix (each 2.5mM)	200µM
180µl H_2O (bi-distilled)	

* Primer sequence DOP 2: (CCGACTCGAGNNNNNNTAGGAG)
**Primer sequence DOP 3: (CCGACTCGAGNNNNNNTTCTAG)

Standard reaction per single reaction for each primer set:
33µl MM + 2µl DNA (approx. 50-100ng/µl) + 15µl H_2O (bi-dist.) + 0.5µl Taq-Polymerase (5U/µl)

Features of DOP2/ DOP3 amplification cycles (see figure 2)

	Initial denaturation	96°C 3'00"
	Denaturation	94°C 1'30"
Low stringency	Annealing	30°C 2'30"
	Time ramp 6°C/min extension	72°C 3'00"

(only for primary amplification) **(10 cycles)**		
High stringency **(30 cycles)**	Denaturation	94°C 1'00"
	Annealing	62°C 1'30"
	Extension	72°C 2'00"
		94°C 1'00"
	Final extension	62°C 1'30"
		72°C 8'00"
	Approximate time	4hr 30'

The amplification product is used for the labeling reaction employing the same two primer sets.

Standard reaction for a single DOP 2 or DOP3 label PCR reaction

47.5µl MM
1-3µl DNA of DOP2 or DOP3 amplification product, (see below)
0.5µl Taq-Polymerase.

DOP2/DOP3 PCR probe labeling with hapten-dUTP (e.g. Biotin-dUTP)

MM for 20 reactions	**Final concentration in reaction**
100µl GeneAmp PCR Buffer 10x (Applied Biosystems, N808-0130)	1x (50mM KCl, 10mM Tris, pH= 8.3)
80µl $MgCl_2$ (25mM)	2mM
100µl DOP2 *or* DOP3-primer (20µM)	2µM
50µl ACG-mix (each 2mM)	100µM
80µl dTTP (1mM)	80µM
20µl Biotin-dUTP (1mM)	20µM
530µl H_2O	

For each primer set use per single reaction: 47.5µl MM + 2µl DNA (primary DOP2/DOP3 amplification, 50-100ng/µl) + 0.5µl Taq-Polymerase (5U/µl)

DOP2/DOP3 labeling PCR with directly labeled dUTP (e.g.TAMRA-dUTP)

For directly labeled nucleotides we prefer to set up first the mastermix and the fluorescent labeled dUTP is added before starting up the reaction.

MM for 20 reactions	**Final concentration in reaction**
100 µl GeneAmp PCR Buffer 10x (Applied Biosystems, N808-0130)	1x (50mM KCl, 10mM Tris, pH= 8.3)
80 µl $MgCl_2$ (25mM)	2mM
100 µl DOP2 or DOP3-primer (20µM)	2µM
50 µl ACG-mix (each 2mM)	100µM
80µl dTTP (1mM)	80µM
490 µl H_2O	

For each primer set use per single reaction: 45 µl MM + 3µl fluorochrome-dUTP (1mM) + 2 µl DNA (primary DOP2/ DOP3 amplification, 50-100ng/µl) + 0.5 µl Taq-Polymerase (5U/µl)

Features of DOP2/DOP3-labeling cycles (see figure 3)

	Initial denaturation	94°C 3'00"
	Denaturation	94°C 1'00"
x20	Annealing	56°C 1'00"
	Extension	72°C 0'30"
	Final extension	72°C 5'00"
	Approximate time	1hr15'

After completion of the DOP2/DOP3 Label-PCR the DOP2 and DOP3 amplification products targeting the same BAC-clone can be merged in one tube since they will be always used together as hybridization probes. See note 7 and note 8.

Small DNA-probes from cosmids or plasmids clones

These kind of probes, especially plasmids, have become out of fashion for 3D-FISH due to their delicate hybridization signals and the lack of a systematic compilation in databases. They are used only in special situations.

We strongly recommend preparing these probes by genomic DNA nick-translation, if not done by sequence-specific primer pairs. Amplification and/or labeling of these probes by universal DOP-PCR did not lead to satisfactory results in our hands (see note 9 and figure 4).

Nick-translation (see comment 2)

100µl NT-reaction mixture		**Final concentration**
xµl	DNA (~2µg)	2µg (less amount and up to 4µg is ok)
10µl	NT-buffer 10x	50mM TrisHCl, 5mM $MgCl_2$, 50µg/ml BSA, pH=7.5
10µl	β-Mercaptoethanol (100mM)	10mM
10µl	dNTP-mix (10x)	50µM dATP, dCTP, dGTP, each 10µM dTTP
4µl	modified dUTP (e.g. Dig-dUTP)	20µM 40µM in case of fluorochrome-labeled nucleotide
xµl ad 100µl	H_20 bi-distilled	
2µl	DNase I (2000 U/ml)	0.016U
2µl	Polymerase I (10 U/µl)	0.2U

* Dilute stock solution (2000 U/ml) 1:250 in ice-cold water and keep it on ice, use 2µl of this dilution per reaction

Incubate 90 minutes at 15°C -> freeze to -20°C and analyze the length of the fragments on a gel (ideal size 400-800 bp) -> further digestion if necessary using 1µl of diluted DNaseI for 15 minutes at 20°C -> finalize the reaction by adding 1µl of 0.5M EDTA (see note 10 and note 11).

Human pancentromeric, mouse major satellite and chromosome specific centromeric probes

To generate pancentromeric and mouse major satellite probes we highly recommend first to amplify the repetitive sequences by the following protocols and then label the primary amplified DNA by nick-translation. For the generation of chromosome specific pericentromeric probes we strongly recommend labeling by nick-translation from centromere specific sequences of genomic DNA. See figure 5.

Specific amplification of a pancentromeric probe (human)

100µl PCR-reaction mixture		**Final concentration**
10µl	GeneAmp PCR Buffer 10x (Applied Biosystems, N808-0130)	1x (50mM KCl, 10mM Tris, pH8.3)
8µl	$MgCl_2$ (25mM)	2mM
2µl	a27 Primer* (100µM)	2µM
2µl	a30 Primer** (100µM)	2µM
2µl	Genomic DNA (100ng/µl)	2ng/µl
5µl	ACGT-Mix (each 2mM)	100µM
70µl	H_2O Bi-distilled.	

0.8µl Taq-Polymerase (5U/µl)

*Primer sequence a27 (5'-CAT CAC AAA GAA GTTTCT GAG GCT TC)
**Primer sequence a30 (5'-TGC ATT CAACTC ACA GAG TTG AAC CTT CC)
Reference: Mitchell *et al*., (1985)

Features of the pancentromeric-amplification cycles

	Initial denaturation	94°C 3'00"
	Denaturation	94°C 0'45"
x35	Annealing	62°C 1'20"
	Extension	72°C 1'20"
	Final extension	72°C 5'00"

Specific amplification of mouse major satellite DNA

100µl PCR-reaction mixture		**Final concentration**
10µl	GeneAmp PCR Buffer 10x (Applied Biosystems, N808-0130)	1x (50mM KCl, 10mM Tris, pH8.3)
8µl	$MgCl_2$ (25mM)	2mM
4µl	Forward primer* (25µM)	1µM
4µl	Reverse primer** (25µM)	1µM
10µl	Genomic DNA (10ng/µl)	1ng/µl
5µl	ACGT-mix (each 2mM)	100µM
58µl	H_2O Bi-distilled.	
0.8µl	Taq-Polymerase (5U/µl)	

*Sequence of forward primer of mouse major satellite DNA: 5'-GCG AGA AAA CTG AAA ATC AC
**Sequence of reverse primer of mouse major satellite DNA: 5'-TCA AGT CGT CAA GTG GAT G
Reference: Horz and Altenburger (1981)

Features of the pancentromeric-amplification cycles

	Initial denaturation	94°C 3'00"
	Denaturation	94°C 1'00"
x35	Annealing	56°C 1'00"
	Extension	72°C 2'00"
	Final extension	72°C 5'00"

We highly recommend to label this primary amplified DNA by nick-translation.

Cell fixation and pre-treatment steps for an efficient permeabilization

Fixed cells require further pretreatment to obtain an efficient accessibility of the DNA probe to the nuclear target DNA. The individual steps listed below should be adjusted to the cell-type and requirements of hybridization probes in order to get an optimal balance between the preservation of the nuclear morphology and hybridization efficiency.

Treatment with the detergent Triton X100 and repeated freezing in liquid nitrogen after incubation in Glycerol helps to make nuclear DNA accessible for FISH probes without strongly affecting the 3D chromatin architecture. These two steps are generally sufficient for hybridization of highly repetitive sequences, e.g. centromeric regions.

Additional deproteinization steps are necessary when single copy DNA sequences are targeted. There are two methods of deproteinization: (i) incubation in HCl and (ii) digestion with pepsin. These pretreatments may be combined or used separately. In many cases, a short incubation in 0.1N HCl makes nuclear DNA sufficiently accessible for probes. Nevertheless, depending on the cell type processed, additional pepsin treatment may improve hybridization signals e.g. cosmid probes. Pepsin incubation should be monitored under the microscope as the duration of pepsin treatment critically affects the preservation of the nuclear morphology. It tends to alter the 3D morphology of the nuclei more than incubation in 0.1N HCl.

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Fixation of adherently growing cells for 3D-FISH

1. Rinse cells in 2-3 changes of PBS at 37°C.
2. Fix in 4% paraformaldehyde in PBS (freshly made, pH 7.0) 10 minutes at room temperature (RT). During the last minute add few drops of 0.5% TritonX-100/PBS.
3. Wash in PBS with 0.01% TritonX-100 -> 3x3 minutes, RT.
4. 0.5% TritonX-100/PBS -> 5-15 minutes, RT.
5. 20% Glycerol in PBS -> at least, 60 minutes, RT (better over night (ON)).
6. Freeze in liquid nitrogen (15-30 seconds), thaw gradually at RT, soak in 20% Glycerol/PBS -> repeat 4 to 6 times.
7. Wash in PBS, 3x10 minutes.
8. Incubate 5 minutes in 0.1M HCl.
9. Rinse in 2xSSC
10. Incubate in 50% formamide (pH = 7.0)/2xSSC (at least 30 minutes at RT, or ON at RT; or few days at +4°C).

Slides can be kept in 50% formamide for several months. (See note 13)

Fixation of cells growing in suspension for 3D-FISH (e.g. lymphoblastoid cells)

1. Incubate dried coverslips (stored in 80% EtOH) 1 hour with ~150µl Poly-Lysine Hydrobromide (1mg/ml). We recommend to put the drop of Poly-Lysine on a piece of Parafilm or in a Petri dish and place the coverslip on the drop;
2. Flush in H_2O bi-dist. and air-dry the cover slips;
3. We recommend to use roughly 1ml of a actively growing cell culture per 22x22mm cover slips -> Spin suspension of cells 10 minutes at 1000rpm -> Discard supernatant and resuspend the pellet in RPMI/50%FCS. In order to obtain an appropriate number of cells on the slide we recommend to resuspend the cell in about ¼ of the initial volume (see comment 3);
4. Place ~200µl of cell-suspension on a coverslip and incubate for 1 hour at 37°C in an incubator -> Check attachment of cells under the microscope, briefly drain off the medium.
5. Incubate 40-60 seconds in 0.3x PBS (this step prevents the shrinkage of these spherically shaped cells that are otherwise prone to collapse during the following fixation. However, keep this step to the time indicated; otherwise the nuclei will increase in size);
6. Fix in 4% Paraformaldehyde/0.3x PBS 10 minutes -> after 8 minutes add some drops of 0.5% Triton-X/PBS solution;
7. Wash 3x5 minutes in 0.05% Triton X/PBS;
8. Incubate in 0.5% TritonX/PBS for 20 minutes;
9. Transfer coverslips to 20% Glycerol/PBS and incubate at least 30 minutes at room temperature (better: incubate overnight at 4°C);
10. Freeze in liquid nitrogen (~30 seconds) and thaw on a piece of tissue paper. As soon as the frozen layer disappears, put the coverslip back to 20% Glycerol/PBS -> repeat 4x;
11. Wash in 0.05% TritonX/PBS 3x5 minutes;
12. Incubate 5 minutes in 0.1N HCl (varies in different cell types);
13. Wash 2x1 minute in 2xSSC;
14. Place coverslips at least 30 minutes in 50%Formamid + 2xSSC (up to a few months at 4°C);
15. Continue with optional pepsin digestion (see note 14).

Pepsin treatment

1. Equilibrate slides (kept in 50%FA/2xSSC) in 2xSSC 2 minutes;
2. Switch to 1xPBS 3 minutes;
3. Pepsin: 3-5 minutes in 0.01 N HCl/0.002% pepsin. (prepare 49.5ml H_2O + 0.5ml 1NHCl at 37°C and add 10ml pepsin solution (stock solution 10%) just before use);
4. 2x5 minutes PBS/$MgCl_2$ (95ml 1xPBS/5ml 1M $MgCl_2$) for inactivation of pepsin;
5. Post-fixation in 1% PFA/1xPBS 1 minute;
6. Wash 1x5 minutes PBS;
7. Wash 2x5 minutes in 2xSSC, then back to 50%FA/2xSSC for at least 30 minutes.

Pretreatment for FISH on tissue sections

We also established working protocols for FISH on paraffin embedded tissue sections and *vibratome tissue sections*. In our hands we prefer FISH on vibratome sections rather than on paraffin embedded sections, but since human pathological material is up to now routinely embedded in paraffin, there may be no option for the tissue of interest. Upcoming procedures from the pathologists should help in the near future (see figure 6 and figure 7).

Treatment of paraffin sections for FISH (see comment 4)

1. Deparaffinize sections in 100% Xylol (3x15 minutes) and re-hydrate in ethanol series: 100% (2x15 minutes), 70% (1x15 minutes), dH_2O;
2. Permeabilize tissue with freshly made 1M NaSCN (sodium isothiocyanate) at 80°C for 30 minutes and then rinse in dH_2O;
3. Digest sections with 14mg/ml Pepsin (in 0.01N HCl) at 37°C for 30 minutes and then rinse in dH_2O;
4. Dehydrate slides in ethanol series (70% and 100% for 10 minutes each) and dry slides at RT.

Treatment of vibratome sections for FISH

1. Vibratome sections are stored in PBS containing 0.04% Na Azide at +4°C. For drying sections on slides, transfer sections to dH_2O for 5 minutes, then dehydrate in ethanol series: 30%, 50% for 10 minutes in each, and then in 2 changes of 70% for 30 minutes each. Then transfer sections on SuperFrost® Plus slides in a drop of 70% ethanol, spread sections with thin brushes, and dry sections at RT for 1-2 days;
2. Re-hydrate sections in 10mM Na-Citrate buffer pH 6.0 for 15 minutes. Place slides in microwave safe plastic container filled with warmed up to 80°C 10mM Na-Citrate buffer, and heat up the preparations for 6 minutes at 700W in the microwave oven. Repeat the heating procedure 5 times, re-filling the container with warmed buffer each time. The slides must be completely covered with liquid to avoid drying of sections during the whole procedure. After heating, cool slides down at RT;
3. Equilibrate sections in 2xSSC for 5 minutes and then in 50% formamide/2xSSC for 1-3 hours.

Probe precipitation

We recommend using 40-60ng/µl hybridization mix of non-repetitive probe DNA. Usually 2µl of labeled PCR-product per 1µl hybridization mix is reasonable for chromosome painting probes or locus-specific probes and 1ng/µl for centromere specific and other highly repetitive sequences. It may be helpful to increase the concentrations for small, non-repetitive probes. The concentration of unlabeled competitor DNA (e.g. Cot-1DNA) added to the probe for suppression of nonspecific hybridization depends on the presence of repetitive sequences in the probe and is around 10 up to 50-fold the concentration of the probe DNA. However, in case of complex probe mixtures it is assumed that probes suppress each other and the amount of COT-1 DNA can be reduced. A volume between 5-10µl of hybridization mix is normally assessed, but larger volumes for frequently needed probes work as well.

Probe precipitation (see note 15 and note 16 and comment 5)

1. Pipette into a 1.5ml screw cap tube all labeled DNA-probes that will be hybridized together. We recommend the following amounts:
    a. 40-60 ng/µl hybridization mix (hybmix) of non-repetitive probe DNA (such as paints, BACs etc);
    b. 1ng/µl hybmix for centromere specific and other highly repetitive sequences;
    c. 80-200ng/µl hybmix for small DNA-probes from cosmids or plasmids clones;
    d. Unlabeled competitor DNA (e.g. Cot-1DNA, 10-50 fold the concentration of the probe DNA);
    e. In case of small amounts of DNA add 20µg unlabeled salmon sperm DNA for efficient precipitation;
2. Precipitate probe DNA in ice-cold 100%EtOH (2.5x volume) and mix well -> at least 30 minutes, better O/N at -20°C;
3. Spin down 20 minutes at 13,000rpm;
4. Discard supernatant and dry the pellet (vacuum if possible);
5. Resuspend the pellet in 50% formamide/2xSSC/10% dextran sulfate as follows: resolve the pellet in the appropriate amount of 100% formamide (shake at 40°C, can take up to a few hours) and then add the equal volume of 4xSSC/20% dextran sulfate;
6. Hybridization probes can be stored at -20°C over a long period of time.

Set up of hybridization

Cellular DNA and probe DNA can be denatured simultaneously even in case of probes that require high excess of Cot1-DNA. The simultaneous denaturation of probe and cellular DNA is quick, simple, and optimal for retention of 3D morphology. We provide protocols for the hybridization set-up for cultured cells as well as for sections, which differ in some aspects depending on how sections were prepared. In most cases separate denaturation can be avoided except for cases where pre-annealing of a given probe DNA provides an extra benefit to reduce nonspecific hybridization (see note 17).

Hybridization set up by simultaneous denaturation (see comment 6)

1. Cultured cells;
    a. Place hybmix on a coverslip (e.g. 4µl per 18x18mm);
    b. Take a slide out of the 50% formamide/2xSSC and quickly drain the excess of fluid off the slide;
    c. Cover the target area of the slide by the coverslip with probe;
    a. Alternative method: In case cells are grown on a small cover slip (e.g. 15x15 or 18x18mm) you can place the drop

3.
    a.

       );
    b. Take out slides from 50% formamide/2xSSC, remove excess of liquid using soft paper, cover section with the chamber, and fill in chamber with hybridization mixture (see scheme below). Typically, 5-10µl of hybridization mixture is sufficient to fill the chamber. Seal mounted chamber with rubber cement; dry the rubber cement at RT;
    c. Pre-hybridize sections with loaded probe for few hours at 40-45°C before denaturation. Denature cellular DNA and probe DNA simultaneously on a hot block at 85°C for 5 minutes;
    d. Hybridize sections for 2-3 days at 37°C in metal boxes floating in a 37°C water bath;
4. For all material;
    a. After hybridization, carefully remove glass chambers with fine forcepsand perform after-hybridization washings of required stringency. Typically, washings include three changes of 2xSSC at 37°C for 10 minutes each and 0.1xSSC at 60°C for 10 minutes.

Separate of probe DNA and target DNA denaturation (see note 18 and comment 6)

1. Denature hybridization mix at 75°C for 5 minutes and allow it to pre-anneal at 37°C for 30-60 minutes. Place the pre-annealed probe on a coverslip just before or during target DNA denaturing;
2. Denature nuclear DNA in 70% formamide/2xSSC for 2-3 minutes at 70°C;
3. Take the denatured slide and quickly drain off the excess of fluid;
4. Cover the target area of the slide by the coverslip with hybridization mix;
5. Wipe off the excess fluid around the coverslip with soft paper and seal with rubber cement;
6. Hybridize at least overnight or better for 2-3 days at 37°C.

Detection

The choice of the detection scheme depends on several factors: (i) the number of haptens and fluorochromes used for probe labeling; (ii) the number of antibody layers required to obtain a sufficiently strong signal; (iii) the colour of nuclear counterstain. Some probes show high hybridization efficiency and require only one detection layer. We even have good results using directly labeled probes for small single copy sequences. The more different labels you want to detect in parallel the more difficult is the procedure to get sufficient stains. One should also plan carefully the detection scheme in advance in order to avoid reactions between antibodies used for the detection of different fluorochromes. In our lab different combinations of up to five or six different fluorochromes were successfully tested with regard to efficient signal intensities and distinct colour separation using a Leica SP2 confocal microscope. For a five colour detection scheme we got best results using Alexa488 (FITC) in parallel with Cy3 (TAMRA), Texas Red, Cy5 (Alexa633) and DAPI as DNA-counterstain. In addition Alexa514 can be implemented, however this in this combination this fluorochrome requires unmixing software for a clear delineation of all colours. In this combination we normally use direct fluorochrome labeling for TAMRA and TexasRed (and optionally FITC) while Alexa514, Cy3 and Cy5 should be detected using the respective conjugates with appropriate antibodies. To our experience in principle all commercially available fluorochrome-conjugated antibodies from the established companies work well. However, one should be aware that the quality of an antibody can vary sometimes depending on the batch provided.

Simultaneous detection of six colours was done by adding Alexa514 to the detection scheme above. Alexa514 is a very bright fluorochrome. It has a broad emission spectrum detectable in the emission spectrum of Alexa488 as well as in settings for Cy3-channel. Therefore the Alexa514 requires unmixing or substraction of the respective channels (see figure 9).

1. After hybridization, peel off rubber cement and strip off the coverslip and transfer it to 2xSSC;
2. Wash 3x5 minutes in 2xSSC at 37°C, shaking;
3. Wash stringently 3x5 minutes in 0.1xSSC at 60°C, shaking;
4. Rinse briefly in 4xSSC/0.2%Tween;
5. Block in 4xSSC/0.2%Tween + 4% BSA (bovine serum albumin) for 10-15 minutes at 37°C;
6. Incubate with the appropriate concentration of primary antibody (first layer) 35 minutes in a dark and wet chamber at 37°C. Antibodies or Avidin are diluted to a working concentration in blocking solution;

7. Wash 3x3 minutes in 4xSSC/0.2%Tween, shaking;
8. Incubate with the appropriate concentration of secondary antibody (second layer) for 35 minutes in a dark and wet chamber at 37°C;
9. Wash 3x3 minutes in 4xSSC/0.2%Tween, shaking;
10. Optional third, forth layer of antibodies, washing in between 3x3 minutes in 4xSSCT, shaking;
11. Stain DNA with DAPI, 0.01-0.02g/ml 4xSSC/0.2%Tween, for 3 minutes;
12. Wash briefly in 4xSSC/0.2%Tween;
13. Mount hybridized areas in antifade (Vectashield);
14. Seal coverslips with colourless nail polish (see note 19 and comment 7).

Below one detection scheme is shown as an example for the detection of five and six different fluorochromes respectively that worked well in our hands

Hapten/ Fluorochrome	Excitation	Emission	Detection
DAPI	350	460	Nuclear counterstain
Biotin > FITC	490	520	1. layer: Av-Alexa-488 2. layer: GaAv-FITC
DNP > Alexa-514*	518	540	1. layer: RaDNP 2. layer: GaR-Alexa514
TAMRA	552	565	direct
Texas Red	580	615	direct
Digoxigenin > Cy5	650	667	1. layer: MaDig-Cy5 2. layer: GaM-Cy5

*In case of 5 colours we recommend to take out Alexa514.

Materials & Reagents

Authors Notes

1. After the fourth round of DOP amplification the probe quality is considerably reduced.
2. Use the low stringency cycles only in case you start with genomic DNA. For all re-amplifications omit low stringency cycles and use only high stringency cycles.
3. In case of the simultaneous labeling of several probes (chromosome specific painting probes) with the same hapten or fluorochrome allow 1µl of unlabeled amplification product from each probe (approx. 50-100ng) and reduce the volume of the mastermix accordingly. This works well for up to several probes.
4. In case of the direct labeling using a fluorochrome bound to dUTP, such as TAMRA-dUTP, FITC-dUTP, TexasRed-dUTP we recommend to increase the concentration to 60µM.
5. The primer DOP1 described in this paper resulted in a poor amplification in our hands, therefore we skipped the DOP1 primer.
6. This approach allows the use of one MM also for very low DNA concentrations of a BAC or for the simultaneous amplification of several BACs by adjusting the amount of DNA (up to 15µl) and water in the individual reaction.
7. In case you prefer a smaller fragment size (approx. 0.3-0.6kb, which might reduce background for some probes) we suggest a short digestion with DNase I as follows:

Amount	Reagent
48µl	Labeled PCR product
10µl	Nicktranslation buffer (see protocol nicktranslation)
40µl	H_2O
2µl	DNase I (1:250 diluted in ice cold water (see nick-translation section))

   Incubate 8 minutes at room temperature, stop reaction by adding 1µl EDTA (0.5M)
8. It is possible and reasonable to generate complex probe sets e.g. BAC-pools containing DNA from up to 20 single BAC-clones (and possibly more) per label reaction. In order to label simultaneously multiple BAC-DNAs within one label reaction we found the following protocol easy to handle and efficient. Prepare a pre-pool of each primary DOP2 and DOP3 amplified BAC-DNAs containing approximately equal amounts of amplified DNA from each BAC (in our hands pre-pools containing DNA from up to 20 BACs worked well). For pools of up to 5 BACs use 1µl in the labeling reaction, for those of up to 10 BACs use 2µl and for more than 10 BACs in the pool use 3µl of the pooled DNA for a labeling reaction.
9. The minimum probe-size reproducibly be labeled and hybridized was 4.5kb. For highly DNaseI sensitive DNA-constructs the labeling-protocol is slightly adapted as follow: the DNaseI has to be diluted 3-5 times more than usually and the incubation time only lasts 45 minutes.
10. Add EDTA only after you have reached the desired fragment size.
11. The activity of DNaseI seems to be variable and may also depend on the DNA. Therefore the amount of DNaseI added and/or duration of incubation time has to be titrated in order to obtain the appropriate size of DNA fragments.
12. Some cell types require longer incubation in 0.1N HCl (10 minutes) or (after 5 minutes of HCl) an additional incubation in

pepsin after the fixation. Digestion with pepsin should be monitored under the microscope because it is easy to over-digest the sample which results in loosing cells or disturbing nuclear morphology. Fixed cells can be kept in 50% FA/2xSSC (at 4°C) for several months. However, long storage often results in a deterioration of the nuclear morphology after denaturation.

13. Growing cells on small cover slips (e.g. 15x15mm) has the advantage that they can directly placed on a microscopic slide for hybridization.
14. Seeding cells on small cover slips (e.g. 15x15mm) has the advantage that they can be directly placed on a microscopic slide for the hybridization set-up.
15. Cot-1 DNA can reduce the intensity of hybridization signal of highly repetitive sequences. This can be compensated by using higher amounts of repetitive probes.
16. Probes containing segments with incomplete sequence homology may require higher concentrations of formamide (e.g. 70%) in the hybridization mix in order to reduce unspecific hybridization. As an example, cross hybridization on different chromosomes of centromeric probes that essentially bind to one chromosome can be prevented by hybridization in 70% formamide.
17. All operations should be done quickly in order not to dry cells. Therefore we recommend to process slides one by one. Furthermore it is crucial to keep temperature and time for denaturation strictly as specified in the protocols. Too short denaturation results in poor hybridization, whereas over-denaturation damages the morphology of nuclei. We recommend incubating slides in a metallic box which is kept in a 37°C water bath. We also want to emphasize that 3D-FISH on tissue sections with a thickness > 10 µm and a best possible preservation of the nuclear and tissue morphology (note: most tissue FISH is done on very thin sections or disseminated nuclei) is not trivial and we are currently still improving our methods. We got good reproducible results for repetitive probes and directly labeled probes in paraffin sections, but found it difficult to detect hapten-labeled probes especially chromosome painting probes. Vibratome sections provide the best morphology and also good reproducible FISH results with the protocol provided, whereas cryo-sections provide good FISH results but are prone to lose their proper morphology.
18. Formamide is toxic, so all the steps that involve the use of this reagent should be performed in a hood and gloves should be worn.
19. In case of tissue sections washing steps should be extended to 10 minutes each and the antibody incubation has to be prolonged up to 2-3 hours. Furthermore the blocking solution (also used to dilute the antibodies) contains 4xSSC with 2% BSA, 0.1% Saponin and 0.1% TritonX100 and the washing solution after antibody incubation is 4xSSC 0.1/ TritonX100.

Reviewer Comments

Reviewed by: Luis Antonio Parada, CIC Biogune, Derio, Spain.

1. In my experience the primers are better preserved when kept at higher concentration (100µM) and different volumes are added according to the required concentrations. Usually the amplification products (unlabeled) are purified by precipitation with ethanol and dissolved in bi-distilled water at high concentration to preserve the DNA. The concentrations of plain and fluorescent nucleotides I use for the DOP PCR labeling reaction are lower than the used by this group without compromising the quality and efficiency of the painting probes. For example the final concentrations are as follow: dNTP=10µM; dTTP=7µM; dTTP-Spectrum Orange=50µM; dTTP-Biotin=30µM; dTTP-Digoxigenin=30µM.
2. I routinely assemble the nick translation reaction on ice. More importantly the working dilution of DNaseI is also prepared with cold water and kept on ice until the moment just before starting the reaction. Apart from adding 1µl of EDTA 0.5M, I also heat the sample at 65°C for 10 minutes to stop the reaction.
3. The concentration of the cell suspension varies according to the size of the coverslips used. Usually 10^5 cells resuspended in 50µl of PBS are enough per 13mm diameter round coverslip. Whereas 2-4x10^5 cells resuspended in 250µl of PBS suffice for one 22x22mm coverslip.
4. In addition to digesting the paraffin embedded tissue sections with Pepsin, pretreatment with 10µg/ml proteinase-K in 1xPBS at 37°C (after washing the pepsin in water) improves the penetration of DNA probes, especially whole chromosome painting probes.
5. Excessive drying of DNA during the precipitation procedure of FISH probes may reduce the solubility of the DNA in the hybridization mix. The probe can also be dissolve by incubating the DNA in an appropriate volume of Formamide 100% pH 7.2 at 37°C, for 30 minutes with strong agitation (vortex) and short centrifugation every 10 minutes. Then equal volume of dextran sulfate 20%/4XSSC is added to reach the appropriate concentration of DNA in a final hybridization cocktail constituted of 50% of Formamide, 2XSSC and 10% dextran sulfate.
6. Hybridization of the DNA probe to the target DNA can also be performed in incubators at 37°C using humid hybridization chambers prepared with large Petri dishes or in staining boxes with light protective tight lid. Denaturation of painting probes addressed to two or three chromosomes (Double or triple painting) can be performed at 80°C for 10 minutes and then pre-annealed 30 minutes at 37°C without damaging the probe. Denaturation of target DNA of tissue sections often requires 5-7 minutes at 75°C. In all cases immersion of the cover slips or slides in ice cold 50% formamide/2XSCC stops the denaturation process and make the target DNA ready for hybridization.
7. The stringency of the washing solutions during the detection procedure may vary with the different type of DNA probes. For example:
   Chromosome painting and BAC clones probes
   1x5 minutes. 50% formamide/2xSSC at 45°C
   1x5 minutes. 1xSSC at 45°C

1x5 minutes. 4xSSC/0.1% Tween 20 at 45°C
1x5 minutes. 4xSSC/0.1% Tween 20 at RT
Pancentromeric probes
1x5 minutes. 50% formamide/2xSSC at 60°C
1x5 minutes. 0.1xSSC at 60°C
1x5 minutes. 4xSSC/0.1% Tween 20 at 60°C
1x5 minutes. 4xSSC/0.1% Tween 20 at RT.

Figures

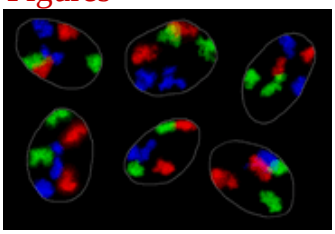

Figure 1:
3 colour 3D-FISH on nuclei of normal diploid human fibroblasts. Maximum intensity projections of confocal serial sections are shown. Chromosome territories3 (green; DNP = detected with FITC), 5 (blue; Dig = detected with Cy3), and 11 (red; Bio = detected with Cy5). Click here for a larger view.

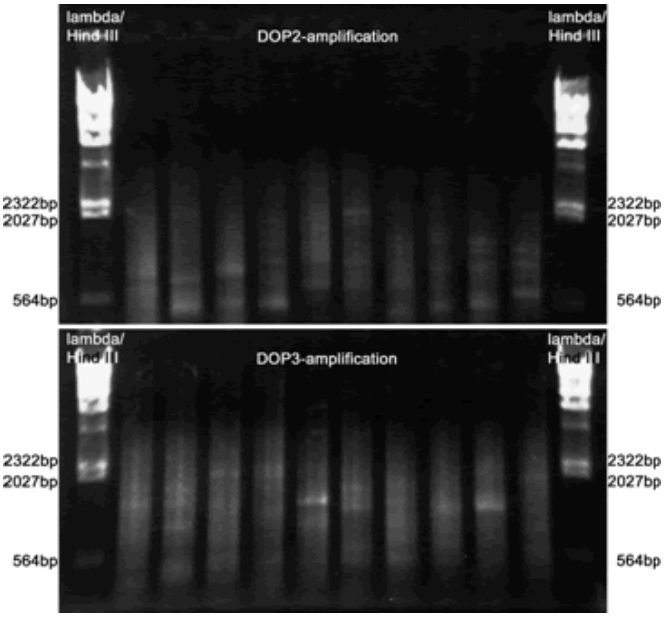

Figure 2:
The gel shows typical patterns after DOP2 (upper row) and DOP3 (bottom row) amplification of the genomic DNA of ten different BAC clones. Proper amplification typically yields a DNA smear between 0.3-4kb. Note within the smear for each BAC fragments of higher intensity which are not the same in DOP2 and DOP3 amplifications for the corresponding BACs.

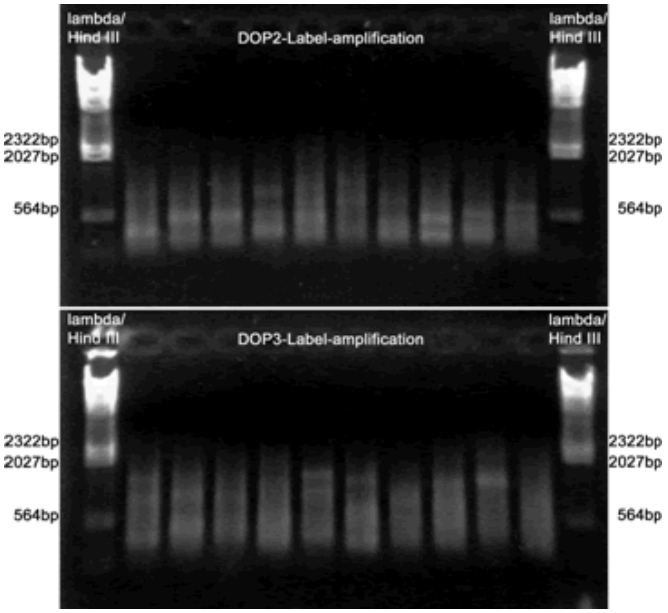

Figure 3:
The gel shows typical patterns after DOP2 (upper row) and DOP3 (bottom row) label amplification of primary DOP2/DOP3 amplification prodcts of ten different BAC clones (same as in protocol 4). Each lane was loaded with 2µl label amplification product. In contrast to the primary amplification the label PCR results in shorter fragments ranging from approximately 0.2-2kb which is an appropriate size for a hybridization probe.

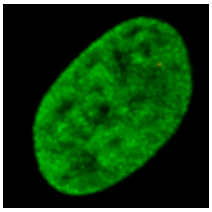

Figure 4:
3D-FISH on HeLa cells using a 10kb plasmid as probe (red). Maximum intensity projection of 8 optical sections of a confocal image stack are shown, green represents the DNA counterstain. Click here for a larger view.

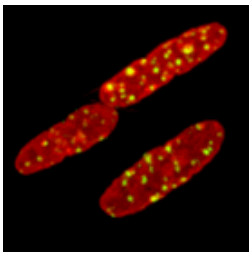

Figure 5:
FISH on a paraffin tissue section of human skeletal muscles. Maximum intensity projection of a confocal image stack showing nuclei counterstained with TO-PRO-3 (red) and centromeres of all chromosomes. Centromeric regions are visualized by a pancentromeric probe directly labeled with FITC-dUTP by nick-translation. Click here for a larger view.

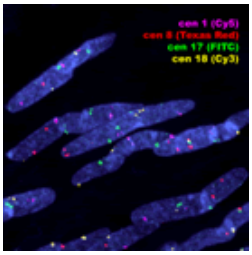

Figure 6:
FISH on a paraffin tissue section of human smooth muscles. Maximum intensity projection of 42 optical sections (c.a. 12.5µm) of a confocal image stack are shown. Nuclei are counterstained with DAPI (blue) centromeres of four chromosomes are visualized by different fluorochromes. Probes for centromeres were alphoid chromosome-specific sequences labeled directly with fluorochromes by nick-translation. Click here for a larger view.

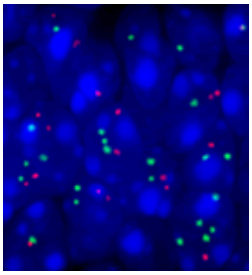

Figure 7:
FISH on vibratome sections of mouse retina. Maximum intensity projection of 7 optical sections (ca. 2µm) of a confocal image stack showing nuclei of neuronal cells counterstained with DAPI (blue) with BAC-probes signals targeting four genes, three in green and one in red colours. Since nuclei representing this tissue type extend over approximately 5-7µm in z-diameter, only a fraction of genes is shown in most of the nuclei. Click here for a larger view.

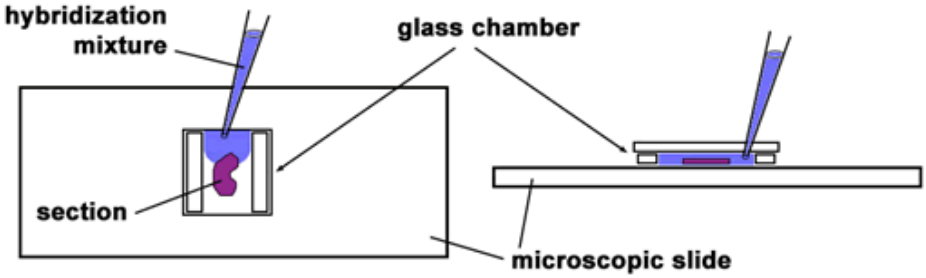

Figure 8: Glass-chamber for hybridization

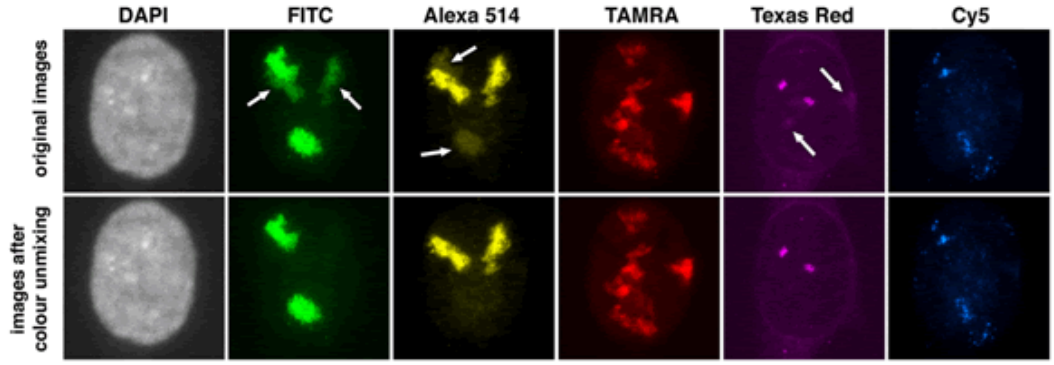

Figure 9:
6 colour 3D-FISH on nuclei of human fibroblasts. Maximum intensity projection of a confocal image stack with 6 colour channels are shown as original images (upper row) and after linear colour unmixing (bottom row) using the software of Leica SP2. The FITC channel delineates the territories of chromosome 12, Alexa514 of chromosomes 11 and TAMRA the territories of chromosomes 17, 19 and 20. Texas Red delineates a BAC contig of chromosome 11 and Cy5 a BAC pool covering different regions of chromosome 12. White arrows point at the image regions generated due to "leakage" of some fluorochromes to the neighboring channels, e.g. Alexa514 to the FITC channel (and vice versa), orTAMRA to the Texas Red channel. Click here for a larger view.

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