# High resolution visual mapping of stretched DNA by fluorescent hybridization

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We describe a method for stretching DNA, which, when combined with fluorescent hybridization procedures, forms a new mapping technology that produces a high resolution, vivid, multi-colour image and map. Restriction fragments and cosmid probes were successfully mapped by this procedure with validation by standard restriction mapping. A long range map of a >200 kilobase region containing five copies of the amplified dihydrofolate reductase gene was easily generated within two days. This DNA mapping procedure offers a significant and rapid alternative to a variety of standard mapping procedures.

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The characterization of gene structure and genome organization has been greatly facilitated by the development of a number of physical mapping technologies. Pulsed-field gel electrophoresis and the use of rare cutting restriction enzymes have provided a means of restriction mapping in the kilobase (kb) to megabase (Mb) size range. For the mapping of larger regions of DNA, the more recent developments of yeast artificial chromosomes (YACs)[1] and radiation hybrid maps[2] have proven to be tremendously useful. Other techniques, such as the "fingerprinting" of repetitive elements[3], have been developed to fill in for long range mapping deficiencies.

A more general approach to physical mapping involves fluorescent *in situ* hybridization (FISH) to identify the position of probes on metaphase chromosomes[4]. The use of this technique allows the rapid mapping of DNA probes with roughly 1 Mb resolution. For higher resolution mapping, FISH has been applied to interphase cell nuclei[5–7]. Since the DNA is less condensed in interphase cell nuclei than in metaphase chromosomes, resolution in the 50–100 kb range can be obtained. However, mapping the distance between two probes in three dimensional nuclei or compressed two dimensional nuclei is complex and requires large data sampling and probability calculations based on a random walk model[8].

Recently, procedures for using FISH to map decondensed nuclear DNA have been developed[9–11]. We now describe a means of forcibly stretching DNA in a linear fashion such that when fluorescent hybridization techniques are used, a high resolution multi-colour map can be obtained.

## A new DNA mapping technology

We have developed a DNA mapping procedure in which duplex DNA is stretched across a slide, hybridized with DNA probes labelled with biotin or digoxigenin and detected via fluorescent avidin or antibody (Ab) binding. The result is a vivid, multi-colour image in which the arrangement of DNA probes along an extended strand of DNA is observed and recorded through a fluorescence microscope. From the images, a map can be constructed, and is referred to as a direct visual hybridization (DIRVISH) DNA map. This mapping strategy is based upon the principle that a small region of DNA (say, 5 kb), when extended to the expected length for relaxed duplex DNA, spans a distance which is visible through a light microscope. Based on the predicted span of 0.34 nm per basepair for relaxed duplex DNA, a 5 Kb DNA fragment would extend 1.7 μm; a 40 kb cosmid would extend 13.6 μm; and a 500 kb YAC would extend 170 μm.

## Mapping restriction fragments and cosmids

The stretching of duplex DNA strands was accomplished by lysing cells with detergent at one end of a glass slide and allowing the DNA in solution to stream hydrodynamically down the slide (see Methodology). Standard FISH techniques were then employed for visualizing the position of hybridized probes on the extended DNA strands through a fluorescence microscope. Two cosmids, c400-30 and c400-13, isolated from the hamster dihydrofolate reductase (DHFR) gene region[12], were used as a source of probes. A restriction map of c400-30, which encompasses most of the 3' end of the DHFR gene, was generated by standard procedures[13] for comparison to the DIRVISH DNA maps. Fig. 1*a* shows the result of a biotin-labelled 4.8 kb *Hind*III

fragment from c400-30 plus a digoxigenin-labelled c400-30 probe hybridized to a hamster DNA stream. The biotin- and digoxigenin-labelled probes were detected by the binding of fluorescein-avidin and rhodamine-anti-digoxigenin Ab, respectively. The DNA stream was stained with DAPI (4',6-diamidino-2-phenylindole) as seen by the blue fluorescence of the background. The large arrow points to a short string of the 4.8 kb fragment's fluorescein signal (green) within a longer string of the cosmid's rhodamine signal (red). During the hybridization, the 4.8 kb probe is in great molar excess of the cosmid probe; thus the 4.8 kb probe, through competition, prevents the hybridization of most of the cosmid probe DNA to that location. The thicknesses of the strings, a product of the layers of avidin and Ab, vary according to the proteins used for detecting either the biotin or digoxigenin labelled probes. The thickness in signals presents a limitation in the resolution of this technique. Background signal, indicated by a small arrow, does not significantly obscure the specific signal and is often not noticeable, depending on probe preparation. The use of DAPI staining in subsequent mapping was discontinued because it obscured the fluorescence of the probes.

The position of the 4.8 kb fragment was mapped with respect to the c400-30 cosmid insert sequence by DIRVISH DNA mapping and compared to the restriction map of the cosmid (Fig. 1*b*). For the interpretation of all the DIRVISH DNA maps in this study, length measurements for each segment of the fluorescent signals were normalized to the signal length of a probe of known size. The 4.8 kb fragment was used for normalization in Figs 1*b*,*c* and 2. An average of the DIRVISH DNA maps, generated by averaging the relative lengths from a minimum of 30 images, was generated for each set of probes. Efficient hybridization was indicated by the finding that 93% of all observed extended c400-30 signals flanked a 4.8 kb probe signal. We did not routinely quantitate the total yield of detectable extended signals; however, one study was observed to yield at least 118 extended signals from a total of 750 possible signals.

The averaged map in Fig. 1*b* is in good agreement with the restriction map of the cosmid. The two small yellow dots of fluorescein signal seen within the c400-30 signal were interpreted as background, since they were never reproduced. The relative lengths of signal appear to remain constant for DNA stretched to various extents, as can be seen from a representative DIRVISH DNA map of DNA stretched to only 40% of the expected length (Fig. 1*c*). Fig. 1*d* shows the graphic representation of the measurements from the 35 images used to generate the averaged DIRVISH DNA map (Fig. 1*b*). Since useful mapping data is derived from DNA stretched to a variety of lengths, the standard deviations for length measurements was not necessarily informative. However, for the large sampling of normalized DIRVISH DNA map data, an acceptable percent standard error of 7–8% was found for the normalized lengths.

A more detailed comparison between DIRVISH DNA mapping and restriction mapping was performed by mapping the positions of three restriction fragments from c400-30 with respect to each other. Using the 4.8 kb fragment described above as a reference, the position of two other restriction fragments (a 6.4 kb *Hin*dIII and a 7.3 kb *Xba*I–*Sal*I fragment) were mapped. A known 0.4 kb overlap between 4.8 kb and 7.3 kb fragments and a 1.8 kb gap between 4.8 kb and 6.4 kb fragments provided a test of the DIRVISH DNA mapping technique for detecting gaps and overlaps. To demonstrate the relative position of all three fragments, we performed a three-probe hybridization utilizing three colours (red, green and yellow). The yellow fluorescence was attained by labelling one of the probes simultaneously with both biotin and digoxigenin. The position of the three fragments determined by DIRVISH DNA mapping is completely consistent with the restriction map with respect to order, gaps and overlap (Fig. 2), however, the thickness of the signals undoubtedly contributes some error in the measurement of the shortest distances. Though we have found that the restriction and DIRVISH DNA maps are generally in close agreement, the DIRVISH DNA averaged maps sometimes portray fragment sizes in slight disproportion, as compared to the restriction map. This may be due to insufficient hybridization of the probes to the target regions that have an abundance of repetitive sequences.

It was remarkable that on occasion the stretched DNA appeared to extend up to twice the expected length for relaxed duplex DNA (0.34 nm $bp^{-1}$). For example, the DNA in Fig. 2 is stretched to a length equivalent to about a 200% extension. We obtained the highest mapping resolution for DNA stretched from 100–200% of the expected length.

### Determining cosmid gap distance and orientation

To demonstrate the usefulness of DIRVISH DNA mapping over longer ranges of DNA for which detailed mapping data was not available, we determined the position of cosmid c400-30 with respect to cosmid c400-13, which was isolated from the 5' end the hamster DHFR gene. Determining the relative orientation and distance between two such cosmids by restriction mapping procedures would normally require the identification of a restriction fragment or fragments that connect the two cosmid sequences, or the isolation and mapping of overlapping cosmid clones. The DIRVISH DNA map was rapidly determined (2 days) for the two cosmids (Fig. 3*a*), which were previously known to be closely linked but non-overlapping, based on Southern blot hybridization (data not shown). In addition to demonstrating that the two cosmids are linked but non-overlapping, we observed an average gap distance of ~6 kb when the gap measurements were normalized to the length of the 38 kb c400-13 signal. Subsequent restriction mapping of this region of the DHFR gene indicated there was a 6.5 kb distance between the two cosmids' sequences (data not shown), thus confirming the DIRVISH DNA map.

Chromosome walking is often used to identify the region between two known probes, such as the two cosmids above. This often involves random walking since the relative orientation of the cosmids is often unknown. Fig. 3*b* demonstrates the usefulness of DIRVISH DNA mapping for determining the relative orientation of two cosmids, thus allowing directional walking. A DNA fragment (labelled with biotin and digoxigenin) from one end of the c400-30 DNA insert was hybridized in conjunction with c400-30 and c400-13. This fragment probe, as seen by the yellow signal, maps to one end of c400-30 and is clearly distal to c400-13. This orientation was subsequently confirmed by restriction mapping (data not shown).

The valleys in signal, seen in the strings of fluorescein

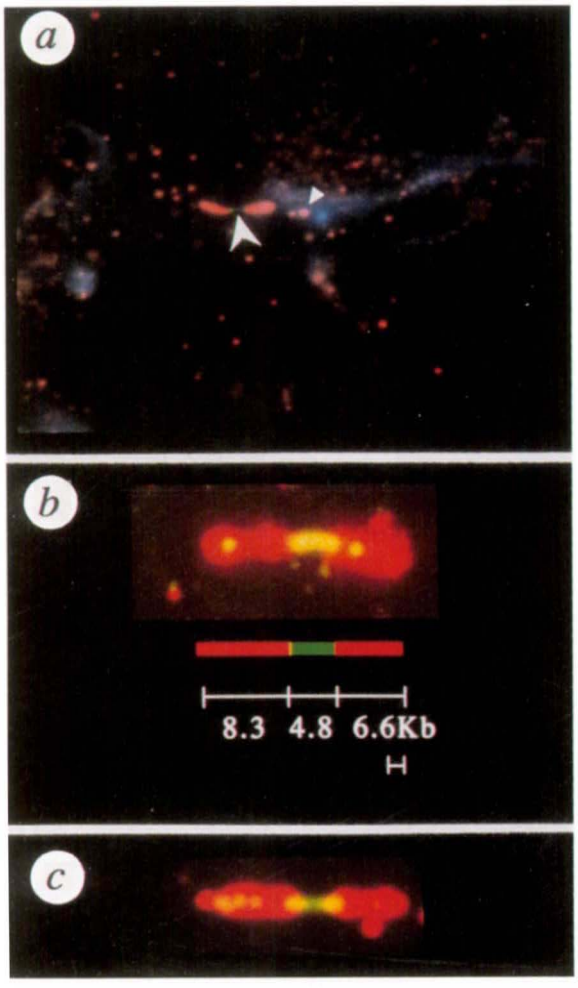



Fig. 1 DIRVISH DNA maps of the hamster DHFR region using cosmid and restriction fragment probes. DNA streams (see Methodology) were prepared from derivatives of the hamster cell line UA21 (ref. 17). The DIRVISH DNA maps in Figs 1–4 were recorded by single exposure film photography. Length measurements used in generating averaged DIRVISH DNA maps were performed by computer on images captured by the American Innovision V150 color imaging system. Scalebars, 1 μm. For *a–c*, digoxigenin-labelled cosmid c400-30 probe and biotin-labelled 4.8 kb *Hin*dIII fragment probe were combined for these hybridizations. Sequences from c400-30 were detected with rhodamine (red) and sequences from the 4.8 kb fragment were detected with fluorescein (green; see Methodology). *a*, The DNA stream was stained with DAPI. A large arrow marks the cosmid and fragment signal. A small arrow marks one of the background signals. *b*, Shown from top to bottom is a representative DIRVISH DNA map, an averaged DIRVISH DNA map constructed from 35 images (see *d*), and a restriction map showing the position of the 4.8 kb fragment within the cosmid insert. *c*, A representative DIRVISH DNA map of DNA stretched to 40% of the expected length for relaxed duplex DNA. The magnification of this image was such that the 4.8 kb fragment appeared approximately the same size as that shown in *b*. *d*, A graphic representation of the 35 DIRVISH DNA images used to construct the averaged map in *b* is presented. The lengths of each representation were normalized to one length and aligned at the right edge of the 4.8 kb signal.

and rhodamine, could conceivably be the sites of repetitive elements which are blocked by the total DNA competitor present during the hybridization. The pattern of these valleys, however, was not usually consistent. Alternatively, the valleys may correspond to sites of DNA anchorage to the slide that are inaccessible to probe hybridization.

## Mapping amplified gene structures

Determining the structure of amplified genes has proven valuable in elucidating the mechanism of their formation[14,15]. However, using standard cloning and restriction mapping techniques, this characterization is usually a time-consuming and difficult, if not impossible task. This is particularly true because of the repetitive and complex nature of amplification arrays. To demonstrate further the application of DIRVISH DNA mapping over longer distances, the structure of an amplified gene locus was studied. We chose to study an amplified DHFR locus in a hamster cell line (75-20S5). These cells have been shown previously by FISH analysis of metaphase chromosomes to contain about eight DHFR genes clustered at the end of one chromosome[12]. The spacing between the amplified DNA units and the arrangement of DHFR genes could not be determined by standard FISH analysis. A DIRVISH DNA image of this region is presented in Fig. 4 as a demonstration of this procedure's potential and is not meant to be a definitive characterization. Fig. 4*a* shows a DIRVISH DNA image of cosmid c400-30 and the 4.8 kb fragment sequences within the amplified DHFR region stretching halfway across the field of view. Fig. 4*b* shows a closer view of this structure. In this image there is an array of 5 units, each with red c400-30 cosmid and green 4.8 kb fragment signals. The higher copy number found with the FISH technique may be an over-estimation due to signal splitting, a FISH artifact where one target copy appears as two signals. We found no evidence of signal splitting using the DIRVISH DNA mapping technique.

Structural details of the amplified array can be derived from the data in Fig. 4. There is a possibility of rearrangement of sequences between one end of c400-30 and the 4.8 kb fragment, as suggested by the two reproducible yellow signals amidst the red signal of c400-30 in addition to that of the 4.8 kb fragment (see the first amplicon on the left). The spacing between each signal cluster of red and green is variable, ranging from roughly 1–16 kb. The size of the largest amplicon is calculated to be ~46 kb. Considerable diversity in the amplification structure was apparent as judged by the diversity in the DIRVISH DNA maps of numerous individual structures.

## Discussion

Techniques for stretching genomic DNA and high resolution mapping of restriction fragments and cosmids along this DNA have been presented. Using procedures comparable to those used in FISH, microscopic multi-colour strings of signal were generated that corresponded to sites of probe hybridization. These images were easily interpretable and gave rise to what we refer to as DIRVISH DNA maps. One extraordinary finding was that some of the DNA was apparently stretched to a length that measures up to twice the maximum expected length for relaxed duplex DNA, as judged by the length of continuous signal from probes of known size. This may indicate that some of the helices are fixed to the slide in a distorted or stressed state. Since this highly extended DNA is capable of hybridizing with the probes, it suggests the possibility that, after stretching, stress release may occur from breaks in the DNA.

Other groups have pursued a similar concept of mapping extended DNA, using either detergent and salt[10], or alkaline solution[9] to decondense DNA in nuclei. The study of Heng *et al.*[9] showed images where separate signals were found for two cosmid probes as little as 21 kb apart.

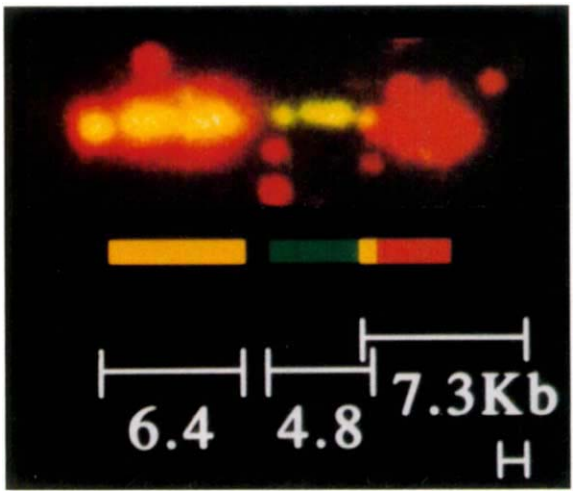



Fig. 2 DIRVISH DNA maps using three restriction fragment probes. Biotin-labelled 4.8 kb *Hin*dIII fragment probe (fluorescein/green), digoxigenin-labelled 7.3 kb *Xba*I–*Sal*I fragment probe (rhodamine/red) and biotin plus digoxigenin-labelled 6.4 kb *Hin*dIII fragment probe (fluorescein and rhodamine/yellow) were combined for this hybridization. The averaged DIRVISH DNA map was constructed from 36 images.

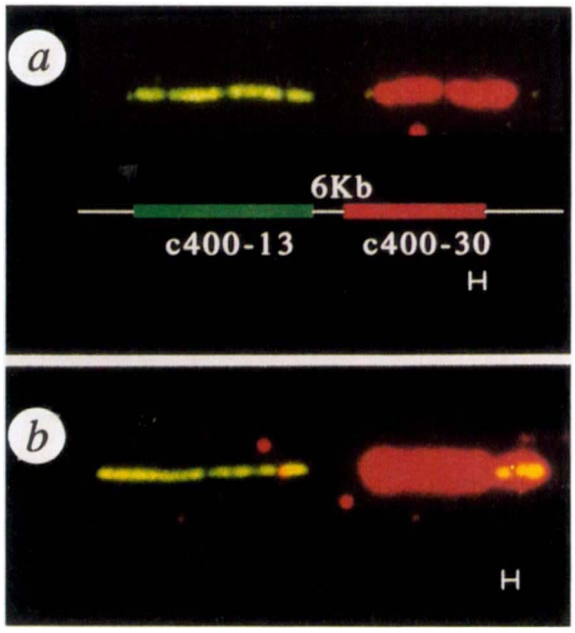
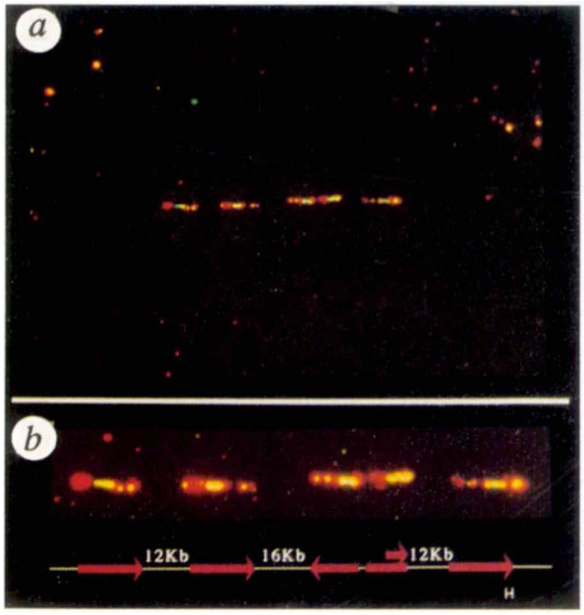



Fig. 3 DIRVISH DNA maps using two cosmid probes. *a*, Biotin-labelled c400-13 probe (fluorescein/green) and digoxigenin-labelled c400-30 probe (rhodamine/red) were combined for this hybridization. The averaged DIRVISH DNA map was constructed from 41 images. *b*, Biotin-labelled c400-13 probe (fluorescein/green), digoxigenin labelled c400-30 probe (rhodamine/red) and biotin- plus digoxigenin-labelled 6.4 kb *Hin*dIII fragment probe (fluorescein and rhodamine/yellow) were combined for this hybridization.

However, each cosmid probe signal still appeared as a small single dot, in contrast to the strings shown in our study. This suggests that their extended DNA was not significantly less condensed than DNA found in interphase cell nuclei. Wiegant *et al.*[10] showed cosmid probe signals sporadically covering approximately the distance for relaxed duplex DNA. The DIRVISH DNA mapping procedure provides the following advantages over these other approaches: (i) the DNA is virtually straight, which simplifies length measurements, as opposed to DNA that winds across the slide, (ii) highly stretched DNA, suitable for high resolution mapping, as well as DNA stretched to a smaller extent for long range mapping are all available on one slide, (iii) the fluorescent signal on a stretch of DNA is continuous or nearly continuous, making it possible to detect minute structural differences.




DIRVISH DNA mapping also has advantages for analysing complex structures such as those generated in gene amplification. The diversity in structure and the complexity that we found in the amplified DHFR array would normally escape detection and proper characterization using standard restriction mapping and cloning, or the techniques of Heng *et al.*[9] and Wiegant *et al.*[10].

The maximum distance possible for DIRVISH DNA mapping was not determined in this study. However, we have observed nearly continuous, straight signals for YAC probes as large as 700 kb (unpublished observations). Therefore, the potential exists for DIRVISH DNA mapping of megabase distances. New applications for DIRVISH DNA mapping are now being explored, such as: (i) determining the position of restriction fragments and cosmids with respect to YAC sequences; (ii) determining the orientation and overlap of YAC sequences; (iii) determining possible deletions, insertions, or complex rearrangements in the genome or cloned sequences; and the generation of fingerprint maps of repetitive sequences. As DIRVISH DNA mapping is explored further, we

Fig. 4 DIRVISH DNA image of amplified DHFR genes. DNA streams containing DHFR amplification arrays were analysed. *a*, Biotin-labelled 4.8 kb probe (flourescein/green) and digoxigenin-labelled c400-30 probe (rhodamine/red) were combined for this hybridization. *b*, A close-up of the image is shown with the approximate spacing sizes.

anticipate finding applications in all aspects of DNA structure analysis.

## Methodology

**Stretching DNA.** Individual cells (100–5,000) in 2 µl of phosphate buffered saline were placed at on one end of a glass slide, air-dried and immediately lysed with 5 µl of a solution of 0.5% SDS/ 50 mM EDTA/ 200 mM Tris (pH 7.4). After 5 min of dissolving, the slide was tilted to allow the drop of DNA to run down the slide. This results in a DNA stream extending down the slide. The DNA stream was air-dried and fixed to the slide with methanol/ acetic acid (3:1) fixative. The DNA was fixed to the slide for a period between 1 and 5 min and air-dried. The slides were stored in a slide box under nitrogen with Drierite at –20 °C until the hybridization step.

**Probe labelling and hybridization conditions.** DNA probes were prepared by nick translation with 30 µM of each of the four deoxynucleotide triphosphates and 100 µM of either biotin-dUTP (Boehringer Mannheim Biochemicals) or digoxigenin-dUTP (Boehringer Mannheim Biochemicals), or both. The single strand size range for the probes was 100–1500 bp.

Hybridization of the probes to the DNA streams followed a modification of standard *in situ* hybridization procedures[16]. Slides were treated with RNAse (100 µg ml$^{-1}$) for 30 min at 37 °C. Slides were then placed in 70% formamide/ 2 × SSC at 70 °C for 2 min to denature the strands followed by immersion in cold 70% ethanol (1 min), 90% ethanol (1 min), 100% ethanol (1 min), then dried. Each hybridization contained 20 ng of each probe plus 10 µg of sheared hamster DNA (used to suppress repetitive sequence hybridization). Probes were denatured at 70 °C for 5 min prior to hybridization. Hybridization occurred in 10 µl with 55% formamide, 10% dextran sulfate, and 1 × SSC, under a coverslip, at 37 °C for 18 h. Slides were washed twice in 50% formamide/2 × SSC for 3 min each, followed by two washes in 2 × SSC for 2 min each, all at 45 °C.

**Fluorescent detection procedures.** The detection of biotin was performed according to standard procedures[16]. A nonspecific pre-avidin block (1% BSA/4 × SSC/5% Carnation non-fat dry milk) was applied to slides and incubated at room temperature for 10 min. Avidin DN (Vector Laboratories), at 5 µg ml$^{-1}$ in 1% BSA/4 × SSC, was applied to slides and incubated at room temperaturefor 20 min. The slides were washed for 2 min at room temperature with each of the following: 4 × SSC; 0.1% Triton ×100/4 × SSC; 4 × SSC; 0.5% NP40/0.1 M $NaP0_4$ at pH 8.0. Amplification was performed by applying a nonspecific pre-Ab block of 4% goat serum (in 0.5% NP40/0.1 M $NaP0_4$ at pH 8.0) to the slides and incubating 10 min at room temperature, followed by goat biotinylated anti-avidin (Vector Laboratories), at 5 µg ml$^{-1}$ in pre-Ab block, incubated for 20 min at room temperature. The slides were washed as described for the avidin step. The pre-avidin block was again applied and incubated as described above. Fluorescein-avidin DN (Vector Labs), at 5 µg ml$^{-1}$ in 1% BSA/4×SSC, was applied to slides and incubated for 20 min at room temperature. The slides were washed as described above. An anti-fade solution, Vectashield (Vector Laboratories), was applied to the slide and covered by a coverslip.

The detection of digoxigenin was performed by a modified standard procedure[6]. A pre-Ab block (4% goat serum/0.5% NP40/0.1 M $NaP0_4$ at pH8.0) was applied to slides for 10 min at room temperature. Mouse anti-digoxigenin Ab (Boehringer Mannheim Biochemicals), at 10 µg ml$^{-1}$ in pre-Ab block solution, was applied to slides and incubated for 20 min at room temperature. The slides were washed as described for the avidin detection. A pre-Ab block was applied for 10 min, followed by digoxigeninylated anti-mouse Ig-$F(ab')_2$ fragment (Boehringer Mannheim Biochemicals), at 10 µg ml$^{-1}$ in pre-Ab block, and incubated for 20 min at room temperature. The slides were washed as described for the avidin detection procedure. A 10 min pre-Ab block was again applied, followed by rhodamine-labelled anti-digoxigenin Fab fragment (Boehringer Mannheim Biochemicals), at 25 µg ml$^{-1}$ in pre-Ab block, and incubated for 20 min at room temperature. The slides were washed as already described and anti-fade solution applied.

**Fluorescence microscopy and imaging.** Fluorescence microscopy utilized a Nikon Labaphot 2 microscope with a Nikon Planapo × 100, N.A.1.4 objective lens and a triple band-pass filter (Omega Optical) which allows the simultaneous visualization of fluorescence by DAPI , fluorescein and Texas red or rhodamine. Film photography was performed using Ektachrome ASA 400 slide film (Kodak). CCD photography was performed using the American Innovision VI50 color imaging system. CCD photography typically required only a 0.5 s exposure versus a 20 s exposure for film photography.

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