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Short communication

3D confocal reconstruction of gene expression in mouse

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Abstract

Three-dimensional computer reconstructions of gene expression data will become a valuable tool in biomedical research in the near future. However, at present the process of converting in situ expression data into 3D models is a highly specialized and time-consuming procedure. Here we present a method which allows rapid reconstruction of whole-mount in situ data from mouse embryos. Mid-gestation embryos were stained with the alkaline phosphotase substrate Fast Red, which can be detected using confocal laser scanning microscopy (CLSM), and cut into 70 μm sections. Each section was then scanned and digitally reconstructed. Using this method it took two days to section, digitize and reconstruct the full expression pattern of *Shh* in an E9.5 embryo (a 3D model of this embryo can be seen at genex.hgu.mrc.ac.uk). Additionally we demonstrate that this technique allows gene expression to be studied at the single cell level in intact tissue.

Keywords: Mouse; Development; Gene expression; In situ hybridization; Confocal microscopy; Reconstruction; 3D

1. Results

1.1. Single-cell resolution

Whole-mount in situ hybridization was performed on mouse embryos using Fast Red (Roche) as the final substrate, and these were then embedded in 5% LMP agarose and cut into 70–100 μm sections using a vibratome. Using a confocal microscope to visualize fluorescence from the Fast Red precipitate we were able to detect a signal resolution which makes it possible to identify the expression pattern of a specific gene at the single cell level.

We first examined the expression of *Shh*, *Gli2* and *Patched*. Although *Shh* and *Patched* are strongly expressed genes which are easy to visualize with in situ techniques, *Gli2* is weakly expressed and therefore a good test of the sensitivity of the assay. All three genes could be well visualized (Fig. 1a–c) and corresponded to their previously described domains of expression (Echelard et al., 1993; Ding et al., 1998; Goodrich et al., 1996).

In another experiment we examined *Pax6* expression in the developing eye of an E10.5 mouse embryo and explored the use of a counter-stain to visualize all the nuclei of the tissue. In addition to the Fast Red staining, the tissue was stained with the nuclear dye YoPro-1 iodide (Fig. 1d,d′). The results show *Pax6* expression in lens, the tips of the optic cup and in the overlying epithelium (Walther and Gruss, 1991; Grindley et al., 1995).

The high resolution offered by this technique provides an ideal tool for studying gene interactions between neighbouring cells. To test this possibility we carried out an experiment looking at the expression of *Shh* and *Patched* in combination with an antibody staining of the homeobox protein PDX1 in the developing gut (Fig. 1e,f). During the development of the pancreas *Shh* expression is excluded from the pancreatic buds, while *Pdx1* is expressed exclusively in this tissue. *Patched* is expressed in the mesenchyme adjacent to the *Shh* expressing endoderm but not in the mesenchyme adjacent to the *Pdx1* expressing endoderm. Our results agree with these observations and clearly demonstrate that this technique can be used to analyze gene interaction at the single cell level in intact tissue.

1.2. 3D data collection

The ability to scan the expression pattern as a fluorescent signal to a significant depth means that complete 3D blocks of high-resolution data can be captured in a single process, as is often done for antibody staining (Mohun et al., 2000). An E10.0 embryo was stained for *Shh* and cut into 70 μm sections. Seventy micrometers is the maximum depth at which good cellular resolution can be obtained (data not

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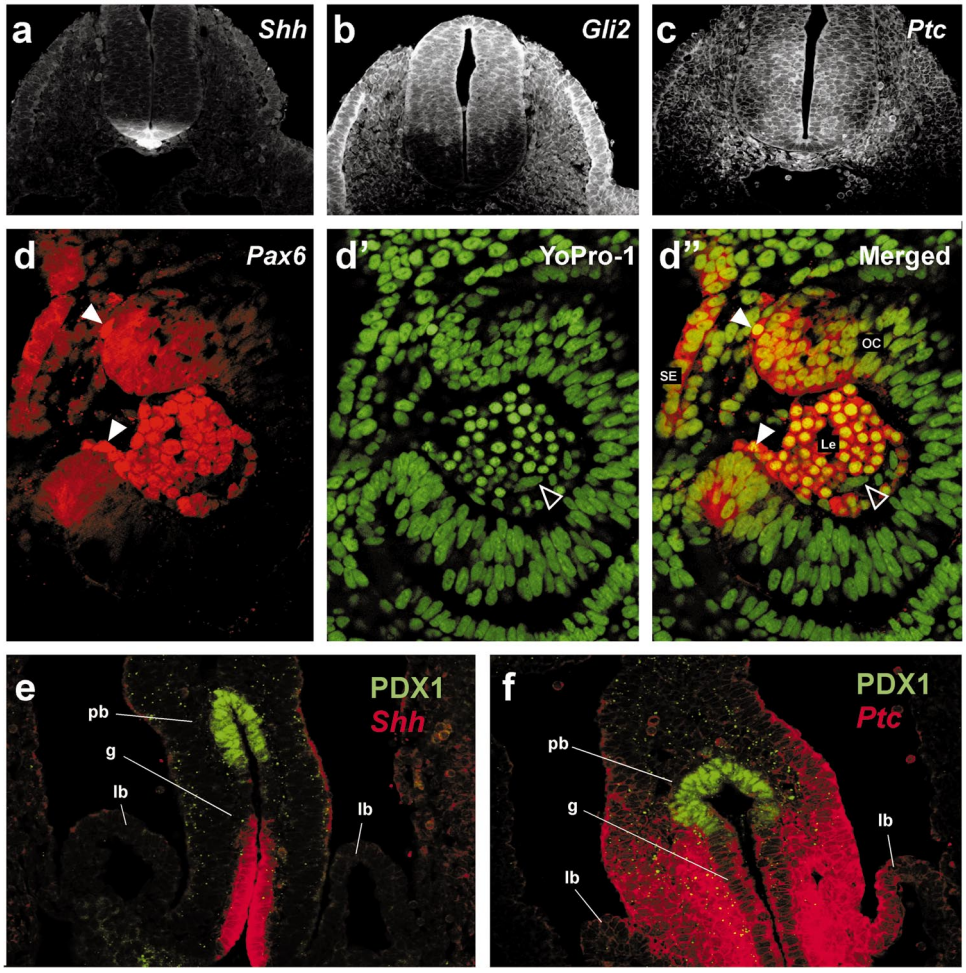

Fig. 1. High-resolution scans of gene expression patterns. (a–c) Expression of *Shh, Gli2* and *Patched*, respectively, in the developing neural tube and floorplate of E9.5 embryos. (d–d″) *Pax6* expression in the developing eye of a E10.5 embryo. (d) shows the Fast Red signal which corresponds to the *Pax6* expression pattern. (d′) shows the signal from the counter-stain YoPro-1 iodide, which highlights all cell nuclei. (d″) shows the merged image. White arrowheads indicate individual isolated cells expressing *Pax6* at high levels. The open arrowhead indicates a non-*Pax6* expressing cell within the lens. SE (surface ectoderm), OC (optic cup), and Le (Lens). (e) Simultaneous detection of *Shh* mRNA and PDX1 protein. (f) Simultaneous detection of *Patched* mRNA and PDX1 protein. pb, dorsal pancreatic bud; g, gut endoderm; lb, liver bud.

shown) The thick section shown in Fig. 2 contains the floorplate of the neural tube at an oblique angle (Fig. 2a). Optical scans where taken at every 1 μm and Fig. 2b shows four optical sections taken at 15, 29, 38 and 47 μm, respectively. Vertical 'virtual' sections can then be viewed from the resulting 3D block (Fig. 2c–e) which provide a resolution almost equivalent to the original horizontal scans. The view in panel c is equivalent to a transverse section (compare with Fig. 1a), and in panels d and e are views equivalent to longitudinal cuts along the floorplate and notochord respectively. Although the original optical sections were taken in an atypical orientation, the virtual sections make it clear that the cells in the neural tube are polarized, such that the nuclei are displaced towards the outer surface and the cytoplasm is displaced towards the lumen. The notochord, by comparison, shows no such polarization (compare Fig. 2d with 2e).

1.3. Reconstructing a whole-embryo expression pattern

It appeared that this technique could be useful for generating large-scale reconstructions of 3D expression patterns, and was likely to make this process faster and easier than traditional techniques, which involve the cutting, and processing of (typically) hundreds of thin sections. As an example we chose to reconstruct the expression pattern of *Shh* in an E9.5 embryo, as it is expressed along the whole length of the body.

The processed embryo was embedded in agarose and cut into 24 (70 μm) sagital sections. Each section was scanned every 10 microns (7 optical sections per vibratome section) using the 10× objective. Fig. 3a shows a whole-mount E9.5 embryo stained with Fast Red, and Fig. 3b shows the 3D reconstruction of the *Shh* expression pattern from a similar embryo. Two major sites of expression can be seen running

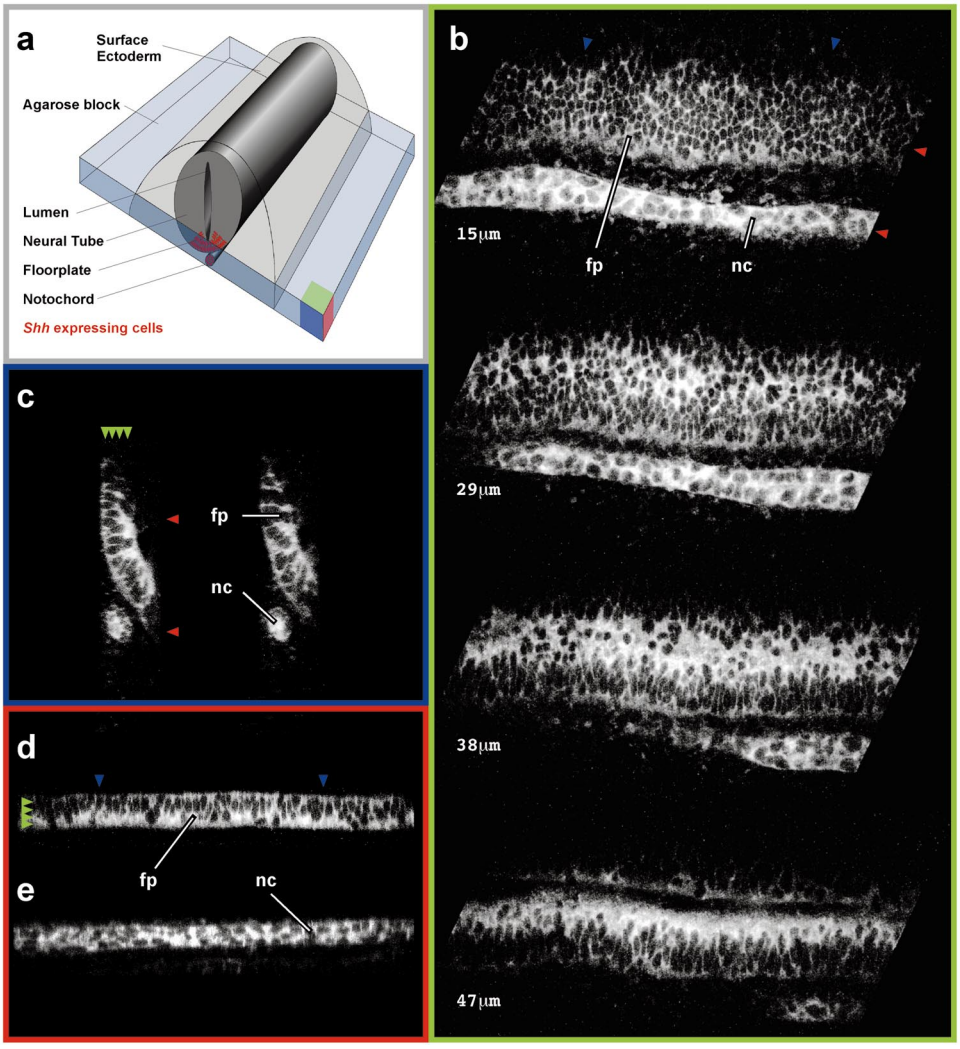

Fig. 2. 3D scanning of *Shh* expression data. (a) Illustration showing *Shh* expression in the neural tube and floorplate. The agarose block represents the thick section described in this figure. (b) Confocal images at various depths of the section (15, 29, 38 and 47 μm). The green outline refers to the coloured box in a, and represents the two dimensions of these sections in relation to the 3 dimensions of the whole block. The blue and red arrows show the positions of the virtual sections in (c–e). (c) Virtual sections cutting transversely through the neural tube. (d,e) Virtual sections cutting along the neural tube and notochord, respectively. In all sections *Shh* mRNA is strongly localized to the cytoplasm. The cell nuclei can be seen as dark spots. fp, floorplate; nc, notochord.

along the body of the embryo: the floorplate of the neural tube extending into the brain, and the embryonic gut including the stomach. At this stage of development *Shh* is expressed at low levels in the limb buds and weak staining was detected in the ZPA of the left forelimb (not visible in this figure). The visible streak of fluorescence in the right forelimb (blue arrowheads) is presumably caused by Fast Red crystals stuck to the surface ectoderm. Towards the posterior end of the floorplate, there appears to be a gap in expression (red arrowhead). This is due to one of the sections being distorted (a potential drawback for any technique that relies on multiple-section reconstruction). The resulting 3D embryo can be viewed from any angle (panel g); a movie of the embryo rotating can be seen at genex.hgu.mrc.ac.uk

Fig. 3c shows an image of one of the original optical sections (which has been patched together from 8 overlapping images). The morphology of most tissues (neural tube, somites, heart) can clearly be seen from the background fluorescence, and give enough information to select anatomical 'landmarks' for the alignment process. Fig. 3d–f show 'virtual' sections equivalent to a frontal view of the embryo (orthogonal to the original section in Fig. 3b), which demonstrate how well the thick sections align with each other. As with all expression analyses, distinguishing between the background and the ISH signal must be performed by selecting an appropriate threshold value. In this case we have made the threshold value (which differentiates between background and signal) more explicit, by artificially colouring everything above the threshold yellow (corresponding to the *Shh* signal).

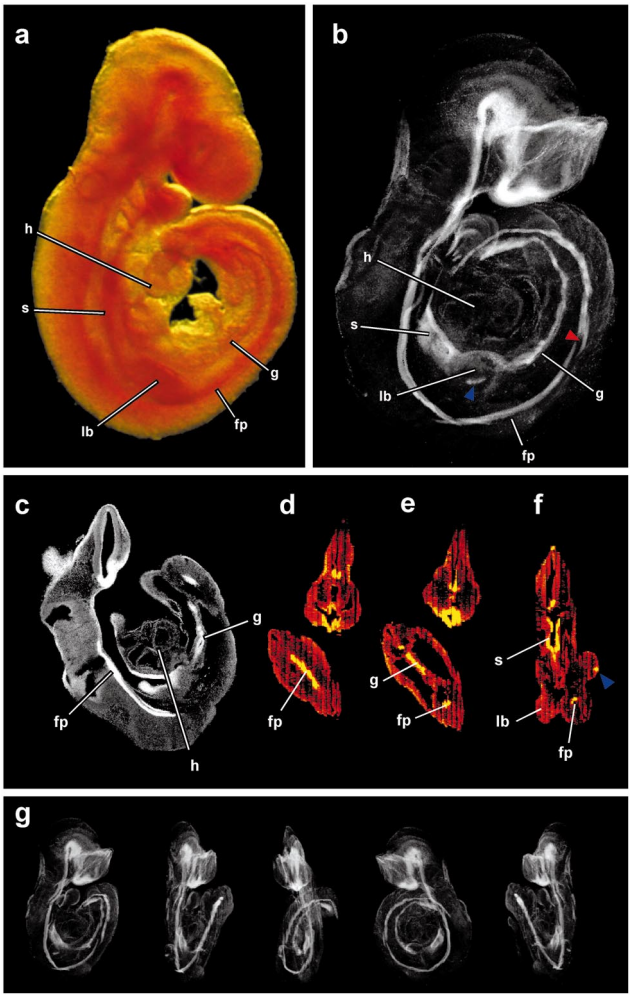

Fig. 3. 3D reconstruction of *Shh* expression in a E9.5 mouse embryo. (a) An E9.5 mouse embryo hybridized with a *Shh* probe and stained with Fast Red. (b) Full 3D reconstruction of *Shh* expression in an E9.5 embryo. The intensity of the image was set high enough to see background staining, so that the general structure of the embryo is visible. One of the sections used for the reconstruction was distorted, resulting in a small gap in the floorplate expression (red arrowhead). (c) Optical section used for the reconstruction. *Shh* expression can be seen as white against the grey background. This particular section has been patched together from eight confocal images. (d–f) Virtual frontal sections through the reconstructed embryo. The sections have been coloured artificially so the *Shh* signal appears yellow and non-expressing tissue as red. (g) Different viewing angles of the reconstructed embryo. The blue arrowheads (b,f) point to a UFO (unidentified fluorescent object) stuck on the surface ectoderm. h, heart; s, stomach; lb, limb bud; fp, floorplate; g, gut.

2. Discussion

3D reconstructions of gene expression patterns are increasingly used by developmental biologists to aid understanding of gene function. However most techniques currently in use are time-consuming or require specialized equipment (Baldock et al., 1997; Streicher et al., 2000; Louie et al., 2000; Palmes-Saloma and Saloma, 2000). A major advantage of the technique presented here is that it is fast, requires only a standard confocal microscope (the results presented here were obtained on an MRC-600) and is simple to perform. Thus 3D reconstructions of expression patterns can be generated from a few thick sections as opposed to the hundreds necessary when using thin-section techniques, and requiring only days to perform rather than weeks. The use of Fast Red as a substrate effectively reduces three techniques into one – whole-mounts, cellular resolution analysis and 3D reconstruction. Although the non-fluorescent Fast Red signal seen in whole-mount embryos is weaker than BCIP/NBT, it allows the expression pattern to be examined before deciding which embryos to reconstruct or analyze at higher resolution.

Another advantage of thick sections is that complete cells with their neighbours can be examined intact. In thin sections (7–10 μm) the majority of cells are cut and become split between two or more sections, thus making it very hard to determine a cell's complete shape or arrangement relative to other cells. The technique presented here overcomes this problem through the use of high-resolution images, and the fact that large regions of the tissue are undisturbed within the thick section.

Finally, thick sections also overcome a problem facing all thin-section techniques: accurately recreating the original shapes in 3D. As thick sections do not crumple or stretch significantly during processing they do not generally need to be 'warped' back into their correct shape using computer programs before being reconstructed. Additionally, thick sections, being 3D, contain information about the angle at which structures pass through them, such that the correct shape can be recreated simply by aligning the bottom of one section with the top of the adjacent one. This is not true for thin-section techniques, which necessitate the use of fiducial markers (Streicher et al., 2000) or other alignment information.

3. Experimental procedures

3.1. In situ hybridization

In situ hybridization was carried out using standard hybridization techniques (Hammond et al., 1998) with the following modifications. Prior to staining the embryos were washed twice in 0.1 M Tris (pH 8.2) for 30 min and the signal was visualized in 2 ml 0.1 M Tris (pH 8.2) containing one Fast Red tablet (Roche).

3.2. Immunohistochemistry and in situ hybridization

Rabbit antibodies against the PDX1 protein (Gift from Novo Nordisk) were added [1:800] together with anti DIG antibodies. The antibody incubation was carried out overnight at 4°C. Unbound antibodies were removed by washing in TNT containing 0.1% BSA 8 times 1 h. The embryos were then blocked for 1 h in blocking solution [TNT, 2% BSA (BDH) and 15% heat inactivated sheep serum (Sigma)] and incubated overnight with Alexa Fluor® 488

goat anti-rabbit [1:200 (Molecular Probes)]. Residual antibodies were removed by washing 5 times 1 h in TNT containing 0.1% BSA. The in situ signal was then developed as described above.

3.3. Embedding, cutting and counterstaining

After fixing the embryos were embedded in 5% LMP agarose in PBS. When the agarose was set the embryos were cut into 70–100 μm sections on a vibratome, and each section was lifted from the vibratome waterbath onto a slide. If counter-staining was performed, the sections were incubated in a 5000-fold dilution of 1 mM Yo-Pro-1 (Molecular Probes, Y-3603). The section was then covered by a thin coverslip, was which glued to the slide at either end – a slight pressure being applied at each end, to ensure that the section lies flat. In order protect the morphology of the tissue the lifting and mounting should be performed very carefully. The mounted sections were stored in PBS to prevent them from drying out.

3.4. Confocal microscopy

We used an MRC-600 confocal with an Argon ion laser (488/514 nm) and the standard A1, A2 filter set (514 nm excitation filter, 540 nm green channel, >600 nm red channel). Image intensity was controlled by turning the gain up to maximum and keeping the pinhole to the minimal size that still provides a bright image. All scanning was performed at the slowest speed (3 s per frame). Low resolution images (i.e. the whole-embryo *Shh* expression pattern) were scanned using the 10× objective, while the cellular resolution images were captured using the 25× objective. As 3D scans progressed from the upper surface of the specimen downwards into the tissue, adjustments were sometimes needed to compensate for the weaker signal captured at lower depths. This was performed either manually, by gradually opening the pinhole during the scan, or automatically using a computer setting in which scanning continues to accumulate data from the same depth, each time improving the image, until a given intensity is reached.

3.5. Computer manipulation

Reconstructing the data from the confocal into 3D images was performed using either software from the Mouse Atlas Project or scripts written for IPLab which can perform the same functions. IPLab is a widely available image-processing program (Scanalytics, Inc.), and the scripts can be obtained from the authors at genex.hgu.mrc.ac.uk. The 3D data was visualized using the software package VTK (Visualization Toolkit, Kitware Inc.), however many other commercial programs exist which can provide similar visualization functions.

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