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Film tomography as a tool for three-dimensional image construction and gene expression studies

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In order to observe three-dimensional (3D) expression patterns of genes in whole animals, whole organs, or whole tissues, *in situ* hybridization (ISH) of many sections must be carried out and then used to construct a 3D image. For this purpose, we have developed an automatic microtome to prepare tissue sections with an adhesive film. We used commercially available film suitable for sectioning and ISH. We constructed a microtome and, after adherence of the film to a paraffin-embedded tissue block, cut the block with a blade to prepare sections on film. Then, the sections-on-film were automatically set in a plastic frame that was the same size as a conventional glass slide. With this automatic microtome, tissue sections can be made for ISH or immunohistochemistry in addition to conventional hematoxylin and eosin staining without specific training. We demonstrate that we can construct 3D images of gene expression patterns obtained by ISH on sections prepared with this automatic microtome. We have designated this method as 'Film Tomography (FITO)'.

Key words: 3D images, adhesive film, automatic microtome, film tomography, *in situ* hybridization.

Introduction

In the postgenomic sequencing era, to understand the functions of genes we need to examine their expression patterns *in vivo* as the first step (e.g. Lein *et al*. 2007). Since it is known that many genes are expressed in various tissues, suggesting that they play multiple roles, we have to know their three-dimensional (3D) expression patterns *in vivo* to elucidate their roles. To establish a 3D expression pattern, we must carry out *in situ* hybridization (ISH) on many sections with the same quality and construct them into a 3D image. In general, conventional microtomes are designed to cut sections from small blocks of tissue and are not compatible with procedures that require generating long serial sections. Furthermore, although tissue sectioning is fundamental to many anatomical and pathological investigations, few automatic microtomes have been developed. The main reason is that well-trained operators are required to prepare tissue sections and thus, it has been thought to be impossible to construct an automatic microtome.

In order to develop an automatic microtome, we used a method with an adhesive film as illustrated in Figure 1A, which was first introduced by Ullberg in 1954 (Kawamoto 2003) to produce frozen sections for whole-body autoradiography. In 1990, Kawamoto (1990) developed a novel adhesive film that was used to make thin stable frozen sections. The sections attached to the adhesive film have been demonstrated to be suitable for conventional staining, immunohistochemistry, and ISH (e.g. Kawamoto 2003). Using this method, Kokubo *et al*. (1996, 2000) showed in principle that sections for microscopic observation could be automatically prepared by slicing a specimen glued to a film. Unfortunately, Kawamoto's film was not adhesive to the paraffin block, while Kokubo's film was adhesive to the block, but not to frozen materials. Thus, we have looked for a new adhesive film for preparation of both paraffin and frozen sections. We found a new adhesive film and designated it as 'HistoFilm' (Fig. 1B,E). Then, we developed a novel automatic

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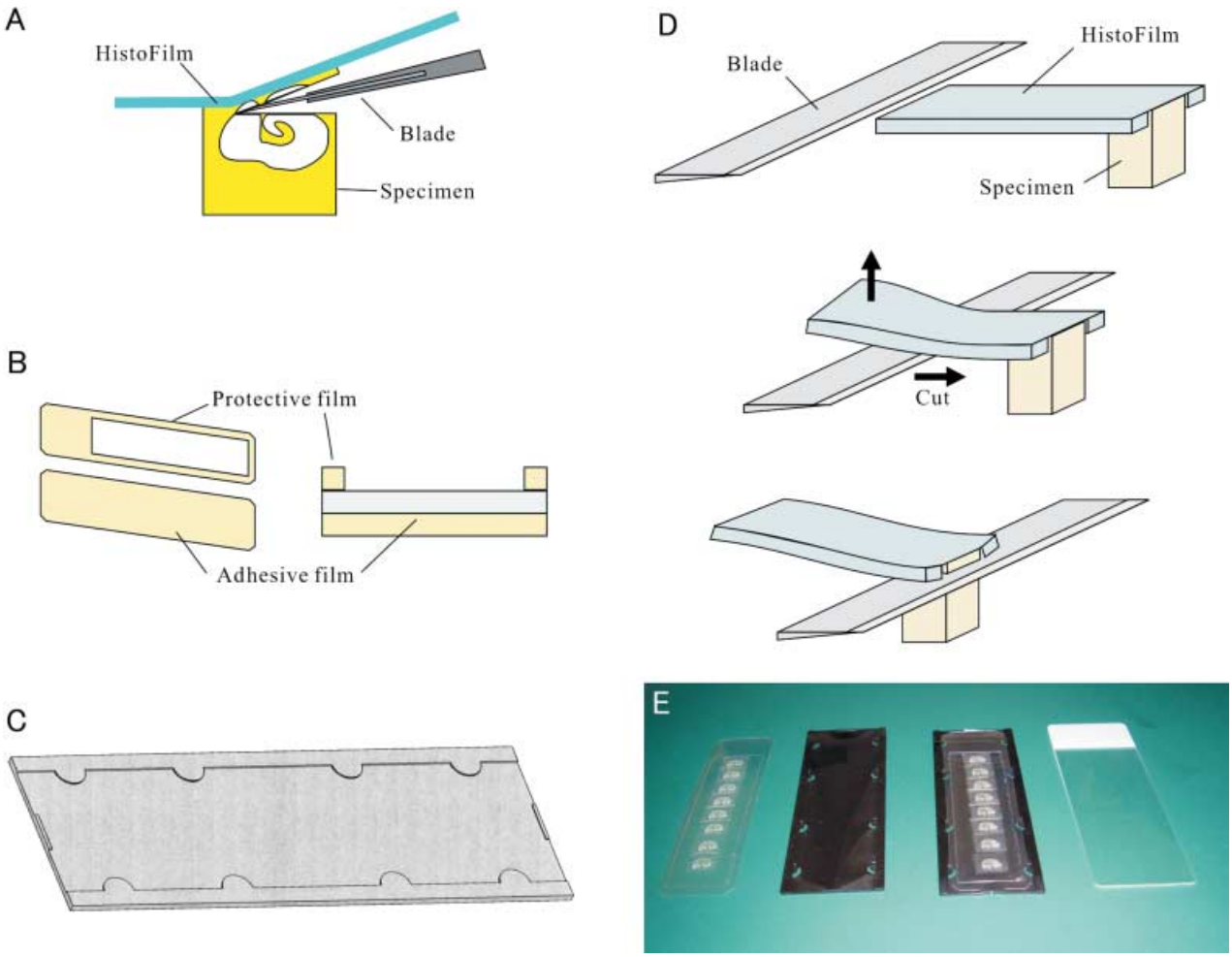

Fig. 1. A new adhesive film for *in situ* hybridization (HistoFilm) and its holder. (A) Schematic illustration showing how to prepare sections with an adhesive film (details in text). (B) New adhesive film with a protective film (P-HistoFilm). (C) HistoFilm holder for easy handling. (D) How to prepare sections on P-HistoFilm with the protective film. (E) Photographs of sections-on-film (left), its holder (left middle), sections-on-film in the holder (right middle), and a conventional glass slide (right).

instrument that can prepare sections on HistoFilm that are suitable for the *in situ* hybridization method.

Here we report that we have succeeded in making an automatic microtome to prepare tissue sections suitable for construction of a 3D image showing the expression patterns of some genes. We have designated this method as 'Film Tomography (FITO)'.

Materials and methods

Adhesive films

We screened five adhesive films kindly provided by Nitto Denko Company (Osaka, Japan) by judging the quality of results obtained with the *in situ* hybridization (ISH) method. One of them was satisfactory for our purposes and was designated as 'HistoFilm'. It is made of polyester (25 μm thick) covered with an adhesive chemical (28 μm thick). We used a protective film (50 μm thick) to make the HistoFilm slide (Fukae Kasei Company, Ltd, Kobe, Japan), which can be used in the plastic holder (Fig. 1E).

Specimen preparation for ISH

In order to carry out ISH, mouse embryos or tissues were fixed in 4% paraformaldehyde and dehydrated before embedding in paraffin or cryo-protected with 30% sucrose before embedding in optimum cutting temperature (OCT) compound (Sakura Finetechnical Japan, Tokyo, Japan) or in 4% carboxymethyl cellulose (CMC).

Sectioning

For manual sectioning with a conventional microtome (Yamato Kohki Industrial Company, Saitama, Japan),

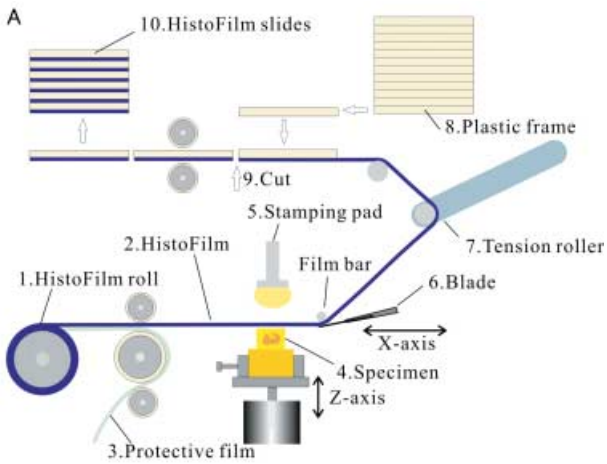

Fig. 2. Adhesive-film mediated automatic microtome (AFMA microtome). (A) Schematic illustration of the film machinery of the AFMA microtome. The HistoFilm (2) from the HistoFilm roll (1), after peeling off the protective film (3), is glued to the surface of a paraffin block (4) by a stamping pad (5), then the block is cut with the blade (6) just under the film to make a section attached to the film. A sections-on-film goes up to the site where a plastic frame (8) is put on the film automatically and this is cut (9) to make a film slide (10), which looks like a glass slide. The tension roller (7) is important to ensure that the film is suitable for sectioning and also works as a tension sensor. If it detects abnormal tension, the machine can stop for safety. (B) A photograph of the AFMA microtome. The numbers in (B) correspond to ones shown in Figure 2A.

we first adhered the P-HistoFilm to the surface of a paraffin block attached to a conventional microtome (Fig. 1D). Then, picking up the film with tweezers, we cut the block to make a section for adhering to the film. The section-on-film was treated using the specific holder (Fig. 1C).

For automatic sectioning, we made an adhesive-film-mediated automatic microtome (Fig. 2B). We used a roll of HistoFilm (20 mm wide, 53 μm thick, 10 m long, available from Fukae Kasei). With this microtome, it takes 4 s to cut a section and an hour to make approximately 900 sections. After sectioning, a fragment of the sections-on-film containing usually four to seven sections was framed with a plastic holder (76 mm long, 26 mm width, 1 mm thick) which is the same size as a conventional glass slide, and designated as the 'HistoFilm slide'. With this microtome, 130 HistoFilm slides were serially made within an hour.

RNA in situ *hybridization*

In situ hybridization was carried out on 10-μm-thick paraffin sections or 10-μm-thick cryo-sections with a semiautomatic hybridization instrument (Aloka HS300, Tokyo, Japan). The protocol was as follows: Paraffin sections were deparaffinized with xylene and rehydrated with ethanol, using standard methods. After washing with phosphate-buffered saline (PBS), the sections were treated with 1 μg/mL proteinase K for 20 min. After washing with PBS again, the sections were dehydrated with ethanol and dried for 10 min and then hybridized for 960 min at 70°C. The hybridization solution was composed of 50% formamide, 2 × standard saline citrate (SSC) (pH 7.0), 1 mg/mL torula yeast RNA, 10% dextran sulfate, and 1% sodium dodecyl sulfate (SDS). After washing with wash solution I containing 50% formamide and 2 × SSC at 70°C for 20 min two times and then wash solution II containing 0.1 × SSC at 37°C for 20 min, the sections were treated with NT (150 mM NaCl, 100 mM Tris–HCl (pH 7.5)) and blocking solution (1.5% casein) at 25°C for 120 min. After incubation with the alkaline phosphatase-conjugated antibody against digoxygenin (DIG) (1/2500 dilution) at 25°C for 120 min, the sections were stained with 4-nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP), using standard methods.

The DIG-labeled RNA probes were prepared according to standard procedure. The corresponding sense probes were used in parallel with antisense probes as negative controls.

Three-dimensional reconstruction of the whole embryo stained by ISH

We made a sequential series of 10-μm-thick sagittal sections of the whole mouse embryo at E10.5 with the present adhesive film mediated automatic microtome.

After *in situ* hybridization using a semiautomatic instrument, they were counterstained with nuclear Fast Red. Every digital image of the section was taken using a VHX-500 Digital Microscope (Keyence, Osaka, Japan) at 1600 × 1200 pixels. After proper alignment of the axes of the tissue sections and the outlines of the sections with Corel Photo-Paint 9 (Corel, Ottawa, Canada) at 360 × 480 pixels, the images were stacked to construct 3D images using ImageJ (W. Rasband, National Institutes of Health, USA).

Results

New adhesive film for carrying out in situ *hybridization*

In order to find a new adhesive film suitable for our purposes, we looked at insulating films because they can be used even in very severe conditions (e.g. at high or low temperature, and in water or oil, etc.). Five insulating films whose characteristics appeared suitable for ISH were kindly provided by Nitto Denko Company (Osaka, Japan). We prepared paraffin sections on a film with the following steps: (i) A film was adhered to the surface of a paraffin block containing tissues attached to the holder of a microtome; and (ii) the block was cut to prepare a section (5 μm thick) by means of a conventional manual microtome with a disposable microtome blade. On the other hand, to prepare frozen sections, the specimen was freeze-embedded with 4% carboxymethyl cellulose (CMC) in a coolant. When we used O.C.T. compound, the films were not adhesive to a specimen in the O.C.T. compound. Then, we carried out ISH with the sections-on-film. We chose the best film for ISH using both paraffin and frozen sections.

Although the film itself had properties suitable for the ISH method, it was very inconvenient to use the film as it was, because it adhered to anything. To circumvent this problem, we developed a protective film covering the edges of the film, designating it as 'P-HistoFilm' (Fig. 1B,E), and its holder (Fig. 1C,E). With this film, one can use the P-HistoFilm for manual sectioning (Fig. 1D,E). The HistoFilm in the holder, designated as the 'HistoFilm slide', can be used in conventional instruments such as a glass slide holder, glass slide container, etc.

We carried out ISH with sections of mouse embryos at E13.5 on the P-HistoFilm, using an RNA probe for *Sonic hedgehog* (*Shh*). A typical result is shown in Figure 3, where the spinal cord and primordial lung tissues are included (Fig. 3A). *Shh* expressions were found in the floor plate of the spinal cord (arrowhead in Fig. 3A,B) and lung epithelial cells (arrowhead in Fig. 3A,C), as reported previously (e.g. Placzek 2000).

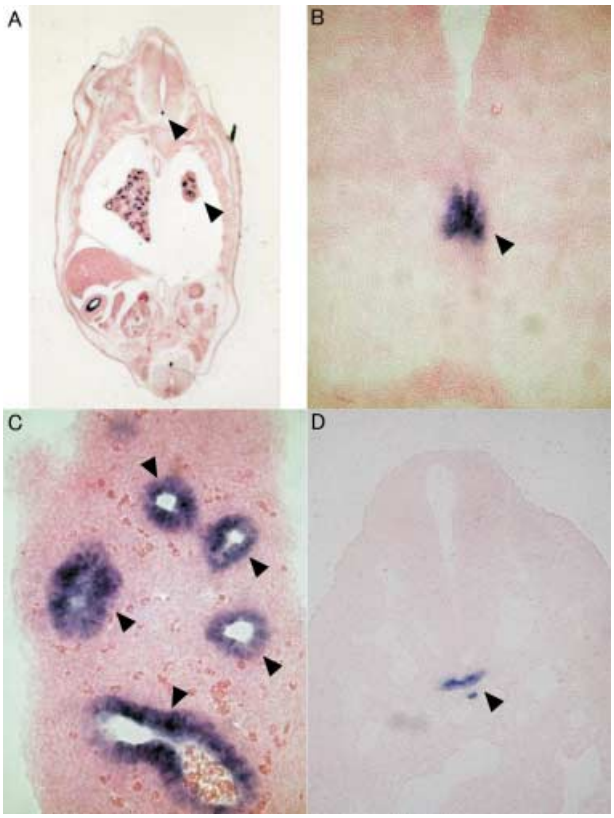

Fig. 3. Typical expression pattern of *Sonic hedgehog* (*Shh*) in a section of a mouse embryo, as revealed by *in situ* hybridization with HistoFilm. The sections were counterstained with nuclear Fast Red. (A) Frontal paraffin section of a mouse embryo at E13.5, showing that *Shh* is expressed in the notochord and the epithelial cells of the lung primordia. (B) High magnification of *Shh* expression in the notochord and (C) in lung epithelial cells. (D) *Shh* expression on a frozen section of a mouse embryo at E13.5.

Signals observed on the P-HistoFilm are similar to or even more intense than those on conventional glass slides.

A typical result with a frozen section is shown in Figure 3D. Signals on the sections-on-film are comparable to those on a glass slide. Thus, we concluded that the P-HistoFilm is likely to be suitable for analysis of gene expression by ISH.

Development of an automatic microtome with the HistoFilm

In order to develop an automatic microtome, we first considered modifying a conventional microtome, in which a paraffin block would usually be designed to move towards a fixed blade to make a section. With this microtome, we had to design it so that a film adhering to the specimen block could move with the block. However, we found that it was mechanically

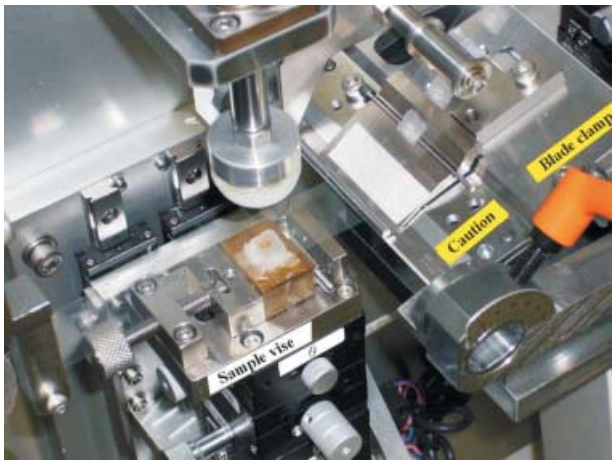

Fig. 4. Photograph of the main apparatus of the adhesive-film mediated automatic microtome (AFMA microtome). The blade on the holder (right) travels to the paraffin block (middle) to cut it. The stamping pad (upper) goes down to push the HistoFilm to the block to glue them together.

difficult to move the film as a whole. So, we decided to design and build a novel instrument in which the blade could move towards the block to cut without moving the film with the block. Our basic design of an adhesive-film mediated automatic microtome (AFMA microtome) is shown in Figure 2A. An adhesive film from a film roll runs in the instrument to make the film slide (Fig. 2A). The film is designed to have v-shaped cuts at a constant interval of 76.5 mm so that by detecting a v-shaped cut, we can feed the film into the microtme at a constant interval. A stamping pad presses the film to the specimen block to glue them together (Fig. 2A,B). A photograph of the sectioning area is shown in Figure 4, where a specimen block, stamping pad, blade, film bar, and film are seen. A blade moves horizontally towards the film-specimen block and cuts it (Figs 2 and 4). A part of the section-on-film is glued to a plastic frame and cut to the size of a conventional glass slide (Fig. 2). Although this AFMA microtome was designed to operate at room temperature for paraffin sectioning, if we could make a cold room in the sectioning area, we would make frozen sections.

In order to obtain serial sections a few micrometers thick, we used an electric micrometer that can travel 25 mm vertically (along the Z-axis) with 1-μm steps to send a specimen block up (Figs 2 and 4). A specimen vice for holding the specimen block was fixed on the top of the micrometer (Figs 2 and 4). Although this method does not normally allow for sections as thin as 1 μm to be cut, thicker sections of tissues from 2 to 20 μm can be produced reliably. The maximum size of a block for this microtome is $14 \times 16 \times 25$ mm, while the minimum size is $5 \times 5 \times 25$ mm.

To adjust the distance between the film and the specimen block, a film bar that can adjust the position of the film above the block was located just above the blade as shown in Figures 2 and 4. To keep the film straight, we used a tension roller, which can change its position to maintain the constant tension of the film (Figs 2 and 4). This also works as a sensor to detect abnormal tension in the film during sectioning. If the sensor detects abnormal tension, the instrument can automatically stop moving for safety. This microtome provides precise control of vertical specimen movements and is capable of producing a sequential series of uniformly thick sections from any specimens attached to the HistoFilm. A photograph showing the complete microtome is provided in the supplemental data.

Three-dimensional images constructed with a series of sections on the HistoFilm

In order to construct three-dimensional (3D) images with ISH data, we first carried out whole-mount *in situ* hybridization (WISH) with a mouse embryo at E10.5, using an RNA probe for *Shh*. *Shh* is expressed in the spinal cord and limb buds (Fig. 5A–C), as reported previously (Placzek *et al*. 2000). We then made sections on the HistoFilm, so we could compare the constructed 3D results with an image obtained by WISH (Fig. 5A). We made serial sagittal sections of the specimen containing 119 sections (Fig. 5C). One of them is shown in Figure 5B. After taking digital images of all of the sections, we cropped the images and aligned the image positions using Corel Photo-Paint 9 (Corel Corporation) and reversed the RGB color to make the boundaries of the sections clear. To produce a 3D image, we used ImageJ (W. Rasband, National Institutes of Health, USA) to stack the images (Fig. 5D). The constructed 3D image is provided in the supplemental data. With these data, we then carried out a 're-slice' to obtain virtual frontal sections (Fig. 5E). As a first step, the virtual sections were very satisfactory to determine expression patterns from a given direction. We have designated this method as 'Film Tomography (FITO)'.

Discussion

We demonstrated here that tissue sections can be made with adhesive thin plastic film, which we have termed 'HistoFilm'. In the conventional method, processes to stretch and flatten sectioned tissue specimens are necessary. However, since sections

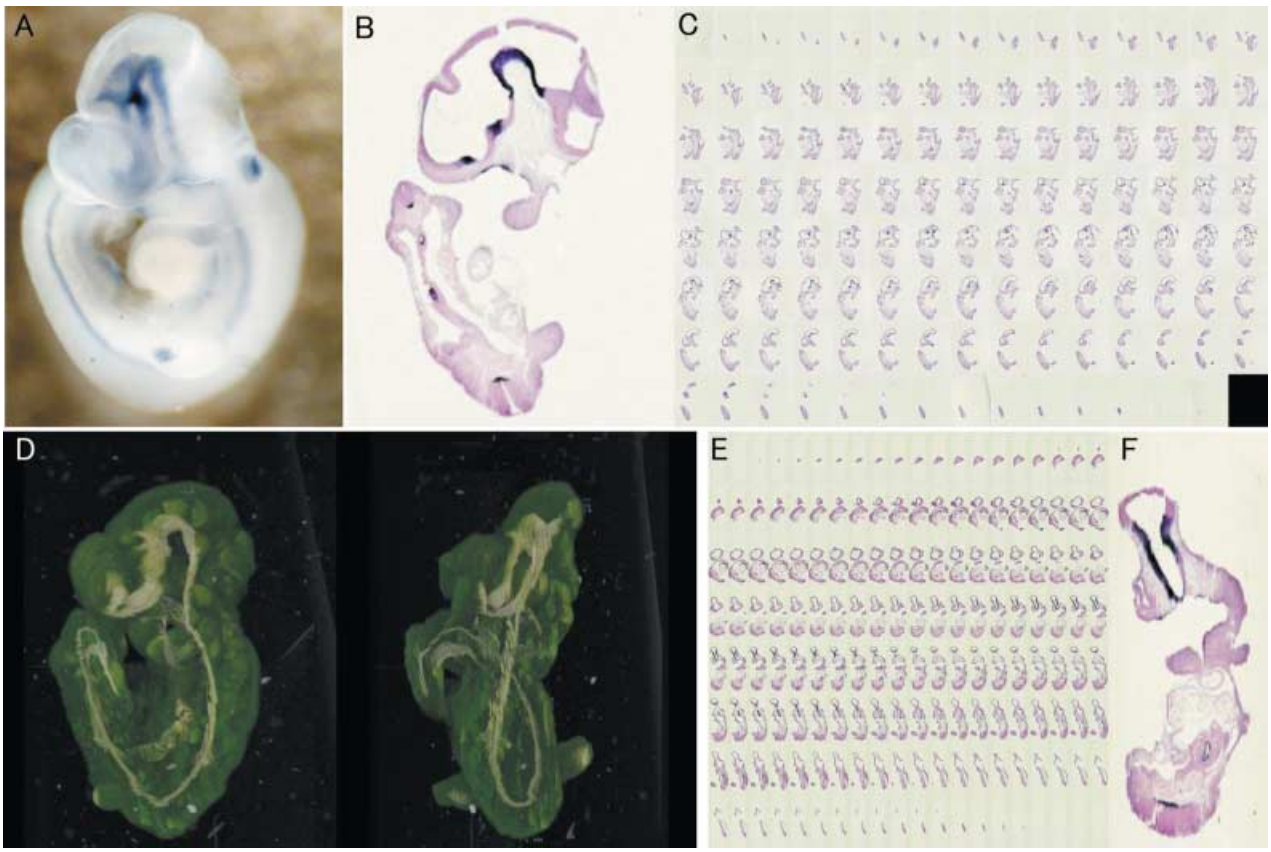

Fig. 5. Film tomography: Construction of 3D images from a sequential series of *in situ* hybridization (ISH) data obtained using sections on HistoFilm made using an adhesive-film mediated automatic microtome (AFMA microtome). (A) Expression pattern of *Sonic hedgehog* (*Shh*) in whole mouse embryo at E10.5, as revealed by whole-mount *in situ* hybridization. (B) Typical image of *Shh* expression pattern on one of the sections in (C). (C) Sequential series of *Shh* expression patterns on sagittal sections of the mouse embryo shown in (A). (D) 3D images (side view [left], front view [right]) constructed from the data shown in (C). (E) Virtual sequential series of the *Shh* expression pattern obtained in silico from the constructed 3D image shown in (D). (F) A typical virtual image of the *Shh* expression pattern on one of the sections in (E).

are made without such processes in the present method, the time and labor for preparing sections can be reduced. Furthermore, the HistoFilm can be used for sectioning of hard tissues or fragile sections, etc., which are difficult using a conventional microtome. The tissue sections on the film can be used for ISH to determine the expression patterns of genes. The HistoFilm can also be used to produce frozen sections embedded in CMC and to carry out ISH. When the film is used with a specific holder, conventional instruments developed for glass slides can be used for the film in the holder.

Microtomes are used extensively in biological research to section specimens. There are a wide variety of commercially available instruments for this purpose, however, they are designed to cut each thin section primarily from a specimen block and it is difficult to make a long sequential series of sections. With the use of the HistoFilm, we showed that a sequential series of specimen sections can be made automatically by the adhesive-film mediated automatic microtome. With the series of ISH data on the sections, 3D images of expression patterns of a given gene can be constructed. 3D images of gene expression patterns have been obtained by means of, for example, optical projection tomography (OPT) microscopy (Sharpe *et al*. 2002) only for optically transparent specimens, like embryos at very early stages. On the other hand, by our present method, virtual 3D images of expression patterns can be obtained essentially for all ISH specimens. An obtained 3D image may be re-sliced in silico to construct virtual sections showing the expression patterns of the gene in a given section, using ImageJ, for instance. Although the quality should be improved, the virtual sections were similar to real sections made with a microtome. The present sections on the HistoFilm can be used for many types of analyses such as histology, general

histochemistry, enzyme histochemistry, immunohistochemistry, etc. as well as ISH analysis. The only problem may be that coloring of the film may occur in HistoFilms that have been stored for more than a year. However, you can save digital images in silico for a longer period of time.

In conclusion, we have developed a new adhesive film (HistoFilm) for making sections to carry out *in situ* hybridization. With the HistoFilm, we constructed an automatic microtome to produce a sequential series of sections to construct 3D images and analyze gene expression. We have designated this method as 'Film Tomography (FITO)'.

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