

Short Technical Reports

Agarose-Embedded Tissue Arrays for Histologic and Genetic Analysis

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ABSTRACT

To facilitate the histologic analysis of large numbers of 7-day-old zebrafish (*Danio rerio*), a method has been developed to process them in agarose-embedded arrays. Using thin tissue sections, the morphology of cells and tissues can be examined microscopically to investigate a variety of biologic processes. Because of their small size, precise arrangement of the larvae is necessary to section them simultaneously. A technique was designed to embed groups of zebrafish larvae in a single plane in agarose before sectioning. Stained tissue sections of thousands of larvae can be examined efficiently using this embedding method. In addition to histologic analysis, PCR-based genotypic analysis of DNA from individual larval sections is also possible. This technique can be modified to accommodate any study that requires the histologic examination of many pieces of tissue.

INTRODUCTION

The histologic analysis of stained tissue sections is an important means for understanding disease. Specific morphologic and staining characteristics of tissues in normal and abnormal states are defined by microscopic examination. For example, most human cancers are graded based on histologic criteria such as nuclear atypia, nuclear-to-cytoplasmic ratio and frequency of mitotic figures (1). This evaluation of cellular malignancy has prognostic and diagnostic implications. The high degree of resolution provided by histopathology makes it essential to the practice of modern clinical medicine.

Histology is also a powerful research tool. For example, the microscopic inspection of tissue histology in 7-day-old zebrafish larvae reveals the details of early organogenesis. Hundreds of larvae are required to detect rare pheno-

types and to determine patterns of heredity. To study such large numbers efficiently, the larvae must be sectioned in groups. Because of their small size ($1 \times 1 \times 4$ mm), the larvae must be precisely arranged to section them simultaneously. We have designed a process to embed arrays of zebrafish larvae in agarose before sectioning.

The visualization of multiple tissue fragments on a single slide requires precise positioning of the tissue in the plane of section. In the case of a whole organism, such as a zebrafish larva, internal heterogeneity makes uniform orientation critical with respect to the plane of section. In addition, the process must not damage the larva, distort tissue architecture or destroy the DNA, which might be extracted for genotypic analysis. Agarose-embedded tissue arrays fulfill these criteria and facilitate histologic analysis of numerous small organisms.

MATERIALS AND METHODS

Acrylic Mold

Acrylic (polymethylmethacrylate) was used to make a mold (Figure 1) because it is sturdy and does not warp or expand significantly at 55°C , the temperature of liquid agarose. A 4×5.6 -cm piece of 1/4-in acrylic was milled on a Bridgeport vertical milling machine (Bridgeport, CT, USA) using 3/64- and 5/64-in, end-mill cutters (Weldon Tool, Valley View, OH, USA). One hundred twenty-eight teeth, each measuring $5 \times 0.8 \times 1$ mm ($l \times w \times h$), were created and arranged in four quadrants of 32 teeth each. Spacing between the teeth was 3/64 in, and spacing between the quadrants was 5/64 in. The mold de-

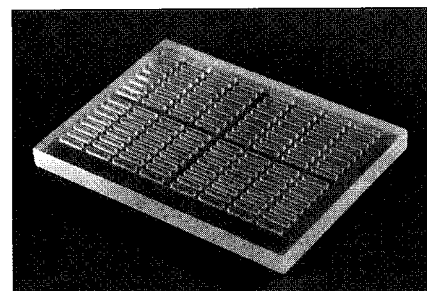


Figure 1. Acrylic mold for making four agarose blocks.

Short Technical Reports

scribed above makes four agarose blocks, each of which accommodates 64 7-day-old zebrafish larvae. The size, number, shape and orientation of the teeth can be altered to meet the needs of different applications.

Agarose as the Embedding Material

For the embedding material, molecular biology grade agarose (Fisher Scientific, Pittsburgh, PA, USA) was chosen because it is soft enough to section easily and has a hardness, after processing, that is similar to fixed zebrafish larvae. A 1% solution forms a gel that is strong enough to handle without breakage and solidifies slowly enough to pipet as needed. Agarose was dissolved at 1% in distilled (d)H₂O by boiling, then cooled and maintained in a 55°C water bath.

Preparation of Agarose Blocks

Four vertical sides were created by securing 1/2-in tape to the edges of the mold. On a level surface, liquid agarose was poured slowly into the mold, avoiding an uneven or excessive meniscus. After removing any bubbles, the agarose was allowed to cool at room temperature for 30 min until completely solidified. The tape was then removed, and the edges of the agarose were trimmed to remove the meniscus. The large block was cut into quarters and removed from the mold carefully to avoid breaking the agarose walls. The gels were stored, immersed in dH₂O to prevent drying and used within 24 h.

Embedding Larvae in Agarose

Zebrafish larvae were fixed in 10% neutral-buffered formalin for at least 24 h. The larvae were positioned in a single plane, dorsal-side-down, in the bottom of the agarose wells. Surface tension holds the larvae in position against the sides of the wells. Because drying adversely affects the histology, a small amount of fixative was allowed to remain in the wells while the larvae were being arranged. To avoid dilution, excess fixative was removed with a pulled glass pipet and absorbent paper just before adding additional agarose. Liquid

agarose was added to the wells to secure the larvae in place, using a warm glass Pasteur pipet. Just enough agarose was used to fill the wells, keeping the surface level, then allowed to solidify at room temperature. The finished quarter block (Figure 2) was placed in a HistoPrep™ OmniSette™ Plastic Tissue Cassette (Fisher Scientific) and immersed in additional 10% neutral-buffered formalin for several hours.

Processing and Sectioning of Embedded Blocks

The embedded quarter blocks were dehydrated and impregnated with paraffin in a Model 166MP Histomatic™ Au-

tomated Histologic Tissue Processor (Fisher Scientific). They were then embedded in paraffin and sectioned, 4- μ m-thick, on a rotary microtome. To cut parallel to the plane of the embedded larvae, care must be taken to align the knife with the face of the block. On 1 \times 3-in glass slides, the sections were stained using a regressive Harris hematoxylin and eosin (H&E) procedure (4) or left unstained for alternative analyses.

Genotypic Analysis

Unstained sections of paraffin-embedded blocks were dewaxed for DNA extraction using a method adapted from S. Thibodeau (5). Individual, 4- μ m-

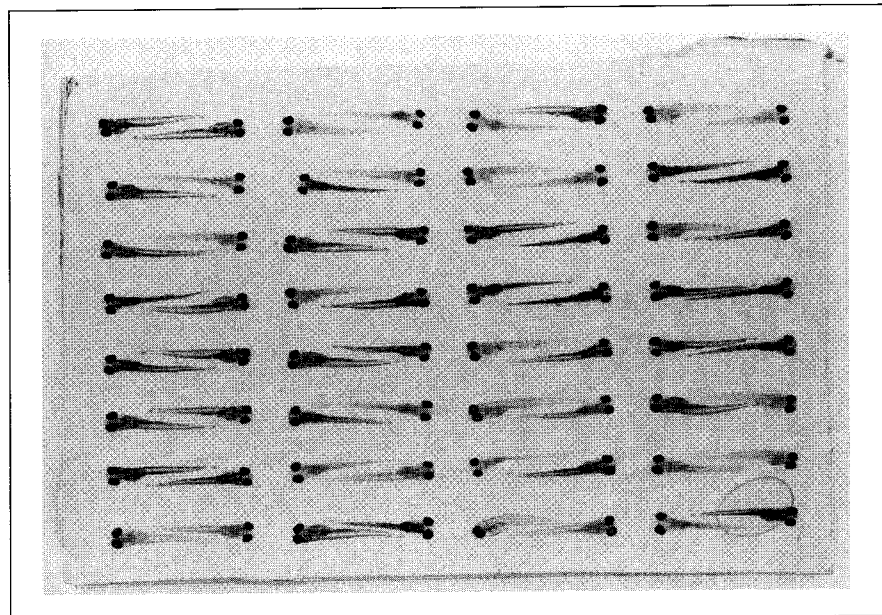


Figure 2. 64 7-day-old zebrafish larvae embedded in an agarose block. Two larvae are placed in each well, with the dorsal surface on the bottom of the well (4 \times).

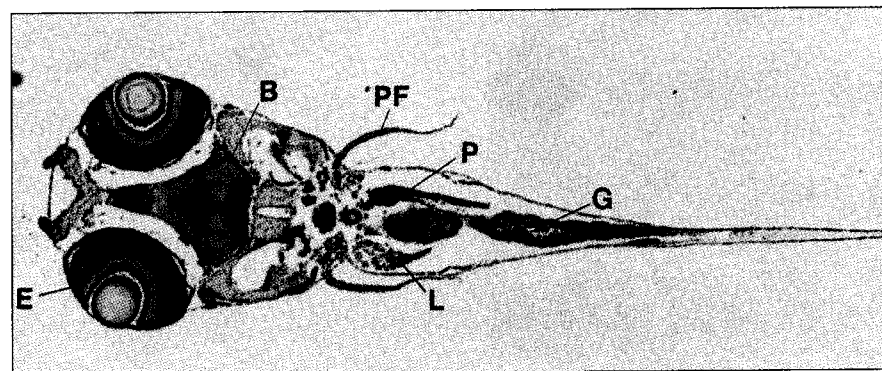


Figure 3. H&E-stained section of a zebrafish larva. E, eye; B, brain; PF, pectoral fin; P, pancreas; L, liver; and G, gastrointestinal tract (30 \times).

Short Technical Reports

thick, larval sections were scraped from dewaxed slides and incubated at 55°C for 48 h in 50–100 µL digestion buffer [0.2 mg/mL proteinase K (Promega, Madison, WI, USA) in TE (10 mM Tris, 0.5 mM EDTA, pH 7.6)]. Proteinase K was inactivated by boiling for 8 min. Two microliters of DNA extract were used in 10-µL polymerase chain reactions (PCR) using (CA)_n microsatellite marker SSR 12 of Goff et al. (3) (forward 5'-TCACTCACTGGG-AATGTAGGG-3'; reverse 5'-ACAAG-CAGAGACATAATCAACTGC-3') as described by Gestl et al. (2).

RESULTS AND DISCUSSION

To histologically study zebrafish tissue differentiation, we designed a method to embed 7-day-old larvae in agarose before sectioning, producing simultaneous coronal sections of up to 64 larvae on a single glass microscope slide.

At a rate of approximately 10 min per 32-well array (64 larvae), over 7500 zebrafish larvae were embedded and sectioned. Four different levels from each block were stained with H&E, and two levels were kept unstained. The stained tissue sections (Figure 3) were used to study the histologic features of multiple tissue types, including skin, brain, eye, intestine, liver, pancreas and

muscle. Using 4 coronal levels, 55%–65% of the larvae embedded could be scored for internal organs. Using 5–7 levels per block increased the scorable percentile to 65%–85%. Sagittal sections can be obtained by positioning the larvae on their sides.

The only significant problem encountered was occasional warping and deformation of agarose blocks in the tissue processor. In sections from warped blocks, only a fraction of the embedded larvae were seen on any single section. Among the 228 blocks processed, 24 (10%) were affected, apparently at random. Varying the block's thickness, size or density of embedded larvae did not seem to influence the occurrence of warping. Because this occurred infrequently, and extra larvae were usually available to embed, no further refinements to the procedure were made.

Individual tissue sections within the arrays can also be used for genotypic analysis. DNA was extracted from eight individual unstained sections and used in PCR analysis with a SSR microsatellite marker (3). Different fixation periods and extraction volumes were compared and found to have no effect on the quality of PCR product (Figure 4). Dilution studies indicate that more than 1000 PCRs of SSRs can be done on an individual larval section (data not shown). Using this technique in genetic screens, mutations causing morphological defects in zebrafish can be mapped, facilitating gene discovery.

This agarose embedding process is applicable to any study in which many small organisms or tissues must be examined. For example, multiple small tumors dissected from mouse lungs were embedded and sectioned, allowing easy visualization of histologically distinct tumor morphologies (Lynn Brown, personal communication). Both histologic analysis and genotypic analysis can be performed from the same tissue samples, facilitating studies linking molecular processes with histologic phenotypes. We have used this method to successfully detect zebrafish mutants with histological defects that are not evident by dissection microscopic inspection of unstained, living larvae (G.S. Tsao-Wu, J.L. Moore and K.C. Cheng, unpublished).

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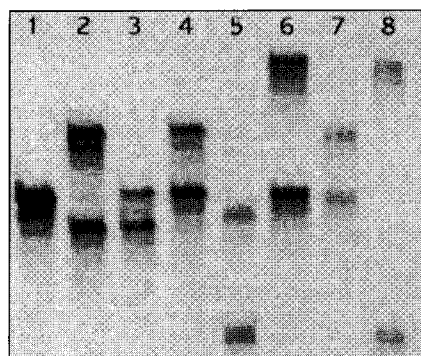


Figure 4. Genotypic analysis using DNA from individual sections of 7-day-old zebrafish larvae. An autoradiogram of ³³P-labeled PCR products using DNA from sections of 8 individual larvae. Primers for SSR12 (3) were used as reported (2) for 28 cycles, yielding products between 135 and 160 bp in length. Larvae were fixed in neutral-buffered formalin for 18 days (lanes 1–4) or for 2 h (lanes 5–8). Larval sections were digested in 50 µL (lanes 1, 2, 5 and 6) or in 100 µL (lanes 3, 4, 7 and 8) of digestion buffer.