

Video Article

SpOT the Correct Tissue Every Time in Multi-tissue Blocks

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Abstract

Multi-tissue paraffin blocks provide high throughput analysis with increased efficiency, experimental uniformity, and reduced time and cost. Tissue microarrays make up the majority of multi-tissue paraffin blocks, but increasingly, researchers are using non-arrayed blocks containing larger tissues from multiple individuals which can provide many of the advantages of tissue microarrays without substantial investment in planning and equipment. A critical component of any multi-tissue analysis is the orientation method used to identify each individual tissue. Although methods exist to maintain proper orientation and identification of tissues in multi-tissue blocks, most are not well-suited to non-arrayed blocks, may consume valuable space within an array and/or are difficult to produce in the standard histology laboratory. The Specimen Orientation Tag (SpOT) is a simple, low cost orientation tool that is clearly visible in paraffin blocks and all tissue sections for reliable specimen identification in arrayed and non-arrayed layouts. The SpOT provides advantages over existing orientation methods for non-arrayed blocks as it does not require any direct modification to the tissue and allows for flexibility in the arrangement of tissue pieces.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52868/>

Introduction

The ability to embed tissue samples from multiple individuals in a single paraffin block enables easy side-by-side comparison between treatments and individuals, eliminates variability between slides, and reduces the cost and workload of sectioning and staining specimens. These multi-tissue blocks are typically produced as either tissue microarrays (TMA) or paraffin blocks containing tissue from multiple individuals in a non-array layout. Maintenance of sample identity is critical to the success of any multi-tissue analysis. Researchers have been using TMAs since their development to improve efficiency of analysis, reduce variation between slides, conserve valuable tissue resources, and reduce the time and cost of experiments¹. Correct orientation of TMAs can be accomplished using a variety of methods, including blank spaces, rows or columns between tissue cores^{2,4}, asymmetric arrangement of core groups^{3,5} (e.g., control vs. treatment), "beacon" cores⁶, and designated orientation cores located outside the TMA matrix³. Although these methods work well for TMAs, most consume valuable TMA core space and TMAs with gaps and spaces as identifiers may become confusing as tissue cores are exhausted over time. Additionally, these methods are not appropriate for use in multi-tissue blocks without an array format because they rely on anomalies in the tightly ordered pattern of uniform microarray cores as identifiers. Most non-arrayed multi-tissue blocks must accommodate non-uniform tissues and, by definition, do not display the structured array grid that makes these aberrations stand out as landmarks.

Although there are many advantages to using a TMA, one of the largest disadvantages is the small size of the cores which is not always representative of largely heterogeneous tissue¹. Non-arrayed multi-tissue blocks provide many of the benefits of a TMA, but contain larger tissue samples, or entire organs from animal studies. Tissue microarrays using single cores have varying concordance with whole tissue sections based on the protein of interest and many require multiple cores for increased concordance⁷⁻¹¹. Due to the complexity and heterogeneous nature of some biomarker phenotypes, TMAs using even large numbers of cores (>10) can still be insufficient and may require methods other than microarrays for analysis⁸. Additionally, TMA construction is time consuming, technically demanding, and requires the initial cost and investment in or access to a tissue arrayer. Non-arrayed multi-tissue blocks can be made in any basic laboratory with significantly less time and effort and is a valid alternative for studies that require more tissue than arrays allow or as a means to cut costs and simplify analysis.

Non-arrayed multi-tissue blocks similarly require a reliable orientation marker to track sample identification, however, the development of these markers has been limited. Much of the literature describing tissue orientation focuses on correct physiological orientation for embedding individual tissue pieces, such as tattooing the tissue with ink¹², pre-embedding tissues in agar-gelatin prior to processing¹³, and marking certain tissues with notches¹⁴ or sutures¹⁵. Although functional, these methods are not ideal as markers in multi-tissue blocks due to their limitations. A suture will be sectioned through quickly and may not be visible in every section. Pre-embedding techniques using agar-gelatin may keep the tissue in proper orientation during processing and embedding, but does not provide a visual cue to differentiate between multiple samples in the paraffin block and slides. Notches or dye on the tissue may complicate analysis or occlude important morphological details. Alternatively,

tissue identity in a non-arrayed multi-tissue block can be maintained through embedding of tissue pieces in an asymmetric arrangement, but this requires 3 or more tissue pieces and may not allow for optimal arrangement of tissues for analysis.

The Specimen Orientation Tag (SpOT) has been developed as an easy and inexpensive method to clearly identify tissues in multi-tissue blocks and offers many benefits over existing orientation methods. The SpOT is a small, colorful, core composed of Hydroxyethyl agarose processing gel and tissue marking dye, and infiltrated with paraffin (**Figure 2C**). The SpOT core is embedded on end next to a single tissue in a multi-tissue paraffin block and appears as a brightly colored dot in the block and in every section (**Figure 1A-D**), clearly indicating the correct orientation of the block and sections for easy tissue identification.

Protocol

1. Construction of the SpOT

1. Add 50 mg of Bovine Serum Albumin (BSA) to 1 ml tissue marking dye in a 15 ml conical tube or 2 ml microfuge tube and vortex for 1 min or until completely dissolved.
Note: For this protocol, use biochemical Grade BSA. While other grades and purities have not been tested, it is expected that grade and purity would not have a significant impact on the success of the SpOT.
2. Heat 9 ml hydroxyethyl agarose processing gel in a 15 ml conical tube in a microwave at 30% power for 10 sec increments until the gel is completely melted.
3. Combine the melted processing gel and dye-BSA solution in a single 15 ml conical tube and mix thoroughly with a pipette. Vortex the solution.
4. Place the conical tube in a refrigerator for a few hrs or until solid.
5. Use a long, thin, metal spatula or probe to gently release the colored gel plug from the conical tube.
6. Use a scalpel or razor blade to cut the gel plug transversely into 5 mm thick sections. Remove the rounded ends and dispose as waste (**Figure 2A**).
7. Place the gel plug sections flat in histology cassettes and hold in 70% ethanol for 1 hr. Change the 70% ethanol solution every 2-4 hr for at least 3 changes over a 24 hr period.
8. Manually process the gel sections through an ethanol series (1 hr each: 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol (3x changes)), then clear in clearing solution 3x for 1 hr each (e.g., aliphatic hydrocarbons (xylenes substitute)) were used in this protocol, xylenes are also acceptable), and infiltrate with molten paraffin (3x for 1 hr each), either manually or in a tissue processor.
Note: Prior to full dehydration in 100% ethanol, the dye may leak from the gel plug which could affect other tissues and the liquid reagents in an automated tissue processor. As such, manual rehydration is highly recommended, however automated processing is equally effective.
9. Embed multiple processed gel sections using standard paraffin embedding methodology (**Figure 2B**). Allow blocks to cool and harden and then come to room temperature.
10. Use a dermal punch needle of desired size to remove SpOT cores from the embedded gel sections until the block is exhausted (**Figure 2C**). A single block may provide up to 20 x 2 mm² cores. Store the cores in a cool area. Use dermal punch in this protocol see Figures, a 1.5 mm (**Figures 1D**, and **2D**) or a 2 mm (**Figures 1B**, **1C**, and **2C**).

2. Using SpOTs to Orient Non-arrayed Blocks

1. Create a tissue orientation diagram to indicate the desired locations and identities of all of the individual tissue pieces to be embedded together, and the location of the SpOT.
NOTE: The tissue orientation diagram is an illustrated map that includes the physical locations of all tissue pieces labeled with identifiers and the location of the SpOT. It can be created electronically or can be hand-drawn. The diagram should be created using whatever method best suits the technician and researcher using the final product. This map will serve as the guide for the researcher so samples can be easily identified during microscopic analysis. The map used in this protocol was hand-drawn. It depicts a tissue block and glass microscope slide with each piece of tissue and the SpOT drawn and labeled in the same position as the actual tissue block and slide.
2. Process the tissue pieces normally, ensuring that they remain properly identified throughout the grossing and processing steps. For this protocol, process the tissues for 1 hr each in: 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol (x3 changes), aliphatic hydrocarbons (xylenes substitute) (x3 changes), and molten tissue infiltration paraffin at 60 °C (x3 changes).
NOTE: All tissues should be processed following standard histology laboratory procedures. This may be variable due to individual laboratory procedure, tissue size and type, but this will not affect the ability to use the SpOT as an orientation tool.
3. Arrange tissue pieces on the embedding station in the correct assigned locations according to the tissue orientation diagram. Use the tissue orientation diagram as a reference to guide the technician on how to arrange the SpOT and tissue pieces in every block.
NOTE: In this protocol the embedding technician put the hand-drawn orientation map next to the embedding station and arranged the tissue pieces on the embedding station in exactly the same arrangement as drawn on the diagram. This ensures that all tissue pieces remain correctly identifiable in the final product. This is absolutely critical to the successful use of multi-tissue blocks.
4. Ensure the SpOT core is taller than all tissue pieces to be embedded in the block.
5. Embed the tissue pieces and then the SpOT on end (transverse) in the proper locations according to the tissue orientation diagram using standard embedding methodology. As needed, hold the SpOT(s) upright with forceps until the paraffin has solidified enough that the SpOT(s) will not fall over (1-2 min).
6. Section and stain paraffin sections with the SpOT using standard methodology.
 1. Allow the sections to float on a water bath at 40 °C and adhered to a positively charged glass slide (uncharged slides have not been tested). Dry the slides in a drying oven at 60 °C for at least 20 min.
 2. Place the slides in an automatic stainer and processed through the following reagents: xylenes (xylenes 1-5 min, xylenes 2 and 3-2 min, each), 100% ethanol (2 min), 95% ethanol (1 min), 80% ethanol (30 sec), 70% ethanol (30 sec), running tap water (2 min), hematoxylin (1.5 min), running tap water (3 min), acid alcohol (1 min), running tap water (2 min), bluing reagent (1 min), running water

(2 min), 80% ethanol (30 sec), eosin (1 min), running water (10 sec), 80% ethanol (1 min), 100% ethanol (3 changes, 2 min each), xylenes (3 changes, 30 sec each).

3. Cover the slides with glass coverslips, mounted with mounting medium.

NOTE: In this protocol, the histotechnician sectioned the paraffin blocks on a microtome in 5 μ m sections. For this protocol we used an automatic stainer, however equal results can be obtained via manual staining.

3. SpOT in TMAs (Manual TMA Kit)

1. Create a tissue orientation diagram, as described in 2.1 and assign the desired location(s) of the SpOT.
2. Fill the TMA mold with melted paraffin at an embedding station. As the mold is cooling, embed the SpOT(s) on end in the margins of the TMA mold at the desired locations indicated by the tissue orientation diagram. As needed, hold the SpOT(s) upright with forceps until the paraffin has solidified enough that the SpOT(s) will not fall over (1-2 min).
3. Assemble the TMA cores according to manufacturer's directions and with regard to the location of the SpOT(s) in the array margins (**Figure 2D**), and follow standard sectioning and staining protocols.

NOTE: Please see step 2.6 to see the sectioning and staining methods used in this protocol.

Representative Results

The SpOT appears as a round, brightly colored dot in the paraffin block (**Figure 1A and 2D**), in all paraffin sections, and remains on the glass slide through the H&E or IHC staining procedure (**Figures 1B-1D, and 2D**). This obvious visual cue aids both the histotechnician and researcher in identifying each individual tissue piece and simplifies communication as the histotechnician can use this visual cue to indicate the arrangement of the tissue pieces to the researcher collecting data from the slide at the microscope. The tissue orientation diagram should be included with the slides provided to the researcher and explained in detail so there will be no confusion during microscopic analysis. **Figure 2A** shows the results of sectioning the solidified, colored agarose gel plugs. **Figure 2B** shows the result of embedding the sectioned agarose gel plugs in a paraffin block. **Figure 2C** shows the results of using a dermal punch to produce the SpOT cores.

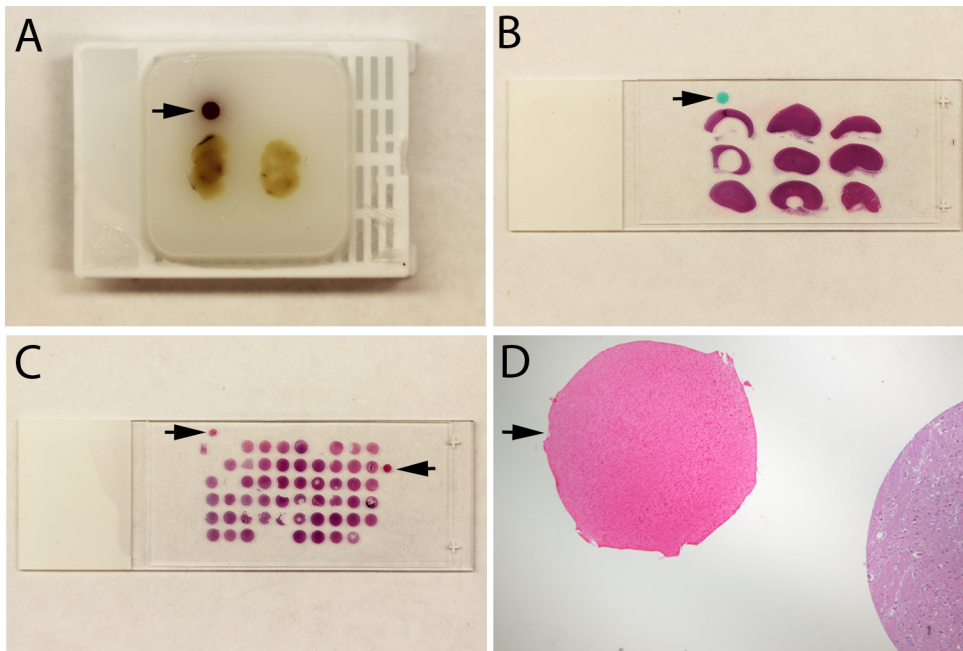


Figure 1: SpOT cores are easily created and readily visible in tissue blocks and slides. (A) SpOTS are clearly visible in the embedded block. **(B)** SpOTS allow for easy orientation and tissue identification for multi-tissue blocks containing similar appearing tissues from different individuals/treatment groups. **(C)** SpOTs allow every core of a Tissue Microarray to be utilized for valuable tissue samples. **(D)** Microscope view of SpOT adjacent to a TMA core. Arrows indicate the SpOT. [Please click here to view a larger version of this figure.](#)

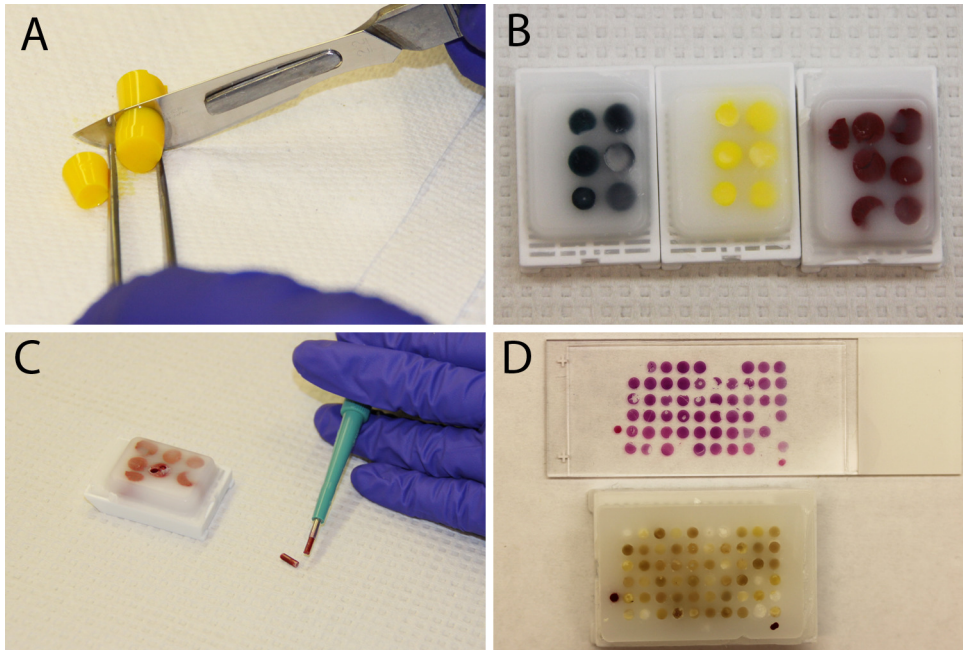


Figure 2: Preparation of SpOT cores. (A) Solidified Hydroxyethyl agarose plugs are released from Eppendorf tubes and cut transversely into 5 mm slices. (B) Multiple slices are embedded in a single paraffin block and (C) a dermal punch is used to remove SpOTs cores. (D) SpOTs are embedded in the margins of a Tissue Microarray. [Please click here to view a larger version of this figure.](#)

Discussion

Successful preparation and utilization of the SpOTs requires careful adherence to a few technical steps. Melting of the hydroxyethyl agarose should be done slowly and at low heat. Melting quickly at high heat can result in some breakdown of the agarose for less than optimal results. When cutting the dye-laden plugs into pieces for processing, ensure that the thickness of each piece is greater than 4.5 mm and does not exceed 7 mm. The rounded ends are removed as waste as they are not uniformly 5 mm in thickness. Pieces less than 5 mm will result in the creation of shorter SpOTs that risk being exhausted by sectioning prior to the exhaustion of adjacent tissue material. To ensure the SpOT is present through the entirety of the block, the pieces need to be at least 4.5-5 mm. To ensure proper infiltration of wax, however, the pieces should not exceed 7 mm. When dehydrating the dye pieces for wax infiltration, the dye will leach slightly out of the pieces in the lower concentrations of ethanol. The leaching does not impact the final product, the SpOTs will still be intensely dyed and clearly visible. The leaching could impact other tissues, though, so tissue sections should not be processed in the same baths as the dye pieces until the pieces have reached 100% ethanol.

When placing the SpOTs into the arrayed blocks, ensure that all other tissue placement is complete prior to adding the SpOT. Since the SpOT is infiltrated with paraffin wax, the molten wax of the recipient block can begin to melt the SpOT core if it is allowed to sit for too long. Arrange all tissue pieces first, add the SpOT core and immediately transfer the block to a cold plate to prevent any melting of the SpOT.

The SpOT can be modified to fit into most histology lab workflows. The color of the dye can be changed for optimal contrast in each application. Red or black dye produces clear high contrast on IHC stained slides, while the red SpOT is not as eye catching in an H&E stained slide of arrayed kidney sections. For H&E slides, green or yellow SpOTs tend to provide higher contrast. Further, under high heat during processes such as heat induced antigen retrieval for immunohistochemistry, the hydroxyethyl agarose melts away. BSA is added to the dye during SpOT preparation to maintain a brightly colored SpOT on the slide even after high heat retrieval methods. If the slides will not be exposed to high heat, the BSA can be omitted for faster SpOT production.

The most critical aspects of the use of SpOT are in the creation and maintenance of a clear and concise orientation map and the faithful adherence to the map when placing the SpOT. When used properly, the SpOT reduces confusion over tissue arrangement and simplifies communication between technicians and researchers, as the embedding technician can simply indicate the location of the SpOT and the relation of the tissues to it on a map. SpOT is clearly visible in all steps of slide preparation allowing for highly consistent section mounting on slides for easier downstream analysis. In contrast to physiological markers, the SpOT is clearly visible in every section and does not modify the tissue in any way, preventing the introduction of artifacts or obstruction of morphology. Unlike the method of embedding the tissues asymmetrically, the SpOT allows technicians and researchers flexibility in the arrangement of tissues to optimize visual analysis under the microscope. In TMAs, the SpOT can be placed outside the main array, eliminating the need for blank spaces, asymmetric assemblies, or beacon cores, allowing the entire array to be composed of valuable tissue sections. The SpOT can also be used to indicate anatomical direction (e.g., distal or proximal) of tissue samples. The SpOT is a simple and low cost solution for easy and consistent orientation of tissue samples during construction and analysis of multi-tissue blocks and TMAs.

Disclosures

The authors have nothing to disclose.

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