1 # Development of a Method to Preliminarily Embed
2 Tissue Samples Using Low Melting Temperature Fish
3 Gelatin Before Sectioning: A Technical Note

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13 **Short Running Title:** Preliminary Embedding in Fish Gelatin

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Abstract

Embedding of tissue samples that maintains a desired orientation is critical for preparing sections suitable for diagnosis and study objectives. Methods to prepare tissue sections include: 1) paraffin embedding or snap-freezing followed by microtome or cryostat sectioning, and 2) agarose embedding followed by cutting on a vibrating microslicer. Although these methods are useful for routine laboratory work, preparation of small and fragile tissues such as mouse organs, small human biopsy samples, and cultured floating spheres is difficult and requires special skills. In particular, tissue specimen orientation can be lost during embedding in molds and subsequent sectioning. Here, we developed a method using low melting temperature (LM) gelatin either alone or mixed with agarose to preliminarily embed collected tissues that are either prefixed or unfixed, followed by conventional fixation, paraffin embedding, freezing, and sectioning. The advantage of the method is that the LM gelatin and its mixture with agarose can be handled at room temperature but quickly hardens at 4 ºC, which allows embedding, trimming, and arranging of small and fragile tissues in a desired orientation and are compatible with traditional stainings. Thus, this method can have various laboratory applications and can be modified according to the needs of each laboratory.

Key words: low melting temperature gelatin, paraffin embedding, preliminary embedding, tissue sample preparation

1 **Introduction**

2 One solution for preserving specimen orientation during embedding in molds and
3 subsequent sectioning is to use agarose (commonly known as agar) to preliminarily
4 embed tissue samples, followed by trimming, formalin fixation, and paraffin embedding
5 or quick-freezing in a semisolid medium such as OCT compound.[1-3] Despite its wide
6 availability and convenience, agarose presents several practical disadvantages,
7 including: 1) biomaterial incompatibility that can promote separation from embedded
8 tissues, 2) a high melting temperature (65-80 ºC) that can result in heat damage to
9 tissues, and 3) rapid solidification that necessitates expedited handling after sample
10 collection.

11 In this study, we developed a method using low melting temperature (LM) gelatin
12 either alone or mixed with agarose to preliminarily embed collected tissues, followed by
13 conventional fixation, paraffin embedding, freezing, and sectioning (patent WO
14 2015199195 A1). Gelatin is obtained by partial hydrolysis of collagen and proteins
15 derived from animal skin, bones and connective tissues. In addition to food, cosmetic
16 and pharmaceutical applications, gelatin has been used for preliminary embedding of
17 biomaterials due to its high solubility and biocompatibility coupled with a low cost and
18 immunogenicity.[4,5] Most gelatins are derived from beef and pork, but a lower-cost
19 alternative is fish gelatin. However, fish gelatin has some disadvantages such as poor
20 gel strength and low melting point (~ 16 ºC) that have limited its applications.[6,7]
21 Previous studies that investigated the physical and rheological properties of fish gelatin
22 revealed that it comprises lower content of imino acids, proline and hydroxyproline,
23 than mammalian gelatin, which results in its low gel modulus and melting
24 temperature.[6,7]

The method we developed capitalizes on the seeming disadvantages of fish LM gelatin to embed tissue samples and cultured cells and spheres. Fish gelatin is liquid at room temperature (RT) and solidifies at 4 ºC, but after formalin fixation fish LM gelatin remains solid, even at RT. LM gelatin also allows embedding, trimming, and arranging of small and fragile tissues in a desired orientation. Meanwhile, a mixture of LM gelatin and agarose at an appropriate ratio (0.5-1 and 0.4%, respectively) is useful for various sample preparation applications. The LM gelatin/agarose mixture can be handled at RT but quickly hardens at 4 ºC, and conformed to both brittle and soft tissues. The mixture was also compatible with paraffin embedding and traditional staining methods such as hematoxylin & eosin (H&E) staining. Thus, this method can have various laboratory applications and can be modified. Here we outline the materials needed for this method and provide representative experimental results.

1 **Materials and Methods**

2 **Preparation of LM gelatin and gelatin/agarose mixtures**

3 Electrophoresis-grade agarose (Agarose S, Nippon GENE, Tokyo, Japan) was dissolved
4 in boiling distilled water (DW) to a final concentration of 0.5%, prior to microwave
5 (500 W) heating for 2 min to achieve complete solubilization (Solution A). Fish LM
6 gelatin (Gelare-blanc, #2809, Nitta Biolab, Osaka, Japan) was dissolved in DW to
7 2.5-5% at 40 ºC and the mixture was stirred for 2 min, allowed to stand for 3 min, and
8 stirred again for 10 sec (Solution B; LM gelatin alone). Solution A was cooled to 40 ºC
9 and mixed with one-fourth (1/4) volume Solution B, followed by stirring for at least 1
10 min to yield a uniform LM gelatin/agarose mixture (gelatin, 0.5-1%; agarose, 0.4%).
11 The mixture remained liquid for several hours at 37 ºC and 10-15 min at RT, but slowly
12 solidified when it is kept at RT and rapidly solidified at 4 ºC. Solution A and B can be
13 stored at 4 ºC for a week. Solution B can also be stored at -20 ºC for at least several
14 months and can be melted in a microwave oven then allowed to cool just prior to use.

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16 **Preliminary embedding, fixation, and sectioning**

17 Tissue samples, which were either prefixed in advance or unfixed, were preliminarily
18 embedded in a LM gelatin/agarose mixture and fixed in 10% buffered neutral formalin
19 solution or 4% paraformaldehyde (PFA) (Nacalai Tesque, Kyoto, Japan) at 4 ºC,
20 followed by trimming, conventional paraffin embedding using a tissue processor
21 (ASP-6025, Leica Microsystems, Bensheim, Germany), and sectioning to 3-4 µm
22 thickness using a conventional microtome (SM2010R, Leica Microsystems). For
23 cryosectioning, tissues preliminarily embedded in LM gelatin (2-5%) were further
24 embedded in OCT compound (Sakura Fineteck, Tokyo, Japan) and snap-frozen in liquid

1 nitrogen, followed by sectioning to 5-10 µm thickness at -20 ºC using a CM3050SIV
2 cryostat (Leica Microsystems). For the fixation of cultured neurospheres before the
3 embedding with LM gelatin, we used 4% PFA throughout the experiment.

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1 **Results**

2 **Experimental result 1. Preliminary embedding of tissue samples with LM**
3 **gelatin/agarose mixture before fixation and paraffin embedding**

4 LM gelatin for preliminary embedding was advantageous due to its transparency and
5 ease of trimming and cropping, as well as its good conformation to soft and fragile
6 tissues. However, LM gelatin tends to shrink and harden following dehydration by
7 alcohol. Therefore, we instead used a LM gelatin and agarose mixture for preliminary
8 embedding (**Figure 1A**). The LM gelatin:agarose ratio was optimized at 0.5-1% gelatin
9 and 0.4% agarose, which allowed maximum manipulability and compatibility with
10 tissues and staining procedures. The gelatin/agarose mixture remained liquid for 10-15
11 min at RT and quickly hardened at 4 ºC, but did not shrink upon alcohol dehydration.
12 The LM gelatin/agarose mixture was harder and more elastic than LM gelatin alone,
13 and had good compatibility with soft and small tissue samples. Preliminary embedding
14 of mouse embryos as well as pituitary, lung, intestine, and skin tissues using the LM
15 gelatin/agarose mixture allowed trimming or cropping of embedded tissues without
16 breakage or loss of desired orientation, which could be useful for subsequent formalin
17 fixation and paraffin embedding (**Figure 1A**). Prepared sections were compatible with
18 conventional H&E, Periodic acid-Schiff (PAS), Alcian Blue, and Van Gieson's staining
19 and immunohistochemistry (**Figure 1B**). The LM gelatin/agarose mixture had no
20 autofluorescence and thus is compatible with immunofluorescence studies (**Figure**
21 **1B**).[4]

22 To prepare lung and intestinal tissues, we filled intratracheal and intraintestinal
23 spaces with the LM gelatin/agarose mixture, followed by preliminary embedding,
24 fixation, and paraffin embedding of collected organs. This approach was useful for
25 cutting samples into preliminary sections that were millimeter-thick and observing the

1 cut section surface. Filling intratracheal and intraintestinal spaces with the LM
2 gelatin/agarose mixture also preserved fine epithelial structures such as tracheal cilia
3 and brush border membranes and likely provided protection from damage due to
4 organic solvents and heat involved in paraffin embedding (**Figure 1C**).

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6 **Experimental result 2. Preliminary embedding of cell spheres and tissues with LM**
7 **gelatin alone, followed by embedding with OCT compound and preparation of**
8 **frozen sections**

9 Preliminary embedding with LM gelatin alone (2-5%) was useful for preparing cell
10 blocks. For example, cultured neurospheres were centrifuged, fixed by 4% PFA and
11 embedded with LM gelatin, followed by snap-freezing, embedding with OCT
12 compound, cryosectioning, and H&E and immunofluorescent staining (**Figure 2A**).
13 Compared with a conventional method to prepare cell blocks that involves sodium
14 arginate and calcium chloride, preliminary embedding with LM gelatin was more useful
15 for trimming samples into arbitrary shapes and was more compatible with
16 cryosectioning and staining. The prepared cell blocks could be fixed by 4% PFA
17 solution at 4 ºC to prepare paraffin-embedded blocks.

18 Preliminary embedding with LM gelatin was also useful for preparing frozen
19 tissue sections. We used LM gelatin to preliminarily embed mouse embryos, which
20 were then fixed at 4 ºC, and embedded further with OCT compound before
21 snap-freezing, cryosectioning to 8 μm thickness, and conventional staining (**Figure 2B**).
22 Compared with a conventional cryosectioning procedure, preparation of tissue sections
23 and section mounting onto glass slides without sample wrinkling or tearing was simpler
24 using LM gelatin.

Discussion

In the present study, we developed a method to preliminarily embed collected samples with either LM gelatin alone or a mixture of LM gelatin and agarose. As described above, one of the advantages of using LM gelatin is its manipulability and compatibility with small tissues and staining procedures. We hope that this method could be widely applied in various applications for routine laboratory work and diagnostics.

The advantages of the use of gelatin and its combination with agarose in preliminary embedding have been described previously.[8-10] Compared with preliminary embedding with agarose alone, which sometimes results in folds and poor adherence of sections, sections pre-embedded with the gelatin/agarose mixture lay flat on the slides and are compatible with many staining procedures.[7] In the method described in the present study, we modified the above method and used LM gelatin derived from fish[6,7] to make a preliminary embedding matrix, taking advantage of the texture of agarose and the low melting point of fish gelatin. One striking feature of our method is that the LM gelatin/agarose mixture can be handled at RT but quickly hardens at 4 ºC, which enables us to arrange small, brittle and soft tissues in a particular configuration or orientation before processing for paraffin embedding or cryosectioning. Another advantage of the method is that the concentration of LM gelatin can be modified ranging from 0.5 to 1% according to user requirements. A general rule of thumb is that 1% LM gelatin is useful for the trimming of preliminarily embedded tissue samples, whereas 0.5% LM gelatin is recommended for filling intratracheal and intraintestinal spaces or vascular perfusion (**Figure 1B, C**).

A seemingly disadvantage of the use of the LM gelatin/agarose mixture to fill body cavities such as the intratracheal space is that the fixation quality of the tissues could be a bit worse than conventional perfusion fixation with formalin. There seems to

1 be a trade-off between manipulability of tissue blocks to preserve fine cellular structures
2 and the penetration of fixatives. Indeed, we found that the nuclei of the tracheal
3 epithelia looked more swollen and condensed compared to the section prepared by
4 conventional fixation and paraffin embedding (**Figure 1C**). Thus, methods for fixation
5 and preliminary embedding need to be chosen and modified according to user
6 requirements.

7 Finally, the method developed in the present study seems to be more applicable to
8 the field of experimental pathology, rather than diagnostic routine work. It needs to be
9 noted, however, that the LM gelatin/agarose mixture can also be used for preliminary
10 embedding of prefixed tissue samples such as biopsy specimens taken from patients.
11 We thus hope that the LM gelatin could be applied for various purposes in biomedicine
12 while taking advantages of various conventional methods.

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Disclosures

The authors declare no conflict of interest. K.U., N.A., and M.T. are inventors of an international patent WO 2015199195 A1 (EP 3163284A1, EP 3163284A4, US 20170160174), named "Embedding medium for specimen preparation, method for preparing curable base material non-penetrating specimen, method for preparing curable base material penetrating specimen, curable base material non-penetrating specimen, thin-slice-performance improver for frozen embedding medium, and frozen embedding medium". All animal protocols were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine. All *in vivo* experiments were performed in compliance with Nagoya University Animal Facility regulations.

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1 **Figure legend**

2 **Figure 1. Examples of applications of LM gelatin/agarose mixtures for sample**
3 **preparation**

4 **(A)** Indicated samples were preliminarily embedded in a LM gelatin/agarose mixture,
5 followed by fixation, paraffin embedding, and sectioning. **(B)** Preliminary embedding
6 with LM gelatin/agarose mixture is compatible with conventional histochemical
7 staining, immunohistochemistry (IHC), and immunofluorescent staining (IF). In IHC
8 and IF, intestinal tuft cells were stained with anti-phospho-Girdin (Y1798) antibody,[10]
9 as denoted by brown and green, respectively. Nuclei were visualized by DAPI
10 (4'6-diamidino-2-phenylindole) staining (blue). Asterisks indicate tissues penetrated by
11 the LM gelatin/agarose mixture. **(C)** Intratracheal perfusion with LM gelatin/agarose
12 mixture enhanced the observation of fine structures such as tracheal cilia (arrowheads)
13 relative to conventional paraffin embedding (open arrowheads).

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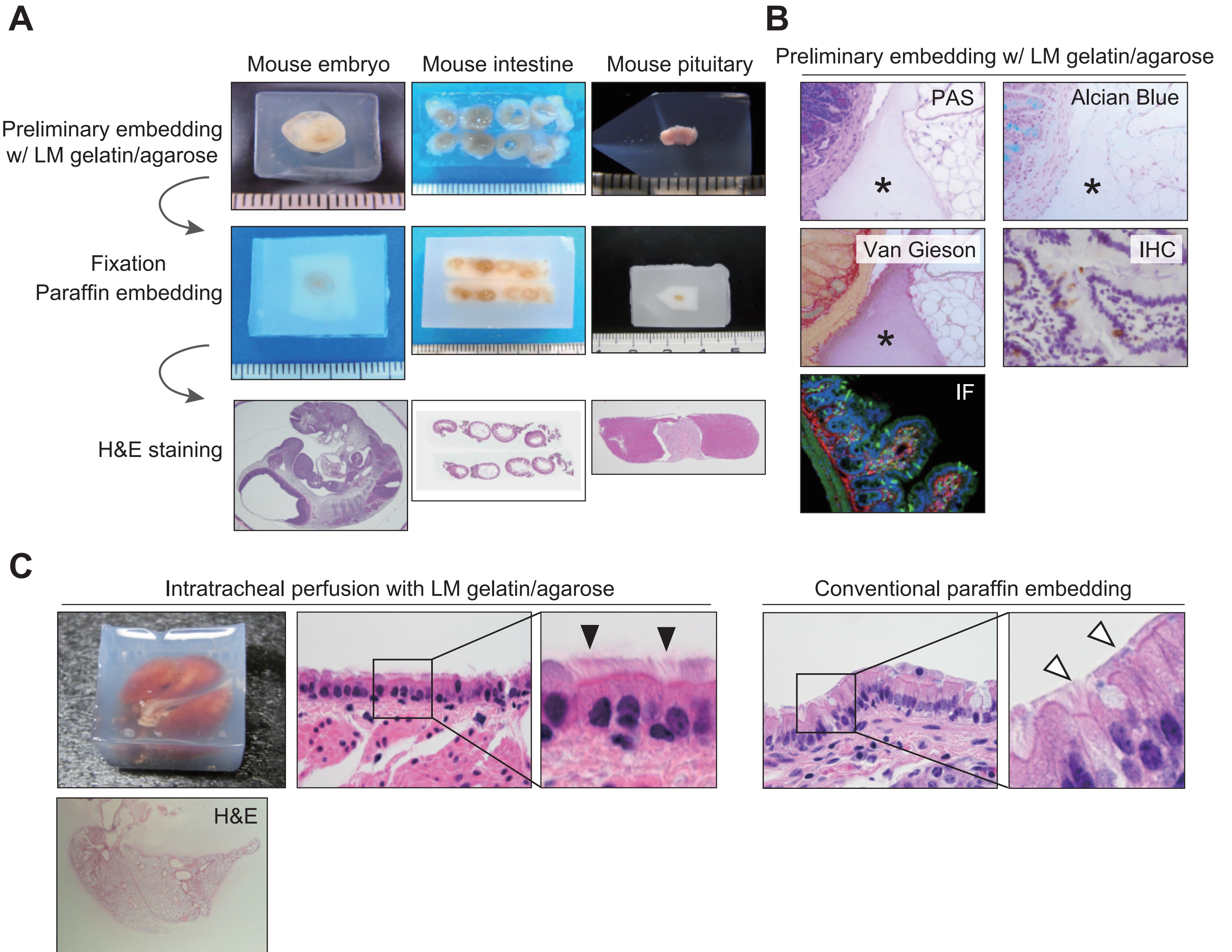
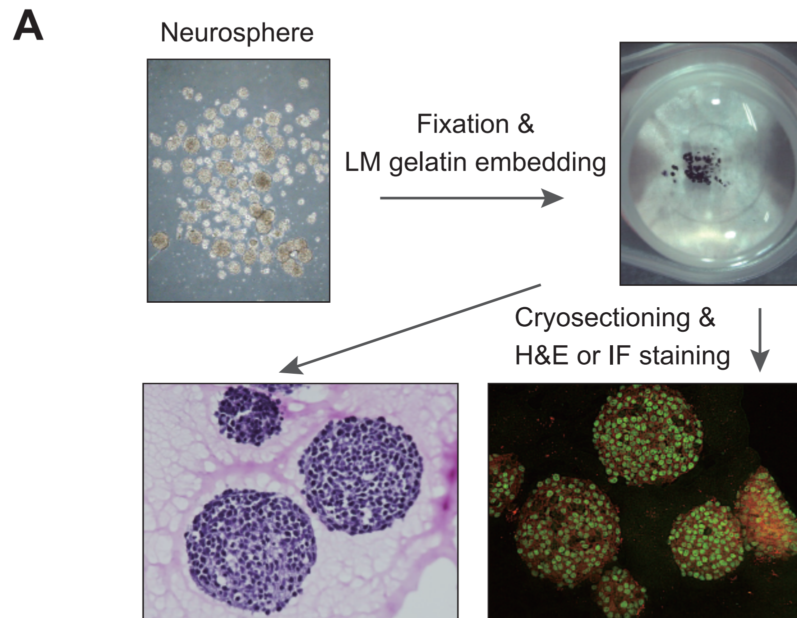
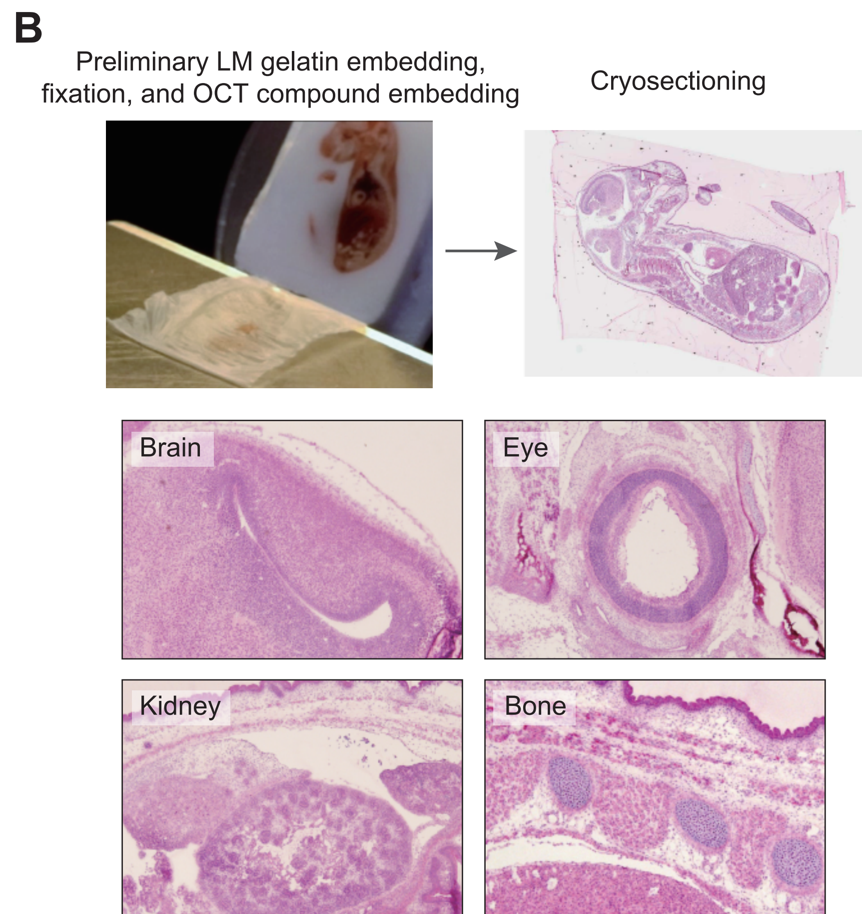
15 **Figure 2. Examples of applications of LM gelatin for sample preparation**

16 **(A)** Neurospheres were fixed and embedded in LM gelatin alone, followed by OCT
17 compound embedding, cryosectioning, and H&E and immunofluorescent staining. **(B)**
18 A mouse embryo was preliminarily embedded in LM gelatin, fixed, and embedded in
19 OCT compound, followed by cryosectioning and H&E staining.



Ushida et al., Figure 1





Ushida et al., Figure 2