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ABSTRACT
Magnetic immunostaining provides a novel technique for rapid intraoperative diagnosis of cancer metastasis and its characteristics in lymph nodes. Previous studies have shown the effectiveness of this technique using the manual staining process. However, to make this technique feasible for use in hospitals, establishing an automatic process is important. Thus, the objective of this study is to realize a device for immunostaining using magnetic fields that could not only automatically stain the samples, but also wash the specimen and change experimental solutions as well. The specifications for our prototype device were determined based on the manually performed experiments. We successfully fabricated a prototype device and demonstrated automatic immunostaining using it. The device completed, within 10 minutes, processes of blocking the specimen, injection of the magnetic beads, antigen-antibody reaction, and washing out the beads. The reaction took 1 minute, which was significantly shorter than that of conventional immunostaining. The device is expected to provide improved reproducibility of staining because of its automated processes.

INTRODUCTION
For stage classification of breast cancer and development of treatment plans, investigating the presence of metastasis in lymph nodes is important. Sentinel lymph nodes refer to one or more lymph nodes that are deemed as the first ones that drain cancer cells directly from the primary lesion via lymphatic flow. These sentinel lymph nodes are identified, and subsequently inspected for the presence of cancer cells to determine metastasis in them. The absence of metastasis in the sentinel lymph nodes indicates that other organs would also have no metastasis; otherwise, other lymph nodes would have to be excised and pathologically investigated to identify the spread of cancer. Currently, the standard procedure is to identify and excise the sentinel lymph nodes during excision surgery for the primary lesion and inspect these excised nodes via rapid pathological diagnosis. The excised nodes are immediately transferred to a pathology laboratory unit wherein frozen sections of the excised nodes are made for staining using hematoxylin-eosin (HE) stain,
which are then used by a pathologist to diagnose for metastasis. Since the pathological examination of the HE-stained node specimens is based on morphological observations of cells and their nuclei, in some cases, pathologists face difficulties in determining metastasis depending on the characteristics of the cancer. Moreover, specimens with micro-metastasis cause additional difficulty for accurate cancer diagnosis. To address these challenges, definitive diagnosis after the excision surgery can be realized using immunostaining. While immunostaining using antigen-antibody reactions facilitates high diagnostic accuracy, this process cannot be completed during surgery because the antigen-antibody reactions are complicated and time-consuming. Furthermore, owing to the lack of resident pathologists, intraoperative diagnosis cannot be conducted in many small- to medium-sized hospitals. Thus, there is a need for an alternative methodology for surgeons to intraoperatively perform metastasis diagnoses during surgery.

In previous studies, our group proposed a novel immunostaining technique involving antibody-labeled fluorescent magnetic beads, which are attracted to the surface of lymph node specimens on application of a magnetic force. Because this facilitated antigen-antibody reaction completes in only 1 minute, this technique meets the abovementioned clinical time requirements. Furthermore, unlike the HE staining approach, the magnetic immunostaining technique can be used to detect cancer cells in the lymph node specimens based on their specific biomarkers. Thus, the magnetic immunostaining technique can be used to realize detailed and highly accurate diagnosis during surgery. In addition to the intraoperative diagnosis of cancer metastasis, many research groups in the world are now investigating new particles, sensors, and applications of immunostaining, which shows a potential of magnetic immunostaining. The objective of this study is to design specifications for an automated magnetic immunostaining device and develop a prototype for use in clinical studies.

RAPID IMMUNOSTAINING USING ANTIBODY-LABELED FLUORESCENT MAGNETIC BEADS

The fluorescent magnetic beads used in our work are not only capable of magnetic accumulation on the surface of a pathological specimen, but also enable fluorescence detection. These beads were based on dispersive polymer-coated magnetic beads that were originally developed as a carrier for isolation and purification of target proteins. For this study, the beads were prepared through introduction of fluorescent dyes into the polymer layer of the dispersive polymer-coated magnetic beads. In particular, the beads were coated with a single organic polymer whose surface could be chemically modified to allow for the attachment of a variety of ligands. Thus, antibodies recognizing specific cancer cells could also be fixed on these beads, allowing for selective staining of specific cancer cells.

In addition, to perform a magnetic immunostaining using the proposed antibody-labeled fluorescent magnetic beads, we developed a prototype for an automated device that could attract the magnetic beads toward the specimen using magnetic force and change chemical solutions according to each process. The locally high concentration of antibodies on the surface of lymph node specimen results in an increased probability of a reaction occurring between these antibodies with the antigens on the cancer cells, resulting in the completion of the reaction in only 1 minute, which allows for its use in intraoperative diagnosis of cancer metastasis. Furthermore, because this technique involves the use of specific antibodies, it can be used to detect specific cancer cells that could not be detected via the HE staining approach.

DESIGNING THE MAGNETIC IMMUNOSTAINING DEVICE

Before designing the immunostaining device, an immunostaining protocol and its associated conditions were established based on the manually performed immunostaining processes. Table I lists the important characteristics and processes to be implemented in the prototype, including shape of permanent magnet used for efficient magnetic accumulation, amount and concentration of beads and other materials for highly accurate immunostaining, conditions for antigen-antibody reaction and washing, and mechanical design of the device. Expert pathologists were consulted in order to design the relevant specifications for the prototype, including number of samples that can be simultaneously processed, volume of chemical solutions for reaction and washing processes, method of introducing chemical solutions and magnetic beads, solution ejection after use, and size of lymph node specimens.

Accordingly, our developed prototype device can simultaneously process a maximum of five specimens because, typically, one to five lymph nodes are excised from one patient for cancer diagnosis. The external dimensions of our prototype device are sufficiently small for benchtop use. A dedicated holder for glass slides with specimens was designed and fabricated. A typical lymph node specimen fits inside an area of 32 × 32 mm² on a glass slide, and 32 mm corresponds to the width of commonly used glass slide. Furthermore, the consumption of antibody-labeled magnetic beads for immunostaining was minimized via preliminary experiments, while that of formalin was also minimized for reducing chemical waste. Figure 1 depicts the automated immunostaining process. The total time for the completion of all automated processes from the injection of magnetic beads in the sample holder to the washing out of residual beads took 10 minutes.

The mechanical system of the device include three components for three different functions, including the injection of magnetic beads and chemical solutions on the specimen, application of magnetic force for immunostaining, and washing out of residual magnetic beads from normal cells. These components were separately developed and tested based on manually performed immunostaining processes. The rest of the prototype system was fabricated after developing these three mechanical components.

FABRICATION OF PROTOTYPE FOR THE MAGNETIC IMMUNOSTAINING DEVICE

As shown in Figure 1, the automated processes for the magnetic immunostaining device consist of injection of skim milk solution on the specimen and subsequent occurrence of the blocking reaction for 5 minutes, ejection of the skim milk solution, injection of the fluorescent magnetic beads on the specimen followed by magnetically-facilitated antigen-antibody reaction, and finally washing out of the residual beads. In particular, the prototype was composed of the following units (a) to (e), which have been described below:
### TABLE I. Protocol and conditions for automated immunostaining using antibody-labeled fluorescent magnetic beads.

| Immunostaining processes          | Materials | Conditions, amounts, and methods | Time          |
|-----------------------------------|-----------|---------------------------------|---------------|
| Infusion of formalin              |           |                                 |               |
| Fixation                          | 10% neutral buffered formalin | Dipping the specimen in solution | 5 minutes     |
| Washing out formalin              |           | Flowing water                    | 15 minutes    |
| Preparation of blocking solution  | 4% skim milk in PBS solution | Mixing skim milk and PBS solution |               |
| Blocking                          | 4% skim milk in PBS solution | Dipping the specimen in skim milk solution | 5 minutes     |
| Injection of beads                | Antibody-labeled fluorescent magnetic beads | Pouring beads in solution on tissue specimen | < 1 minute    |
| Antigen-antibody reaction         | Cylindrical permanent magnet | Placing magnet below specimen    | 1 minute      |
| Pre-wash                          | PBS solution | Pouring PBS solution on specimen using pipette | ...           |
| Washing out                       | Washing with magnetic force | Placing magnet above specimen    | 1 minute      |
| Post-wash                         | PBS solution | Pouring PBS solution on specimen using pipette | ...           |

**FIG. 1. Automated magnetic immunostaining.**

(a) Pipette:
The chemical solutions, including blocking solution and magnetic beads, were injected onto the specimens through a commercially available, electronically controlled, semi-automatic pipette. The pipette can be moved through horizontal (x-axis) and vertical (z-axis) guides so that it can be positioned above each specimen. The motions in x and z axes were realized via the use of belt and trapezoidal thread mechanisms, respectively, which were driven using a step motor. In particular, the mechanical switch on the pipette was operated via a push rod that was driven by the step motor too; a worm gear was used to generate linear motion of the push rod owing to the rotation of the step motor. Positive and negative pressure application was controlled through this switch.

(b) Glass slide holder:
The glass slide holder housed five glass slides arrayed in the x direction. The angle of the holder could be changed from 0° to 60° to enable ejection of the solutions. The tilts in the holder were realized via the belt thread mechanism using a step motor.

(c) Magnets and magnet holder:
The permanent magnets used in the device were positioned below the glass slides to apply a downward magnetic force on the magnetic beads. Owing to the applied magnetic...
force, the density of magnetic beads increased on the surface of the specimens, thus facilitating antigen-antibody reactions. In particular, five neodymium cylindrical magnets arrayed in the x-direction were placed on the magnet holder that could be moved in the y-direction such that the magnets can be positioned beneath the glass slides for the reaction process to occur. The magnets were oscillated in the y-direction during the reaction using a dedicated drive unit to homogenize the magnetic force applied over the specimen.

(d) Wash out shower:

The prototype was equipped with a 500-ml reservoir filled with phosphate-buffered saline (PBS) solution. The PBS solution was poured onto the glass slide holder through a dumper using a pump for absorbing pulsation of flow.

(e) Electronics:

Motor drivers, controllers, and power supply units were placed in the upper back side of the fabricated prototype device to ensure that they were not in the vicinity of the liquids in the device.

Figure 2 shows photographs of the exterior of the prototype device, while those of the pipette, magnet holder, and glass slide holder are shown in Figure 3.

The area of the lymph node specimen placed on the glass slide was $36 \times 18 \text{ mm}^2$. To ensure homogenous application of magnetic force on the magnetic beads distributed on the slide, a cylindrical magnet of diameter $\phi 16 \text{ mm}$ was oscillated with a stroke of 10 mm during the reaction. This mechanism allowed us to use smaller magnets in our device.

Figure 3. Components of the magnetic immunostaining device prototype. (a) Pipette for providing solutions, (b) permanent magnet array for applying magnetic force on magnetic beads, (c) glass slide holder, and (d) washing out nozzle.
to seal the chamber. The inner width of chamber was 18 mm. For washing out the beads after completion of the reaction, PBS solution was supplied to the chamber through a shower nozzle that had three outlets of diameter φ1.4 mm for each slide. The PBS solution was pumped with a sufficiently large flow rate for washing out.

**EVALUATION OF THE PROTOTYPE DEVICE**

Magnetic immunostaining of tissue samples containing cancer cells was performed using the prototype device. A431 (epithelioid histiocyte, highly expressing epidermal growth factor receptor: EGFR) or H69 (lung cancer cells, no EGFR expression) cells were injected in nude mice, and excised when their tumors grew to diameters of about 5 mm. These samples with and without the expression provided positive and negative controls of immunostaining, respectively. Frozen sections were then made from the excised tumors. Figure 4 shows the results of immunostaining using magnetic beads with anti-EGFR antibodies. The cancer tissue expressing EGFR (upper samples in the photographs) caused the beads to express red fluorescence; in contrast, the cancer tissue without EGFR expression (lower samples) exhibited no accumulation of magnetic beads. Furthermore, blue fluorescence was generated by 4′,6-diamidino-2-phenylindole (DAPI), which was used to stain cell nuclei. The five samples that were processed simultaneously all showed quite similar staining results; moreover, the reaction process was completed in just 1 minute. Thus, these results demonstrate that our approach enabled magnetic immunostaining in a relatively shorter time that was clinically viable for intraoperative diagnoses; in addition, it indicated that the designed processing conditions were feasible and suitable. Background fluorescence was also sufficiently low, indicating that the antibody had high selectivity towards the target marker and the washing out conditions were suitable. As future work, we might perform a clinical study using human lymph node samples to demonstrate the clinical effectiveness of our proposed device.

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