デジタル正立顕微鏡を用いた複数免疫染色標本の位置合わせ機能の開発†

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Position Alignment Function Development of Multiple Immunostained Specimens using a Digital Imaging Microscope

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病理診断では病変部位から細胞や組織のサンプルを採取し，薄くスライスして染色を施すことで病理標本を得て，病理医が顕微鏡を用いて細胞や組織の形態を観察することで，病気の原因や進行状況を明らかにする．特定のたんぱく質やその他の物質を抗体を用いて特異的に染色する免疫染色では，組織のサンプルを連続に薄くスライスすることで近接した位置の連続標本を得て，各標本 1 枚ずつに 1 種類の染色を施し，総計 3 ～20枚程度の免疫染色標本を観察して診断に供する．さらに各標本で同じ位置を顕微鏡で観察しながら探索して結果を記憶する必要があり，病理医の負担となっている．

デジタル正立顕微鏡では標本全体のデジタル画像を取得可能である．標本全体の画像を用いて標本間の位置ずれを自動検出し，同じ位置を自動探索する位置合わせ機能を開発した．さらに各標本の同一位置における拡大画像をモニターに並べて表示する機能を開発することで，顕微鏡観察時の探索や記憶といった病理医の負担軽減を目指した．

本稿では，複数免疫染色標本の位置合わせ機能に関して，デジタル正立顕微鏡の特徴及びデジタル画像を用いた位置合わせ方法について解説する．

In pathological diagnosis, pathologists routinely examine stained cell or tissue samples, extracted from lesion sites, to identify disease causes and assess progression. This process often involves immunohistochemical staining, where antibodies are used for selectively staining specific proteins or other substances within the samples. However, this approach can be demanding for pathologists as locations must be consistently observed and the results from identical locations should be memorized across multiple specimens.

To mitigate this challenge, we leveraged digital imaging microscope capable of capturing digital images of the entire specimen in a single shot. We developed a function that uses these images to detect any positional shift between the specimens automatically and, subsequently, automatically search for identical locations for alignment. Furthermore, we aimed at alleviating the burden on pathologists during microscopic observation by developing a feature that displays microscopic images of higher magnification from identical positions across different specimens side by side on a monitor.

In this study, we present digital imaging microscopic features and the alignment approach using digital images related to the alignment function for multiple immunostained specimens.

Key words 顕微鏡，位置合わせ，自動化，病理切片，デジタルパソロジー
microscope, alignment, automation, pathological specimen, digital pathology

1 Introduction

Pathological diagnosis entails cell or tissue sampling from the affected area, which are then thinly sliced and stained to prepare pathological specimens. Pathologists then use a microscope to observe the morphology of the cells or tissue and clarify the cause and progression of the disease. Developments of immunohistochemistry techniques that use antibodies to selectively stain specific proteins and other substances, have enabled a more detailed understanding of various pathological conditions and more precise diagnoses [1]. Immunostaining involves the staining of 3–20 or more

† This article is not intended to advertise any specific medical devices.

different proteins and other substances, which requires the same number of pathological specimens to be prepared. To do so, tissue samples are sliced in succession to obtain consecutive specimens at the same tissue coordinates, and each specimen is stained with a different immunostain for observation. However, the task of observing the same tissue position for each specimen and recording the results from memory imposes a large burden on pathologists.

Nikon has developed a digital upright microscope that does not require an eyepiece and allows the observation to be viewed on a display screen. This digital upright microscope acquires images of the entire specimen in a single shot, and we have developed an alignment function that automatically searches for the same position between different specimens using the acquired images. An additional function has also been developed to display the magnified microscope images of the same position of each specimen side by side on a monitor, with the aim of reducing the burden imposed on pathologists.

In this paper, we explain the alignment function of multiple immunostained specimens using this digital upright microscope, with a focus on the alignment method using digital images.

2 Uses and Challenges of Immunostained Specimens

Immunostaining, which selectively stains specific proteins and other substances, has contributed to the development of medical research and diagnosis / treatment. The basic principle of immunostaining is the use of specific bonds formed between antibodies and antigens. Antibodies have a high affinity towards specific antigens, so they can selectively target antigens. Immunostaining is used in many fields owing to its ability to clearly determine the presence and location of specific proteins. Immunostaining has become a particularly indispensable technique in medical applications such as disease diagnosis, treatment effectiveness predictions, and new drug development.

Increasing the number of proteins that can be identified by immunostaining in medical research and pathological diagnosis could lead to the elucidation of new mechanisms and improved diagnostic accuracy. Below, we describe the outline of the process from specimen preparation to evaluation in pathological diagnosis and medical research, as well as the associated issues (underlined parts A–D).

■ Specimen preparation (Fig. 1)

① The sample extracted from the target organ is fixed and embedded in paraffin to prepare a block.

② Slices that are cut to a thickness of about 4 μm are attached to a glass slide (block is cut into thin slices and about 3–20 slices are prepared).

③ The slices are stained on the glass slide (HE staining, immunostaining with Ki67, CD3, etc.).

④ The slices are protected using a cover glass and mounting medium.

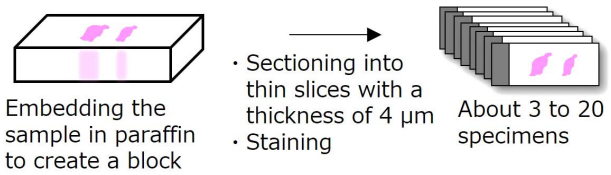

Fig. 1 Sample preparation

■ Specimen observation and evaluation (Fig. 2)

The following process is usually conducted using an upright microscope.

① Observe the HE-stained specimen and determine 3–5 points of interest. At this time, the observer remembers the position and appearance of the points of interest (A).

② The first immunostained specimen is observed. Based on the position and appearance that were memorized in the HE stain, the observer makes observation by looking for the same position (B). The findings (e.g., presence of target proteins) are memorized (C).

③ The second immunostained specimen is observed. Based on the position and appearance that were memorized in the HE stain, the observer makes observation by looking for the same position (B). The findings (e.g., presence of target proteins) are memorized (C).

④ The aforementioned pattern of observations of immunostained specimens is repetitively conducted on up to about 20 specimens, and the results are analyzed. Given the

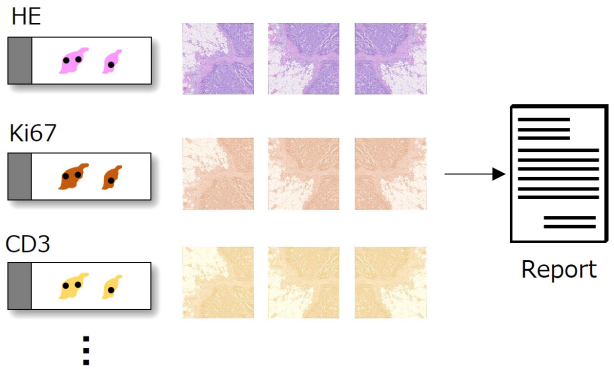

Fig. 2 Observation and evaluation of the specimens with HE staining and immunostaining

difficulty of memorizing all the findings at once, a report could be written for every few specimens, for example. Observers who would like to confirm again can re-observe the specimen (D).

This heavily memory-reliant type of specimen observation and evaluation method is repeated on a daily basis in pathological diagnosis, which imposes a heavy burden on pathologists.

3 Alignment Function using Digital Upright Microscope

The specimen is set on the digital upright microscope, and the load button is pressed to take a macro image that gives an overview of the entire specimen. When the specimen is placed at the observation position, the image obtained through the objective lens (micro image) is displayed live on the display, and the macro image is also displayed simultaneously (Fig. 3). The system can memorize the observation position of the macro image, and the micro image at the observation position can be saved.

We devised a method that uses the macro image that is acquired when loading specimens into the digital upright microscope to detect and correct the misalignment of multiple specimens, enabling the exploration and display of the same locations. This method achieves the system workflow shown in Fig. 4. Each step is explained below.

Fig. 3 Display of digital upright microscope
(Center image is micro image (top) and macro image (bottom)

①–②: Acquisition of macro image of first specimen

The observer sets and loads the first specimen into the digital upright microscope. The system acquires a macro image of the first specimen (Fig. 5). Usually, HE stain is

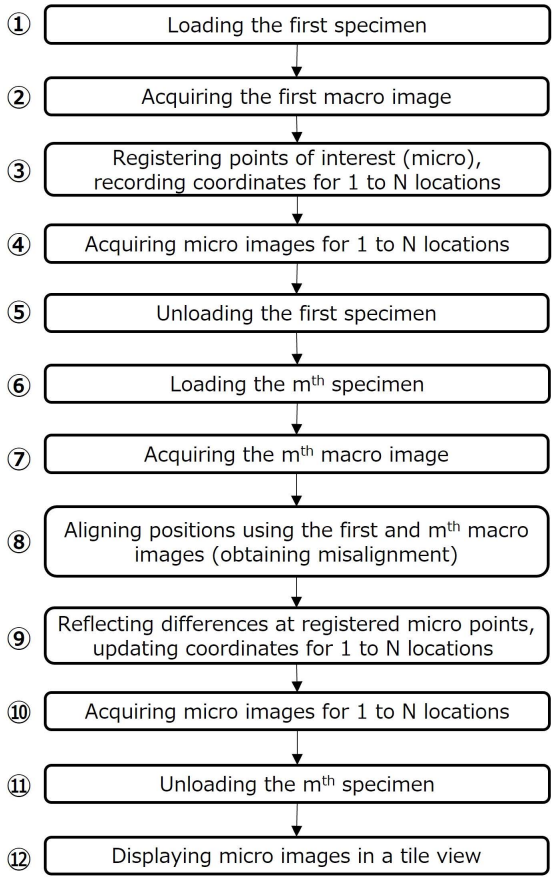

Fig. 4 Alignment function workflow overview

selected for the first specimen to understand the tissue morphology.

③–④: Registration of points of interest of first specimen

The observer visually checks the micro image and registers the points of interest in the first specimen in the system. The system captures and stores the micro images of the registered points (Fig. 5). Multiple points of interest, usually around 3–5 points are often registered (up to a maximum of 12 points can be registered).

⑤: Unloading of first specimen

The observer unloads the first specimen.

⑥–⑦: Acquisition of macro image of m^{th} specimen

The observer sets and loads the m^{th} specimen into the digital upright microscope. The system acquires a macro image of the m^{th} specimen (Fig. 5). Immunostained specimens are often selected for the second and subsequent images. Up to approximately 20 immunostained specimens are prepared in pathological observation.

⑧ Alignment using macro images

The system aligns the first and m^{th} specimens using the

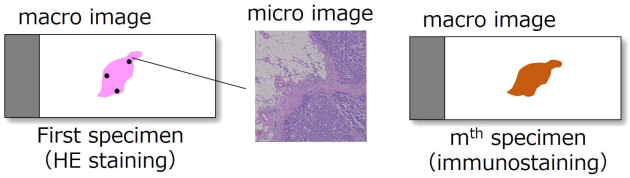

Fig. 5 Macro and micro images

macro images (Fig. 6). The alignment algorithm uses the Scale Invariant Feature Transform (SIFT) [2] to calculate the positional and angular shifts (ΔXY, Δθ) between the macro images. Details on SIFT are presented in Section 4.

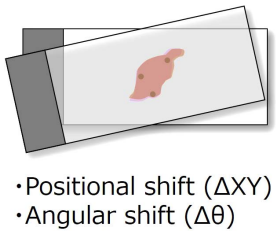

Fig. 6 Detection of extent of misalignment in macro images using SIFT

⑨–⑩: Acquisition of micro image of m^{th} specimen

The system reflects the positional shift ΔXY obtained in step ⑧ to the coordinates of the point of interest in the first specimen in step ③, which allows acquisition and updating of the coordinates of the same point of interest in the m^{th} specimen. The system acquires a micro image at the new coordinates and stores the rotated image that reflects the angular shift Δθ. Steps ⑥–⑩ are repeated for the second to m^{th} specimens to acquire a micro image of the same point of interest in each specimen.

As shown in Fig. 7, the first and m^{th} micro images are in the same position, so their morphology is similar, and the m^{th} micro image is recorded as a rotated image according to the extent of rotational misalignment.

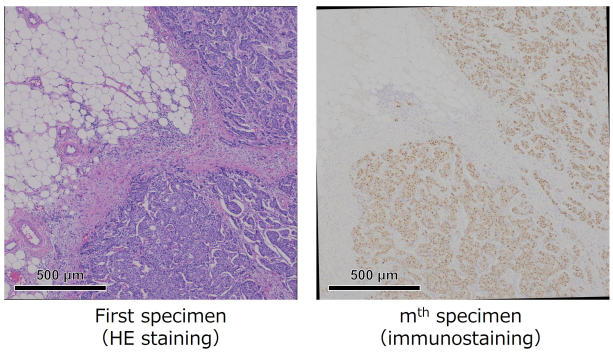

Fig. 7 First and m^{th} micro images

⑪: Unloading of m^{th} specimen

The observer unloads the m^{th} specimen.

⑫: Display of micro images in a tile view

The observer selects up to 10 micro images from all the recorded micro images and displays them in a tile view. The system displays micro images of the same point of interest for each selected specimen in a tile view on the display (Fig. 8).

When any of the images displayed side by side are zoomed in or out or moved laterally in the XY direction by the observer, then all the images move in tandem (Fig. 8). The observer can compare the details of each point while zooming in on the images.

Fig. 8 Display of micro images in a tile view
(Top: standard, bottom: digital zoom)

The above workflow of the alignment function of the digital upright microscope provides solutions to the problems of HE stained and multiple immunostained specimens, as shown in Table 1. Particularly, it is expected to reduce the burden imposed on pathologists in pathological observation.

Table 1 Problems and solutions for HE stained and multiple immunostained specimens

Problem/ Solution	Content
Problem A	The observer memorizes the position and appearance.
Solution A	The system memorizes the position and appearance.
Problem B	The observer searches for the same position.
Solution B	The system searches for the same position.
Problem C	The observer memorizes findings from the observed images.
Solution C	The system memorizes the images.
Problem D	The observer re-examines the specimen.
Solution D	The system displays the images in a tile view.

4 Alignment Function Algorithm and Accuracy Evaluation

SIFT was adopted as the alignment algorithm for the digital upright microscope. SIFT is a feature detection method for image recognition that was published by David G. Lowe in 1999 [2]. The method is widely used for image alignment and object recognition because it has the ability to detect features that are strongly invariant to changes in scale, rotation, viewpoint, and lighting. The algorithm for the alignment function in sample images is described below.

First, feature points of the macro image are extracted. As shown in Fig. 9, the macro image is smoothed and a difference of Gaussian (DoG) image is created, which is the difference between images with the scale (blur intensity) changed. A comparison is made between the pixel value of target and the pixel values of 26 neighboring pixels to identify those pixels with extreme DoG values, including the scale direction. Pixels with extreme DoG values exhibit a remarkable change in contrast and are expected to contain a high volume of information. The above process is used to extract pixels with a high volume of information as candidates for feature points, and top feature points are used for alignment.

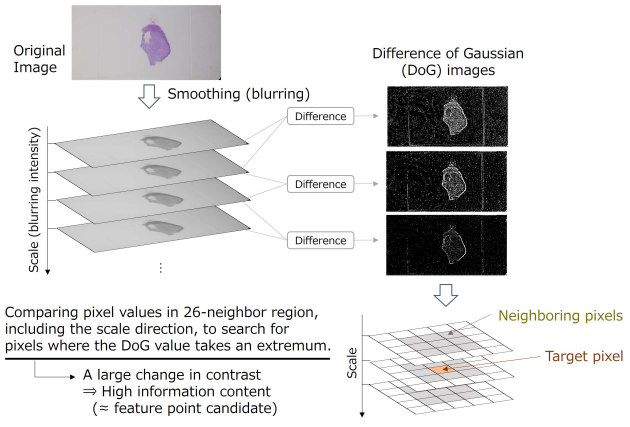

Fig. 9 Extraction of feature points in specimen images using SIFT

Next, the feature values of the feature points are calculated. As shown in Fig. 10, the area around the feature points is divided into 4 × 4 blocks, and the gradient intensity of brightness is calculated in the eight directions for each block. The number of blocks and gradient intensity are used to create a feature value as 128-dimensional gradient intensity information.

Finally, the feature points are matched, and the positional and angular shifts (ΔXY, Δθ) between the two images are calculated. As shown in Fig. 11, the Euclidean distance between one feature value of HE stained specimen and all

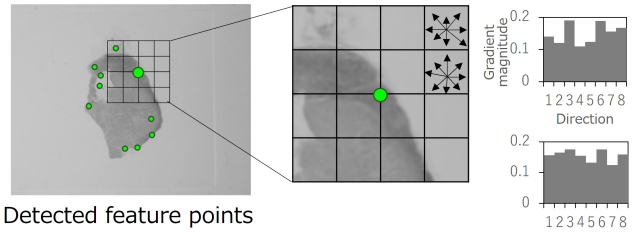

Fig. 10 Calculation of feature values of specimen images using SIFT

feature values of immunostained specimen is calculated. The feature point with the lowest Euclidean distance is determined as the corresponding feature point. The lowest Euclidean distance means that the difference is the lowest in comparison with the aforementioned 128-dimensional gradient intensity.

In cases where there are at least two corresponding feature points, the positional and angular shifts (ΔXY, Δθ) between the two images are obtained by overlaying the images so that these feature points match.

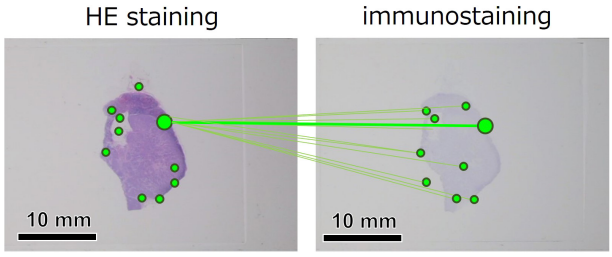

Fig. 11 Matching feature points of specimen images using SIFT

In the alignment algorithm for the digital upright microscope, the parameters for each of the aforementioned steps were optimized using numerous specimens. Evaluation of the accuracy of this alignment function is explained below.

For accuracy evaluation, the images obtained through the 10x objective lens of the digital upright microscope were used. The micro images of the two specimens obtained after alignment were manually superimposed by visual inspection so that the fine structures of the images were completely overlapped. As shown in Fig. 12, the overlapping area (blue diagonal line) was divided by the total area of the micro images (green frame) to determine the accuracy index. This index ranges from 0 to 1, and an index value closer to 1 implies higher alignment accuracy.

A set of 22 specimen pairs was prepared from 44 specimens, and the alignment accuracy of the micro images obtained by the alignment function was evaluated. An alignment accuracy of approximately 0.95 was attained, as shown in the box-and-whisker plot in Fig. 12. It is worth mentioning here that specimens have a wide range of shapes and colors, and we will continue to investigate ways to improve the accu-

racy and robustness so that this method can be utilized for such specimens as well.

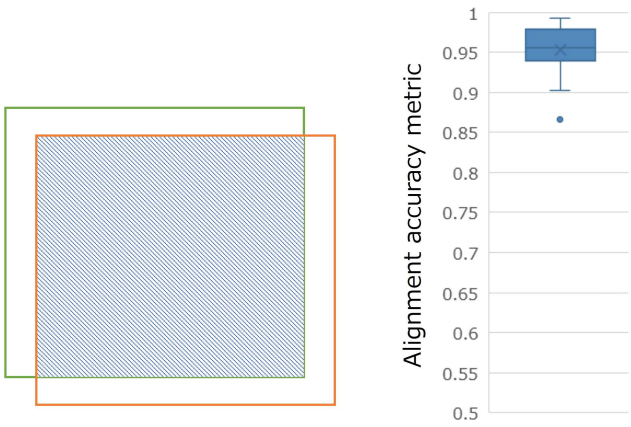

Fig. 12 Alignment accuracy evaluation index (left) and results (right)

5 Conclusion

The digital upright microscope can obtain macro images of the entire specimen and enlarged micro images of the point of interest. Through this study, we demonstrated the possibility of accurately and quickly extracting micro images on multiple immunostained specimens at the same site on the tissue of interest to the observer using the alignment function with the macro images. Further, the alignment algorithm has also been explained in detail. The observation support provided by the alignment function is expected to reduce the burden imposed on observers. In future, we will further improve the accuracy and robustness of the alignment function to increase the accuracy of the observations and evaluation of multiple immunostained specimens for smaller lesions for which there is current demand in the field. We expect this study to make a positive contribution to the development of medical research and pathological diagnosis.

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