Fluorescent Staining for Detecting Larvae of the Japanese Scallops Mizuhopecten Yessoensis*

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In farming Japanese scallops *Mizuhopecten yessoensis*, larvae investigation and efficient seed collection are very important processes. During yearly larvae investigation from May to June, fishery managers count the number of larvae and measure the shell size. Identification technology has been developed using immunostaining to mark scallops. The resulting fluorescence images contain both fluorescence-stained and autofluorescent larvae. However, there is currently no technology for evaluating these images automatically, so the experts have to perform these tasks manually.

Our overall aim is to develop a system for automatically measuring the number and size of scallops. In this paper, we propose a method for detecting fluorescence-stained larva and measuring the items in the fluorescence images. We will present the experimental results and discuss the effectiveness of the proposed method. We will also introduce an application that facilitates measurement of scallops.

1. Introduction

Larvae investigation and efficient seed collection are very important parts of farming Japanese scallops. Seed collection is a process of selecting the seeds of scallop larvae between the floating-larva period and the bottom-landing period. In order to estimate the growth of scallop larvae, we must distinguish them from other bivalve larvae that are present in the field [1].

There are several periods in the life cycle of the bivalves: floating-larva, bottom-landing, young-clam, and adult clam [1]. Larval development can be classified into the following four stages from the viewpoint of the umbonal prominence: D-shaped, early umbo, umbo, and fully-grown [2]. The optimum date for the seed collection can be estimated from the shell length (SL) and shell height (SH). The intermediate breeding larvae are stocked in the sea.

In Hokkaido, Japan, larvae investigation involves measuring the number of larvae, SL, and SH from May to June every year. The samples are collected using plankton nets at the depth from 5 to 30 meters. The SL and SH of scallop larvae are measured using microscope images. The investigation results are publicly announced in each region [3].

However, it is almost impossible to visually recognize scallop larvae from other bivalve larvae. Shimizu et al. developed an immunostaining method to mark scallop larvae (*Mizuhopecten yessoensis*) [4] in a sample. Fluorescent images can be captured by using a fluorescent staining technique and a fluorescence microscope. A fluorescence image may contain both fluorescence-stained and autofluorescence larvae. Currently, there is no technology for automatically evaluating the data from these images, so manual measurement by experts is required.

Lojk et al. proposed a method for counting the cells in a fluorescence microscope image [5]. Delves et al. presented the development of a method for accurately and consistently determining oocyst burdens on mosquito’s midgut using green fluorescent protein (GFP)-expressing malarial parasite *Plasmodium berghei* and a custom-written macro for ImageJ [6]. Thomas et al. measured the distribution of pearl oyster (*Pinctada Margaritifera*) larvae using NIvision software [7]. However, these methods do not distinguish fluorescence-stained cells from autofluorescent.

Our overall aim is to develop a system for automatically measuring the number and size of scallops. We also need to identify fluorescence-stained and aut-
of fluorescence larvae in the single-wavelength fluorescence images. In this paper, we propose a method for detecting only fluorescence-stained larvae and measuring the investigation items from fluorescence images. We will present the experimental results and discuss the effectiveness of the method using a sample. We will also introduce an application to facilitate the measuring of scallops.

In the next section, we describe larvae investigation, fluorescence imaging, and design considerations for detecting fluorescence-stained larvae in fluorescence images. We present the details of our proposed method in Chapter 3. In Chapter 4, we discuss the parameters set from the preliminary experiments. We explain the experimental method and results obtained from applying our method to fluorescence images and discuss its effectiveness in Chapter 5.

2. Design Consideration

2.1 Larvae Investigation

In fishery resource management and marine biology, various bivalve larvae are investigated, for example scallops (Mizuhopecten yessoensis and Mytilus edulis) [2, 8], pearl oysters (Pinctada margaritifera) [7], and surfclams (Spisula solidissima Dillwyn) [9].

In larva investigation of the aquaculture industry in Hokkaido, Japan, the measurement targets are scallops (Mizuhopecten yessoensis). Mytilus is a cosmopolitan genus of medium to large-sized edible, mainly saltwater mussels, and marine bivalve mollusks in the Mytilidae family is the same as scallops [10]. Two mytilid species, Mytilus galloprovincialis and M. trossulus, are distributed around the coastal areas of Hokkaido. For seed collection, we need to collect scallop larvae without collecting too many mytilus larvae.

The larvae investigation process is as follows.

1. Getting the samples of seawater in target areas
2. Staining scallops in a sample with fluorescence markers
3. Capturing fluorescence images
4. Counting and measuring scallops in fluorescence images
5. Aggregating data using spreadsheet software

The samples are collected in steps 1 and 2. In steps 3 to 5, fluorescence images are captured in the green or red wavelength ranges and the SL and SH are measured in the images. The optimum date of seed collection is assessed from these data.

2.2 Fluorescence Staining Technique

Immunostaining is a general term in biochemistry that applies to any use of an antibody-based method to detect a specific protein in a sample. Fluorescence staining with immunostaining involves fluorescent dyes being recognized as antigens. A fluorescence image is captured at an optimum wavelength for each fluorescent dye. Usually, images from larval investigation include autofluorescence larvae that should not be counted in the investigation. One of the methods to avoid contamination with autofluorescence larvae in the investigation images is to use a particular wave length that autofluorescence larvae do not emit. However, to apply that wave length to the larvae investigation, expensive dyes or expensive spectra analyzers would be required. We developed an inexpensive method for detecting fluorescence-stained larvae in the images that include autofluorescence larvae.

Fig. 1 shows digital photographs in the green and red wavelength ranges (1392 × 1040 pixels in 24-bit color). We stained only the scallop larvae in the two wavelength ranges because of the shapes of scallops. The fluorescence images were captured using a 50x microscope at 1.724µm/pixel. The fluorescent dyes were AlexaFluor488 (wavelength laser: 488nm) and AlexaFluor594 (wavelength laser: 561–594nm). Figs. 2 and 3 show fluorescence-stained and autofluorescence larvae. The fluorescence-stained larva areas are uniformly luminescent and have clear contours, as shown in Figs. 2(a), (b) and Figs. 3(a), (b). The autofluorescence larva areas are unevenly luminescent and have blurry contours, as shown in Fig. 2(c) and Fig. 3(c). These areas are mixed and overlapping. There are also larvae with non-uniform dyeing, i.e., extremely luminous or dark.

2.3 Design of Automatic Measuring System

Fig. 4 shows a system that we are developing for measuring the SL and SH of scallops. The system involves steps 3 to 5 in Sect. 2.1, i.e., capturing fluorescence images and measuring the SL and SH of larvae.

Fluorescence images are automatically captured using an electric XY stage, a charge-coupled device (CCD) camera, and a PC (Fig. 4). Scallops larvae are counted from the fluorescence images. Our proposed method detects fluorescence-stained larvae and measures the SL and SH. These data are exported as a comma-separated values (CSV) file compatible with spreadsheet software.

We have to assume several scenarios of fluorescence imaging because the distributions of scallop larvae are unknown. A fluorescence image does not always contain both fluorescence-stained and autofluorescence larvae. Moreover, in some cases all areas in an image may contain fluorescence-stained or autofluorescence larvae.

Fig. 5 shows our proposed method for detecting fluorescence-stained larvae. First, a wavelength range is differentiated using a fluorescence image (Sect. 3.1). Next, the candidate areas are detected using the Hough transform (Sect. 3.2). The target regions are extracted from the candidate areas using seeded region growing (Sect. 3.3). Finally, the fluorescence-stained larvae are detected by threshold processing using certain features (Sects. 3.4–3.6).
3. Proposed Method for Detecting Fluorescence-stained Larvae

3.1 Differentiation of Wavelength Range

Fluorescence images are captured in the wavelength ranges of green or red. A wavelength range is differentiated as follows. The sum of the RGB elements in an image $I$ is denoted as $S(ch)(ch = R,G,B)$. A wavelength range is defined as

$$\text{Color} = \begin{cases} \text{Green} & (G = \arg \max_{ch=R,G,B} \{S(ch)\}) \\ \text{Red} & (R = \arg \max_{ch=R,G,B} \{S(ch)\}). \end{cases}$$ (1)

In this paper, we use only the $\arg \max \{S(ch)\}$ element of a fluorescence image.

3.2 Detection of Candidate Areas

Scallop larvae are shaped like a circle [2,10]. In a fluorescence image, there are many overlapping areas due to larval density.

We define the shape of larvae as a circle and extract it using the Hough transform. This method is effective against noise and can be set at arbitrary sizes. The features used to detect a circle are edge magnitude and direction. The extracted circle region is defined as the candidate area $C$. The size regions of the larva are finished surveying [2] and the actual scale can be converted to an image scale. Therefore, we can set the diameter to 100–330 $\mu$m.

Fig. 6 shows the candidate areas detected with the Hough transform. The fluorescence-stained and autofluorescence larvae were accurately detected, but some autofluorescence larvae areas were not detected (Fig. 6). We do not need to detect excessively dark regions by using the edge features because these areas obviously do not contain any fluorescence-stained larva.

3.3 Binarization

In this section, the object images are binarized to the detailed areas. However, there are not always fluorescent-stained and autofluorescence larvae in fluorescence images. We must assume that all areas either contain or do not contain fluorescence-stained larvae. Binarization is difficult using dynamic threshold processing and contrast correction.

Our method binarizes a fluorescence image using improved seeded region growing, which is a region segmentation method proposed by Kiyono et al. [11]. This method can be applied to images in which the areas are unevenly luminescent and have blurry contours. Moreover, we adapted to a multi-class extended this method.

We will now briefly explain the principle of improved seeded region growing [11]. An image is denoted as a graph $G = \langle V,E \rangle$, where $V$ is a set of nodes and $E$ is a set of edges. A set of nodes $V$ equivalent to a pixel set. We defined the foregrounds
Fig. 4 System for automatically measuring scallop larvae

Fig. 5 Proposed method for detecting fluorescence-stained larvae

as $FG$, backgrounds as $BG$, unknown regions as $N$, and sets of neighbor pixels as $Q$. A set of nodes is denoted as $FG_0 \subset V, BG_0 \subset V (FG_0 \cap BG_0 = \emptyset)$. Here, $FG_0$ and $BG_0$ are the initial foregrounds and backgrounds. Variables are first initialized as follows.

$$FG_0 = FG_0$$
$$BG_0 = BG_0$$
$$N = V - (FG \cup BG)$$
$$Q = \{ t \in N | e(t) \cap (FG \cup BG) \neq \emptyset \},$$

where $e(t)$ is the source node of neighbor $t$. Next, the segmented node $t_m = \arg \min_{t \in Q} CST(t)$ is selected. Here, $CST(t)$ is the cost function. The node $t_m$ is segmented $S(s)$ ($FG$ or $BG$), which belongs to the source node $s \in e(t_m)$. The $Q$ is updated as

$$Q = Q \setminus \{ t_m \}$$

where $n(t)$ is the neighbor and the unknown node is $t$. Determine whether $Q$ is $\emptyset$. If not, repeat the procedure (more information is available in [11]).

In this paper, the $FG_0$ and $BG_0$ are given as follows. First, we define $FG_0$. The $FG$ of the fluorescence-stained and autofluorescence larvae are brighter than the $BG$. Therefore, $FG_0$ are defined as the pixels in the top $RT$ of the candidate area $C$. This process is done in all $Cs$. Next, we define $BG_0$. With improved seeded region growing, if the $BG$ contains a foreground, it affects the outcome of the segmentation. Therefore, we denote a circular region $NC$ as about $N$ times of the radius of the candidate area and define $BG_0$ as

$$Q = Q \cup \{ t \in n(t_m) \},$$

where $n(t)$ is the neighbor and the unknown node is $t$. Determine whether $Q$ is $\emptyset$. If not, repeat the procedure (more information is available in [11]).
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where \((x,y)\) is a coordinate and \(L(x,y)\) is a brightness value. In this paper, we set parameter \(N=2\) and discuss parameter \(RT\) in Sect. 4.1. The seeds are set as the obtained \(FG_0\) and \(BG_0\) and the fluorescence image is binarized using improved seeded region growing. Additionally, we corresponded to the foreground area of the candidate area number.

3.4 Measuring Shell Length and Height

Larvae investigation requires the measuring of the SL and SH of larvae from fluorescence images. In clams, the SL is measured from the siphon edge across the valve to the longest point, and and the SH is measured over the umbo across the widest spot. Maru summarized the morphological characteristics of observed larvae using SL and SH [2].

We propose to measure the SH and SL in digital images, which are defined as follows.

**SH** The minimum length of a line through two contour points and gravity point

**SL** A line perpendicular to the SH through the gravity point

Fig. 7 defines the SL and SH of a larva. The ratio of the SL to SH is roughly constant regardless of growth [2]. Shape is defined as

\[
F_{LH} = \frac{SH}{SL} \quad (0 < F_{LH} \leq 1).
\]  

3.5 Fluorescence

The fluorescence-stained larva areas are uniformly luminescent and have clear contours (Figs. 2(a) and (b) and Figs. 3(a) and (b)), but autofluorescence larva areas are unevenly luminescent and have blurry contours (Fig. 2(c) and Fig. 3(c)).

We discuss fluorescence using the edge magnitude. The candidate areas discussed in Sect. 3.3 are calculated from the contour points. Next, an edge magnitude image is obtained from the original image using the Sobel mask. Fig. 8 shows the edge magnitude images of fluorescence-stained and autofluorescence larvae. The edge magnitude on the contour points of the candidate area is high in the fluorescence stained larvae (Figs. 8(a) and (b)) and low in the autofluorescence larvae (Fig. 8(c)).

We define the fluorescence feature as follows. When the contour points \(P=P_1,P_2,...,P_N\) and the edge magnitude function is \(M\), fluorescence \(F_f\) is defined as

\[
F_f = \sum_{i=1}^{N} \frac{M(P_i)}{N}.
\]  

The higher the \(F_f\), the stronger the \(P=P_1,P_2,...,P_N\) and \(M\) of a candidate area.

3.6 Detection of Fluorescence-stained Larvae

The recognition equation \(D\) is defined as

\[
D(F_{LH},F_f) = \begin{cases} 
    Fluoro. & (Th_{LH} \leq F_{LH}, Th_f \leq F_f) \\
    Autofluoro. & (otherwise)
\end{cases}
\]  

where \(Th_{LH}\) is the threshold for \(F_{LH}\) and \(Th_f\) is the threshold for \(F_f\). We set \(Th_{LH}=0.7\) [2] and discuss \(Th_f\) in Sect. 4.2.

3.7 Application for Measuring Larvae

A system for automatically measuring larvae should be simple to use. Therefore, we developed an application that makes measuring larvae easy. The development environments were Microsoft Windows10 64bit, Microsoft Visual Studio Ultimate Visual C # 2013, Aforge.NET as the image processing library, and OpenCVSharp [12] of OpenCV wrapper for the .NET Framework platform.

Fig. 9 shows a screenshot of our application. The application was designed to be easy to use via a graphical user interface (GUI). It supports the selection of images and folders, automatically counts, converts scales, and outputs the measurement results to a CSV file. These tasks are required by fishery researchers.

4. Preliminary Experiments

4.1 Verification and Evaluation for Binarization

The extracted regions are used to seed for improved seeded region growing. We examined parameter \(RT\) to set the optimum values and evaluated the performance of our method.

We used four fluorescence images taken on 16 August 2014 and 27 September 2014: two images in
the green wavelength range and two in the red wavelength range. The four images contained 55 scallop larvae. Parameter \( RT \) was verified by the extraction rate of the fluorescence-stained larva region with a change value 0.0001–0.1 at an interval of 0.0001. For comparison, we used the binarization algorithm Otsu method [5] and the same four images.

Fig. 10 shows the preliminary experimental extraction results. When \( 0.011 \leq RT \leq 0.0175 \), the extraction rate was 100\%. Therefore, we set \( RT = 0.0144 \) because of the median value in this region.

Fig. 11 shows selected experimental results from the proposed method and Otsu method. All larvae were correctly extracted using the proposed method (Fig. 11(a)), but the arrowed larvae were extracted incorrectly using the Otsu method (Fig. 11(b)). The extraction rate of the Otsu method was 94.5\%(52/55).

The proposed method extracted the fluorescence-stained larva, but the Otsu method did not in certain cases, such as in areas containing extremely luminous larvae (dyeing unevenness). Additionally, Otsu method can not adapt depending on the ratio of the fluorescence-stained larva and the autofluorescence larva contained in the fluorescence microscope. We used shape and brightness in our method; therefore, it was effective.

4.2 Parameter Verification for Larva Detection

We examined the \( Th_f \) to set the optimum values. We used the same images discussed in Sect. 4.1.

The \( Th_f \) was verified from a receiver operating characteristic (ROC) curve of the detection rate when a change value \( 0 < Th_f \leq 1 \). Fig. 12 shows the ROC curve. When \( Th_f \) was 0.1215, AUC value was maximum 0.97. Therefore, we set \( Th_f = 0.1215 \).

5. Experiment

5.1 Method

We used 26 fluorescence images taken on 16 August 2014 and 27 September 2014: ten images in the green wavelength range and 16 in the red wavelength range. The images in the green wavelength range contained 123 larvae and those in the red wavelength range contained 185 scallop larvae. These images contained 276 autofluorescent larvae, determined by visual observation.

The evaluation methodology is defined as follows.
If a fluorescence-stained larva was correctly detected in all extracted areas, we determined the result to be true-positive (TP).

If a fluorescence-stained larva was not detected, we determined the result to be false-negative (FN). Furthermore, if a non-fluorescence area was detected incorrectly, we determined the result to be false-positive (FP). Other results were determined to be true-negative. Precision, recall, and F-measure are defined as

\[
\text{Precision} = \frac{TP}{TP + FP} \quad (12)
\]

\[
\text{Recall} = \frac{TP}{TP + FN} \quad (13)
\]

\[
F\text{- measure} = \frac{2 \cdot \text{Recall} \cdot \text{Precision}}{\text{Recall} + \text{Precision}} \quad (14)
\]

5.2 Results

Selected experimental results from the proposed method are shown in Fig. 13. Ten fluorescence-stained larvae were correctly detected and their SL and SH were measured. Therefore, the object located upper-left is a fluorescence-stained larva in Fig. 13, but it leans. A case does not measure such a thing by the sampling research, because we should measure the SL and the SH of the scallop.

These experimental results are also listed in Table 1. The precision, recall, and F-measure were 0.992, 0.967, and 0.979, respectively in the green wavelength range. In the red wavelength range, they were 0.957, 0.973, and 0.965, respectively.

5.3 Discussion

We developed a new method for measuring scallop larvae from fluorescence images using shape and fluorescence. The proposed method can be used for detecting with a high degree of accuracy fluorescence-stained larvae in fluorescence images containing both fluorescence-stained and autofluorescence larvae. The proposed method defines the shape features, such as the ratio of SL to SH, and fluorescence features of edge magnitude.

Table 1 shows that the results in the green wavelength range are more accurate than those in the red wavelength range. This is because the fluorescence images in the red wavelength range had a large variation in fluorescence intensity. Therefore, the proposed method is effective for larvae investigation.

Even though the accuracy was sufficient for measuring larvae, there were some errors. Fig. 14 shows sample images involving errors. The autofluorescence area was detected incorrectly from the images in Figs. 14(a) and (b). In Fig. 14 (a), this region was detected during the recognition process of the candidate area. In Fig. 14 (b), the bright region was detected when the detection process of the candidate areas involved the Hough transform. We may be able to address these errors using other features such as larvae morphology.

6. Conclusion

We proposed a method for detecting and measuring fluorescence-stained larvae in fluorescence images. We used two wavelength ranges to detect larvae;
fluorescence-stained larvae were detected by threshold processing using shape and fluorescence. The experimental results showed that our method is effective in detecting fluorescence-stained larvae. We also introduced an application to facilitate the measuring of larvae during larval investigation. In the proposed system, the accuracy of the statistic becomes higher as the number of samples increases, because it is possible to measure a large number of images. We are preparing to offer the proposed system to collaborative research institutes.

As future work, we plan to develop a fisheries system and a highly accurate estimation method for seed collection. Our method can be applied for other bivalve larvae [7–9]. We will also develop a method for detecting larvae from stereoscopic microscope images, because many research institutes have stereoscopic microscopes and can observe larvae without using fluorescent dyes.

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