Target Area Extraction Algorithm for the In Vivo Fluorescence Imaging of Small Animals

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ABSTRACT: Bio-optical imaging can noninvasively describe specific biochemical reaction events in small animals using endogenous or exogenous imaging reagents to label cells, proteins, or DNA. The fluorescence optical bio-imaging system excites the fluorescent group to a high energy state by excitation light and then generates emission light. However, many substances in the organism will also emit fluorescence after being excited by the excitation light, and the nonspecific fluorescence generated will affect the detection sensitivity. This paper designs and develops a set of high-level biosafety in vivo fluorescence imaging system for small animals suitable for virology research and proposes a target area extraction algorithm for fluorescence images. The fluorescence image target extraction algorithm first maps the nonlinear separation data in the low-dimensional space to the high-dimensional space. Then, based on the analysis of the characteristics of the fluorescent region, a method for discriminating the target fluorescent region based on the two-step entropy function is proposed, and the real target fluorescent region is obtained according to the set connected region. Based on the experiment of collecting and analyzing the in vivo fluorescent images of mice, it is verified that the proposed algorithm can automatically extract the target fluorescent region better than the classical linear model. It shows that the proposed algorithm is less affected by background fluorescence, and the estimated separated spectrum based on this method is closer to the real target spectrum.

INTRODUCTION

As a new type of optical imaging technology for the in vivo imaging of living animals, bio-optical imaging uses endogenous or exogenous imaging reagents to label cells, proteins, or DNA, thus noninvasively describing specific biochemical reactions in small animals. Due to the advantages of simple operation and intuitive response results, bio-optical imaging has become an ideal method for studying in vivo imaging of small animals and thus has been widely used in life science research. Using this kind of imaging technology, the interaction between fluorescently labeled cells or proteins can be directly observed in real time. Therefore, bio-optical imaging technology is widely used to study the occurrence and development of diseases, drug screening, gene expression, gene therapy effect evaluation, tumorigenesis, and metastasis evaluation.

In recent years, with the development of biology, in vivo imaging systems for small animals have become more and more widely used. As one of the most important instruments for live animal experiments, it can achieve real-time live imaging, no damage to animals, and long-term observation and monitoring. In vivo imaging systems could guarantee the normal physiological activities of the experimental animals as much as possible and avoid the interference and destruction of human factors, making it closest to the real pathological phenomenon. In contrast, animal dissection and pathological section experiments require the animal to be sacrificed first and then a series of mechanical cutting and chemical treatment of its organ tissues. This process may cause tissue damage and deformation, apoptosis, and necrosis during sampling and sample preparation, which destroys the true physiological state and produces false-positive or false-negative results, and causes misjudgment for wrong experimental conclusions.

At present, the in vivo imaging system mainly adopts two kinds of technologies: bioluminescence and fluorescence. The difference between these technologies is the labeling substance used. Bioluminescence is used to label cells or DNA with luciferase gene, and fluorescence technology uses small-molecule fluorescent reporter group (GFP, RFP, dyes, etc.) markers. The basic steps of bio-optical in vivo imaging can
be divided into fluorescent substance labeling, fluorescent imaging, analysis of fluorescent images, and further analysis of the obtained data. This technology is extremely sensitive to the detection of small tumor metastases, does not involve radioactive materials and methods, and is less harmful. It has been widely used in life sciences, medical research, and drug development.

The labeling substances used in the fluorescence technology mainly include green fluorescent protein (GFP), red fluorescent protein (DsRed), and other fluorescent reporter groups. It excites the fluorophore by excitation light to reach a high-energy state and then generates emitted light, which has the advantages of low cost and simple operation. Similar to the penetration of bioluminescence in animals, the penetration of red light in the body is much better than the penetration of blue and green lights, and near-infrared fluorescence is the best choice for observing physiological indicators. However, many substances in the organism will also emit fluorescence after being excited by the excitation light, and the nonspecific fluorescence generated will affect the detection sensitivity. Especially when the luminescent cells are buried deep inside the tissue, a higher energy excitation light source is needed, which also generates a strong background noise.

In view of the wide and uneven distribution of background fluorescence, researchers proposed methods to deal with background autofluorescence, including adding blue-shift background filters and non-negative matrix factorization. Although these methods can achieve background fluorescence removal, image acquisition equipment needs to add additional channels to collect background fluorescence. This requires some additional work, and sometimes the hardware conditions do not meet our requirements. In this paper, based on the original classic spectral separation model, a background fluorescence matrix is added. On the basis of no need for hardware acquisition or tissue-specific estimation of background fluorescence, a new linear spectral separation model based on background fluorescence reconstruction is proposed.

This paper designs and develops a set of high-level biosafety in vivo fluorescence imaging system for small animals suitable for virology research and proposes a target area extraction algorithm for fluorescence images. The core components of the fluorescence imaging system include a small animal safe bin for meeting biological safety requirements and a spectral fluorescence imaging device adapted to the safe bin. The advantage of this product is the use of spectral high-power semiconductor filters to expand the spectral imaging function, which can support spectral imaging to distinguish a variety of fluorescently labeled viruses. The inspection chamber and the imaging system are relatively independent and maintain a negative pressure, which is suitable for the requirements of biosafety laboratories, and the inspection chamber has a life-sustaining function and can anesthetize small animals to maintain a fixed posture for a long period of time. Based on the spectral separation model, a new linear spectral separation model based on background fluorescence reconstruction is proposed by introducing fluorescence matrix. The fluorescence image target extraction algorithm first maps the nonlinear separation data in the low-dimensional space to the high-dimensional space, and then based on the fluorescent region feature analysis, a target fluorescent region discrimination method based on the second-order gradient entropy function is proposed. The algorithm can obtain the real target fluorescence area according to the set connected area.

## RESULTS AND DISCUSSION

In this experiment, we injected five fluorophores into the bottom, middle, neck, abdomen, and underarms of the mouse. Among them, a fluorescent agent with a concentration of 1.5 μg/mL and a volume of 1 mL is injected into the bottom, middle part, and neck of the mouse; a fluorescent agent with a concentration of 150 ng/mL and a volume of 1 mL is injected into the mouse abdomen; and a fluorescent agent with a concentration of 15 ng/mL and a volume of 1 mL is injected into the underarms of the mouse. The injection depth is about 5 mm under the skin. Image information was collected 1 min after injection.

During the process image acquisition, the mouse was anesthetized with ether to ensure that the image acquisition position in the dark box was unchanged. The load-carrying platform of the dark box is set to keep warm to ensure that the mouse maintains a normal physiological state during anesthesia. Figure 1 shows the fluorophore injection locations and the fluorescence image of 509 nm emission light collected by 472 nm excitation light.

![Figure 1. Fluorophore injection location and concentration.](https://dx.doi.org/10.1021/acsomega/6b01733)

As shown in Figure 1, although the background fluorescence is relatively weak, it is widely distributed on the surface of the mouse.

The target area extraction algorithm is used for further analysis based on the acquisition of fluorescent images to extract clearer and more accurate fluorophore light-emitting areas. In order to compare the performance of the target region extraction algorithm proposed in this paper with other algorithms, Figure 2 shows the fluorescence regions obtained by using different target region extraction algorithms. Among them, Figure 2a adopts the algorithm, which is proposed in this paper with Gaussian kernel function as the kernel function, and the highlighted area in the figure is the target area. We show the results of the other algorithms using the classic models from Figure 2b–f: (b) nsNMF (c) NMFsc, (d) MCR-ALS, (e) CAMNS, and (f) nLCA-IVM. From these, we can see that the results of these methods all contain the effect of background fluorescence, making the results difficult to identify.

In the calculation process using the classic model, under normal circumstances, we can only get two fluorescent end-member images. If the background fluorescence is regarded as a terminal element, there are three fluorescent terminal elements for our experimental data, but in the calculation results, the image result of the third terminal element is often the same or similar to the other two.

Before the fluorescent labeling of mice, we used the same excitation light to irradiate the mice and collected the original tissue spontaneous fluorescence, that is, background fluorescence, and the background fluorescence matrix \( B \) is
established. After the fluorescence image acquisition, we use the labeled fluorescence image to subtract the background fluorescence matrix $B$, and get the “target spectrum”, which is close to the reality. Then, we calculate the joint correlation coefficient between the separated spectrum of estimated fluorescence image and the target spectrum and evaluate the performance of the proposed algorithm. According to the correlation coefficient calculation method defined in formula 1, we calculate the joint correlation coefficient for the target spectrum and the estimated separated spectrum obtained by the algorithm.

We used the joint correlation coefficient between the target fluorescence image estimated by the algorithm and the real target fluorescence image to evaluate the algorithms. Before labeling the mice, the original tissue autofluorescence of the non-fluorescent labeled mice is collected under the same excitation light emission filter channel at the same position, that is, background fluorescence. According to the calculation method of the correlation coefficient defined by formula 1, our real target fluorescence and the target fluorescence calculated by the algorithm are combined to calculate the correlation coefficient.

In general, the larger the value of the joint correlation coefficient, the better the result of the algorithm. This paper compares and analyzes the joint correlation coefficients of several algorithms in spectral abundance and spectral coefficient.

After calculation, the average joint correlation coefficient of the fluorescence image target region extraction experiment can be obtained. Assuming that the joint correlation coefficient of abundance is denoted as $\text{coef}_1$ and the spectral coefficient is denoted as $\text{coef}_2$, Figure 3 shows the joint correlation coefficient of the results obtained by the above several algorithms.

From this, we can see that the proposed algorithm shows great advantages over other traditional linear separation algorithms. These evaluation results indicate that the proposed algorithm is less affected by background fluorescence and the estimated separated spectrum is closer to the target spectrum. In general, the larger the joint correlation coefficient, the stronger the correlation between the two images, so the proposed algorithm can be well applied in practice.

## CONCLUSIONS

In this paper, a set of in vivo fluorescence imaging system for small animals is designed and implemented. On the basis of fluorescence image acquisition, an automatic extraction algorithm of target fluorescence area is proposed. Due to the influence of various factors on the background fluorescence intensity, the fluorescence image shows a diffused spatial
region and uneven brightness distribution. Fluorescence image target extraction can be regarded as an autocorrelation function that maximizes the linear combination. The algorithm maps the nonlinear separation data in the low-dimensional space to the high-dimensional space, which can be expressed as a linear separation. Then, in order to realize the automatic extraction of the target fluorescent region, this paper proposes a two-step gradient entropy function for discriminating the target fluorescent region by analyzing the characteristics of the fluorescent region and obtains the real target fluorescent region according to the set connected regions.

Based on the experiment of collecting and analyzing fluorescent images of mice in vivo, it is verified that the proposed algorithm can automatically extract the target fluorescent region better than the classical linear model. It shows that the proposed algorithm is less affected by background fluorescence, and the estimated separated spectrum based on this method is closer to the real target spectrum.

## EXPERIMENTAL AND COMPUTATIONAL METHODS

**In Vivo Fluorescence Imaging System for Small Animals.** The images used in this paper are acquired based on the self-developed in vivo fluorescence imaging system for small animals. This system uses adjustable gain sCMOS as the core image acquisition original, which can quickly achieve high-quality image acquisition and processing. The developed in vivo fluorescence imaging system includes a hardware framework controlled by various devices, user-oriented computer software applications, and image data processing methods. The system provides a reliable and practical method for observing living body fluorescence imaging, which can be effectively applied to actual biochemical imaging experiments. The system structure is shown in Figure 4.

After the xenon lamp is reflected by the concave mirror, the light beam is coupled into the optical fiber through the coupling lens, and an 8-bit filter wheel is placed at the front end of the optical fiber so that the required excitation light can be coupled into the optical fiber through the filter. The four corners of the chamber are provided with optical fiber fixed joints. After the four beams are transmitted through the optical fiber, they are irradiated to meet the needs of fluorescence imaging. Most in vivo imaging systems use reflected illumination to excite the fluorescence signal in the body. This method has the defects that the excitation light energy is dispersed and the spontaneous background fluorescence of the whole body tissue is strong. This system adds a transmission excitation mode, and the energy of the light source is led to the bottom of the experimental animal through the optical fiber, and then a multipoint projection excitation scan is performed from the bottom of the animal, thereby reducing the generation of spontaneous background fluorescence.

The in vivo fluorescence imaging system adopts a multi-spectral microimaging subsystem based on a filter module to perform spectroscopic processing. The filter module is composed of an excitation light filter module and an emission light filter module. Using the filter module component light can directly analyze the wavelength dimension of the spectral image, collect one band of the emission spectrum, and obtain the information of spatially resolved data and then obtain the image information of the entire band through continuous collection. By adjusting the filter module, a specific wavelength can be switched from 420 to 950 nm. In addition to wavelength adjustment, different half-height widths can also be selected for each center wavelength. Choosing a wide half-height width can increase the light transmittance from a broadband light source, while a narrow half-height width can only transmit a few wavelengths of light from a broadband light source. In this way, the imaging optical path of the spectral microscopic in vivo imaging fluorescent imaging system can be realized. The instrument is designed to use a spectral imaging system, but the focus of the work in this paper is the extraction algorithm of the fluorescent target area. So, only one wavelength of the fluorescent image was used for comparative analysis of the algorithm.

**Fluorescent Probe Reagent.** In this experiment, DiO was used as the fluorescent probe reagent. DiO, called 3,3′-dioctadecylxarboxycanine perchlorate (DiOC18(3)), is one of the most commonly used fluorescent probes in cell membranes, showing green fluorescence. DiO is a lipophilic membrane dye, and the molecular structure of DiO is shown in Figure 5a. After entering the cell membrane, it can diffuse laterally and gradually stain the entire cell membrane.

DiO fluorescence is very weak before entering the cell membrane, and only after entering the cell membrane can it be excited by strong fluorescence. DiO can emit green fluorescence after being excited, and the excitation spectrum and emission spectrum after the combination of DiO and phosphoric acid bilayer membrane are shown in Figure 5b.

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**Figure 4.** System structure of the in vivo fluorescence imaging system.

**Figure 5.** (a) Molecular structure and (b) spectrogram of the fluorescent probe reagent.
Among them, the maximum excitation wavelength is 484 nm, and the maximum emission wavelength is 501 nm.

**Extraction Algorithm of the Target Fluorescence Area.** In the actual image acquisition process, due to the influence of various factors on the background fluorescence intensity, the fluorescence image appears to be spatially dispersed and the brightness distribution is uneven. In the fluorescence image, the target area is defined as the small animal area injected with a fluorescent reagent. In general, the spontaneous fluorescence of the target area will be excited by the target fluorescence and then show a slightly stronger spontaneous fluorescence photon count than other unlabeled areas. Based on this, it can be considered that the fluorescence intensity of the target area is generally a little larger than the background fluorescence, and it is the main fluorescence intensity change area. In this paper, the target area we extract is the main change area of fluorescence intensity. In the image pre-processing stage, the average gray value of all pixels in the binary image is calculated first, and then the region composed of pixels larger than the average gray value is selected as the candidate target region for further separation.

In this paper, we use kernel maximum autocorrelation analysis (kMAF) to extract the target fluorescence area. First, the image information is transformed into the high-dimensional space by the kernel method, and the autocorrelation analysis and maximization processing are carried out, which makes the correlation between the target fluorescence signals greater and the correlation between the background signals smaller so that the algorithm can better separate the target fluorescence area.

In the maximum autocorrelation factor algorithm, there are spatially collected data \( x(r) \) with an average value of 0, and its linear combination is \( a^T x(r) \). Our goal is to maximize the autocorrelation function of the linear combination. Assuming that \( s(r) \) is the multivariate observation at position \( r, s(r + \Delta) \) is the observation obtained at position \( r + \Delta, \) and \( \Delta \) is the spatial displacement. The autocorrelation expression of the linear combination \( a^T s(r) \) is

\[
\begin{align*}
    r &= \text{Cov}(a^T s(r), a^T s(r + \Delta)) = a^T C_{\Delta} a
\end{align*}
\]

One of the key points of the kMAF algorithm is the design of kernel function. Suppose there is some implicit method \( \theta \) to map a matrix to a high-dimensional N-dimensional space, which is \( x \rightarrow \theta(x) \). The basic idea is shown in Figure 3. The nonlinear separation data in the low-dimensional space can be expressed in the form of linear separation by mapping to the high-dimensional space (Figure 6).

![Figure 6. Mapping of nonlinear separation data in the low-dimensional space to high-dimensional space.](image)

Each element of the mapping matrix can be expressed as a function expression of \( \theta(x) \):

\[
\theta = \begin{bmatrix}
\theta(x_1) \\
\theta(x_2) \\
\vdots \\
\theta(x_n)
\end{bmatrix}
\]

Based on formula 2, the low-dimensional nonlinear observation value is converted into a high-dimensional linearly separable result so as to distinguish the background and target fluorescence values. Based on this, the row data of \( X \) is mapped to the high-dimensional space by nonlinearity, so the problem of target region extraction can be transformed into the problem of autocorrelation analysis. The maximum autocorrelation analysis can identify the signals with high correlation in multispectral images. By maximizing the autocorrelation coefficient, the autocorrelation vector is obtained and the regional change detection is analyzed by using it. In order to realize the autocorrelation vector analysis, this paper analyzes the characteristics of the fluorescent region and proposes a two-step gradient entropy function for discriminating the target fluorescent region and obtains the real target fluorescent region according to the set connected regions.

The automatic extraction method of the target fluorescent area is equivalent to a feature extraction technology, and the target fluorescent area is the area to be extracted. The target fluorescent area has the following obvious characteristics: (1) In the target fluorescent region, the gray gradient between pixels is small. (2) Around the fluorescent area of the target, the change gradient of the gray value of the pixel is large. In the image, the expression of this feature is obvious fluorescence contrast and random gray distribution. (3) The area formed by the dots in the target fluorescent area has connectivity, that is, the pixels that meet characteristics (1) and (2) are not isolated dots, and there must be a similar pixel distribution around them. Using the above three features, the nontarget fluorescent region and the target fluorescent region can be divided.

In this paper, we used the Laplacian operator to process the in vivo fluorescence images of small animals, with the purpose to retain the characteristics of the fluorescent target area and remove the influence of the background. The Laplacian operator is a two-step operator. For a continuously differentiable image gray function or surface \( f(x, y) \), the Laplacian image \( g(x, y) \) is defined as

\[
g(x, y) = \frac{\partial^2 f(x, y)}{\partial x^2} + \frac{\partial^2 f(x, y)}{\partial y^2}
\]

Based on the above formula, the Laplace operator is used to assign a new gray value to each pixel of the image. If the difference between the gray value of a pixel in the image and the gray value of the adjacent pixel is larger, the gray value of the point will be larger after Laplacian processing. After being processed by the Laplace operator, the region with obvious jump between the image edge and gray value will be retained.

Because the fluorescence target area has lower feature contrast than other areas, the gray-scale distribution is narrower and contains less information. Assuming that the amount of fluorescence information contained in point \( (x, y) \) is \( l(x, y) \), the judgment value of the target fluorescence region in a certain neighborhood centered on point \( (x, y) \) is defined as
R(x, y). The larger the value of R(x, y), the greater the probability p(x, y) of the point in the target fluorescence region. The determination value R(x, y) of the target fluorescence region is related to the fluorescence information I(x, y) as follows:

$$R(x, y) = \varphi[I(x, y)] \quad (4)$$

In this paper, \( \varphi \) takes the Heaviside function. If it is larger than the threshold set value, it is considered as the decision value \( R(x, y) = 1 \); if it is less than the threshold value, it is considered as \( R(x, y) = 0 \). Finally, all the points with a decision value of 1 are connected to form the target fluorescence region.

In order to count the amount of information contained in the fluorescent target area, this paper introduces the concept of entropy. Assuming that the probability of the occurrence of the random variable of the information source of point \((x, y)\) is \( p(x, y) \), then the information entropy of the information source is defined as

$$E(x, y) = \sum_{x=0}^{M} \sum_{y=0}^{M} p(x, y) \log_2 p(x, y) \quad (5)$$

The fluorescence information \( I(x, y) \) is expressed by the information entropy, which is \( I(x, y) = E(x, y) \).

The above formula reflects the average amount of information in the image. For pure black or pure white images, the image information entropy value is zero, and it can be considered that the amount of information carried by the image is very small. However, when the gray histogram is stable, that is, the probability of gray values in the picture is equal, then the value of the image information entropy is relatively large; at this time, it can be considered that the image contains a large amount of information.

In this paper, we proposed a function suitable for extracting the features of the fluorescent target area from the image features of the fluorescent target area, and the function is the second-gradient entropy function \( Q_M \). Based on the elimination of background effects, \( Q_M \) is used to determine the fluorescent target area in the image:

$$Q_M = \sum_{x=0}^{M} \sum_{y=0}^{M} P_{V(x,y)} \log_2 \sum_{x=0}^{M} \sum_{y=0}^{M} P_{V(x,y)} \quad (6)$$

Among them, \( M \) represents the size of the side length of the test subregion, and \( P_{V(x,y)} \) is the probability that the second-order grayscale gradient value of the current pixel \((x, y)\) appears in the \( M \times M \) size subregion. By traversing the picture with the subarea of the area \( M \times M \), the second-order gradient entropy value \( L_0 \) sequence of the image can be obtained. The maximum value \( Q_{\text{max}} \) can be found from the sequence \( L_0 \).

Set the entropy range of the target area to be \( [Q_{\text{max}} - k, Q_{\text{max}}] \). When the second-gradient entropy value \( Q_{(x,y)} \) corresponding to the neighborhood centered on point \((x, y)\) in the image is at \( [Q_{\text{max}} - k, Q_{\text{max}}] \), set the point to 1, otherwise set the point to zero so that the segmented binary image images can be obtained. In the binary image images, an area array \([S_1, S_2, ..., S_N]\) of connected areas can be obtained according to the area where the value 1 is located. Arrange the areas of the connected areas from large to small. If the number of speckle areas is set to \( N \), the final area where the speckle is located is \([S_1, S_2, ..., S_N]\).
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