A novel method for eliminating autofluorescence of small animals in fluorescence molecular imaging

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ABSTRACT

As a newly emerged optical imaging method, fluorescence molecular imaging technique has been receiving increasing attention for its ability of non-invasive visualization of the cellular and molecular activities. However, as a kind of background noise, autofluorescence is a major disturbing factor in fluorescence molecular imaging. In this paper, we proposed a novel method to eliminate autofluorescence of small animals. The method is based on the fact that most autofluorescent signal has a broad excitation and emission spectrum, whereas specific fluorescent probe has a narrow one. First, two fluorescent images are obtained at two different excitation wavelengths. Then we divide the two obtained fluorescent images into blocks with the size of 8×8 pixel. The two blocks from the same position of the two different images respectively constitute a block pair. The ratio of one block's summation of total pixel value to that of ther other block belonging to the same block pair is calculated. After that, we classify all block pairs into fluorescent and non-fluorescent ones by ratio. The former are considered to be actual fluorescent regions. In next step, we adopt an adaptive cluster analysis method to classify all fluorescent block pairs into multiple interest regions. A general centroid algorithm is then applied to locate the center of each interest regions. We recover the fluorescent interest regions using flood filling algorithm. Finally, we choose a GFP-transfected tumor mouse model and a GFP-transplanted mouse skin model to validate our algorithm.

Keywords: Autofluorescence eliminating, fluorescence molecular imaging, GFP mouse model

1. INTRODUCTION

Fluorescence molecular imaging is a newly emerged gene expression analysis and detection technology. The development of fluorescence molecular imaging benefits from achievements in terms of fluorescent protein, fluorescent dye and molecular probe. It promotes both the *in vivo* and non-invasive study of gene expression, protein function and interaction between proteins. Nevertheless, autofluorescence caused by endogenous fluorophores intrinsic small animal when excited by certain waveband light source, becomes main interference for useful getting fluorescent signals and even submerges the target signals sometimes [1].

It is reported that the utilization of a broad bandpass filter is not recommended, for the reason that the overall signal-to-noise ratio becomes lower since the increase of autofluorescence outweighs the target fluorescence. Therefore, the use of a narrow bandpass filter will restrain the growth of autofluorescence to some extent [3]. In 1986, Steinkamp and Stewart brought up a measurement that using different lasers to provide two different excitation wavelengths to reduce interference of background autofluorescnece in flow cytometry experiments [2]. But the method may not be genreral enough to be extended to other application fields, such as the autoflluorescence elimination of small animals in fluorescence reflection imaging.

It is well known that most autofluorescent signal has a broad excitation and emission spectrum, while certain specific fluorescent probe may have a narrow one. So two images are collected, one at a wavelgenth exciting both the autofluorescence and the fluorescence, the other only contatining autofluorescence. Then substraction between two images is implemented. With the operation that the image mainly containing autofluorescence is corrected by multiplying a coefficient, and the coefficent is calculated by non-specific fluorescent region. This technique can reduce lots of autofluorescence [4]. However, some useful fluorescenct signals are also impaired, especially when signal-to-

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noise ratio is at low level. In this paper, we provide a method to enhance the signal-to-noise ration, and simultaneously with no sacrifice of target fluorescence. Furthermore, this technique's practicablilty and efficacy is proved in mouse experiments with the fluorescence dye GFP.

2. METHODS

2.1 Background

In our method, two filters of different wavelengths are adopted. One called primary filter is to collect target fluorescent signals, while the other called assistant filter is mainly used to gather background autofluorescence signals.

f_1, f_2 both denote components from fluorescent parts, while both a_1, a_2 are autofluorescent parts; I_1 is summation of f_1 and a_1, when I_2 is summation of f_2 and a_2. f_{1m}, f_{2m} denote components from fluorescent part, while a_{1m}, a_{2m} are autofluorescent parts. I_{1m} is summation of f_{1m} and a_{1m}, when I_{2m} is summation of f_{2m} and a_{2m}. I_1 and I_{1m} are both obtained by primary filter; I_2, I_{2m} are both obtained by assistant filter. So we have:

$$I_1=f_1+a_1,\ I_2=f_2+a_2,\ I_{1m}=f_{1m}+a_{1m},\ I_{2m}=f_{2m}+a_{2m}$$

If fluorescent area, we believe that $f_1>>f_2$, $a_1<a_2$; if not fluorescent area, we assume that $f_{1m}=f_{2m}=0$, $a_{1m}<a_{2m}$. Then we easily get that:

$$\frac{I_1}{I_2}=\frac{f_1+a_1}{f_2+a_2}>\frac{f_{1m}+a_{1m}}{f_{2m}+a_{2m}}=\frac{I_{1m}}{I_{2m}}$$

From the reduction process above, a conclusion can be drawn that ratios of fluorescent area to non-fluorescent area in two different excitation images are of much difference, which can be used to distinguish fluorescent regions with others.

Steps of proposed algorithm

Here, we note image that got by primary filter as F, image got by assistant filter as B. F and B have the same width and height.

Step 1: we divide F and B into identical blocks.. That is, every two blocks at the same position of F and B is one-to-one correspondence and called a block pair. The size of each block pair is 8×8 pixel.

Step 2: i denotes the row index number of block pair, when j denotes the column index number of block pair. $S_1(i,j)$ is summation of each block in F, while $S_2(i,j)$ in B (As in Figure 1). $r(i,j)$ is the mentioned ratio:

$$r(i,j)=\frac{S_1(i,j)}{S_2(i,j)}$$

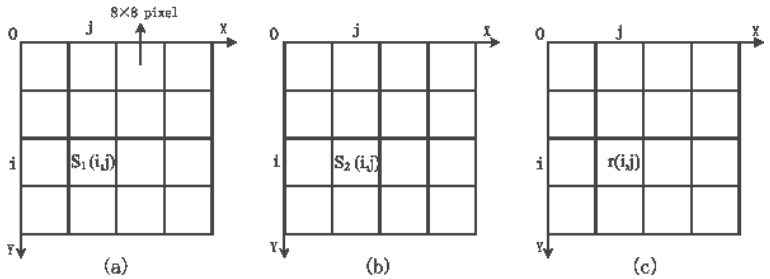

Figure 1. Images dividing and ratios calculation. (a) and (b) demonstrate the divding process in F and B; (c) is ratio matrix.

Step 3: Block pairs which ratio value above 0.9 times as large as maximum ratio of all are taken as trustworthy fluorescent ones. Next, we acquire center coordinates of selected fluorescent block pairs. These center coordinates of fluorescent block pairs are classified to several categories. Each category is a fluorescent region. We exploit an adaptive cluster analysis method to complete classification by distance between center coordinates.

Step 4: Center of each category are by centroid method and ratio is selected as weighted value. (x_k, y_k) are coordinates of the kth member of each category; r_k is the ratio value of each block pair; (x, y) is the desired center coordinates of each category and can be got by following formulations:

$$x = \frac{\sum x_k \times r_k}{\sum x_k},\ y = \frac{\sum y_k \times r_k}{\sum y_k}$$

Step 5: Take center coordinates of fluorescent regions as seeds, to find fluorescent regions using seed flood filling algorithm in F. The value that 1.3 times as large as the average pixel value is a threshold, and when achieved, the searching process is finished.
Step 6: Calculate and remove the contributions from autofluorescent part to present target fluorescent signals.

3. EXPERIMENTS AND RESULTS

In this section, we implement two experiments to validate our method. Since fluorescent dye GFP's optimal excitation wavelength is around 488 nm, it is unavoidable that autofluorescence is strong enough to interfere target fluorescent signal and even submerge it. We choose GFP as fluorescent dye to prove that our technique is practicable and beneficial, for its ability to extract useful fluorescent signal from image severely contaminated by autofluorescence. In the following part, we will introduce more details about our experiments. Experimental results are also analyzed.

3.1 Experiments for GFP-transfected mouse tumor model

In this experiment, The HCC-LM3-fLuc tumor model was established by subcutaneous injection of from 1×10^6 to 7×10^6 HCC-LM3-fLuc tumor cells into the right upper flanks of BALB/c mouse. In this model, both fluorescent and and bioluminescent signals of GFP are emitted at the same position. Since bioluminescence has much low background noise, it is accepted as a golden rule to locate the actual position of fluorescence. Here, we evaluate validity of the proposed tecnique by contrast of the fluorescent region obtained by our algorithm and the bioluminescent area.

In the experiment, we choose 488 nm, 410 nm and 525 nm as the center wavelength of primary excitation filter, assistant excitaion filter, and the emission filter, respectively. The FWHM (full width at half medium) is 20 nm. Furhtermore, the exposure time of fluorescent image is one second, and bioluminescent image one minute, both with a perture f/2.8, focus 55 mm. Operation is done when the temperature of CCD chip is locked at -70℃, which can restrict thermal noise to a very low level.

We take three out of seven sets of data from mice of different tumor size to illustrate the effectiveness of proposed method. In figure 2, we can see clearly that our algorithm successfully recovers the actual fluorescent regions that seriously stained by autofluorescence. It is also seen that the locations of bioluminescent region and fluorescent region are nearly the same.

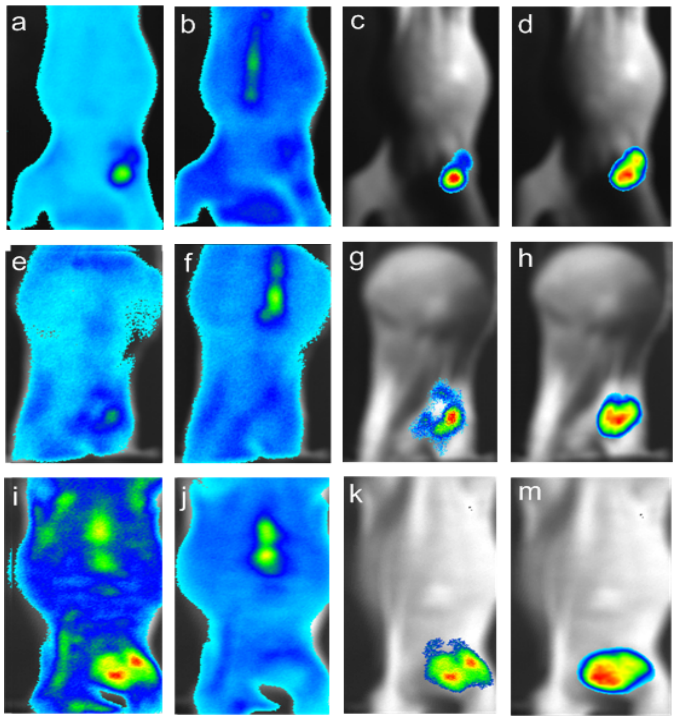

Figure 2. Experiments with GFP-transfected mouse tumor model. (a), (e) and (i) are fluorescent images obtained by primary filter; (b), (f) and (j) are fluorescent images obtained by assistant filter; (c), (g) and (k) are results processed by our algorithm; (d), (h) and (m) are bioluminescent images for verification.

With the purpose of getting quantitative comparison results, we analyzed the linear correlation of bioluminescent intensity and fluorescent intensity without and with our alogrithm corrected. Here, we suppose that which pixel value is over 1.5 times as large as average pixel value, is regarded to fluorescent region. In figure 3, we can get the linear correlation of the latter is 0.8773, while the former is only 0.3141. So we can arrive at a conclusion: the non-corrected fluorescent regions fail to reflect the true situation because of interference from autofluorescence; the algorithm extracts most of the fluorescent signals, which is closer to the truth.

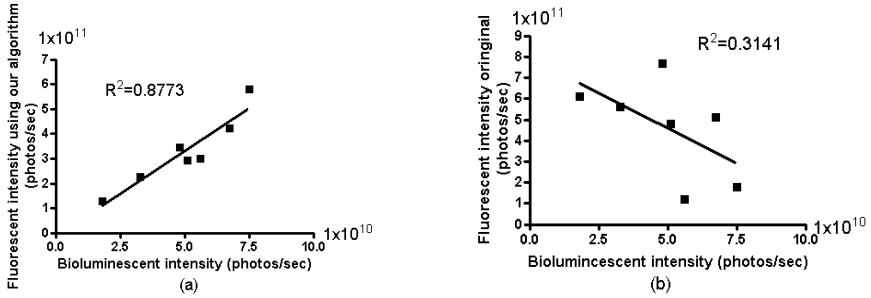

Figure 3. Linear correlation of fluorescent intensity and bioluminescent intensity. (b) denotes the statistical results from original fluorescent images, while (a) from fluorescent images corrected by proposed algorithm.

3.2 Experiments for GFP-transplanted mouse skin model

In this experiment, we developed a GFP-transplanted mouse skin fluorescence model. We transplanted a piece of fluorescent skin with a size of 1×1.5 cm to establish this model. The transplanted skin emits GFP fluorescent signals.

However, the autofluorescence is as strong as true fluorescence, especially interferences from tail, legs and ears. So we are not able to distinguish target fluorescent signal with autofluorescence. In this case, our method can deal with the problem effectively. As seen in figure 4, fluorescent images of different exposure time (1s, 2s, and 5s) are applied by our algorithm. White circles denote the actual locations of transplanted skin, which encirlce the corresponding selected fluorescent signals.

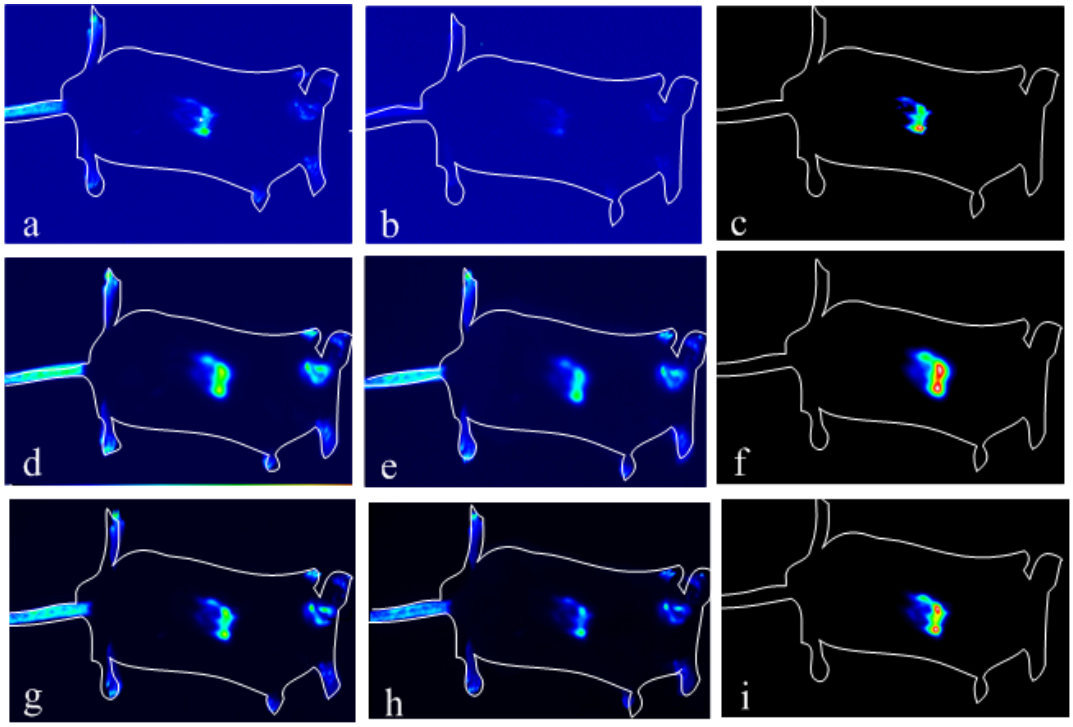

Figure 4. Experiments with GFP-transplanted mouse skin model. (a), (d) and (g) are fluorescent images obtained by primary filter; (b), (e) and (h) are fluorescent images obtained by assistant filter; (c), (f) and (i) are results processed by our algorithm.

4. CONCLUSIONS

In the fluorescence molecular imaging experiments on small animals, background fluorescence is so strong that interferences with the desired target fluorescent signals. The probed is proved to be the main factor to determine the results of experiments. We record two images of different wavelengths, and the ratios of useful fluorescence to autofluorescence of the two are different. In this paper, Based on the information mentioned above, a novel method is proposed to eliminate the autofluorescence of small animals. To validate our algorithm, we established two mouse models. Both the experimental results well demonstrate this validity of our method.

In the next work, we will try to apply this method on other flluorescent dyes, such as DsRed, FITC, RFP, and so on. Otherwise, we attend to explore the method for parameters selection, and hope to develop an adaptive parameter selection strategy.

Besides, more experiment models on fluorescence imaging will be set up to validate and improve our method.

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