REDUCING INCIDENTAL FLUORESCENCE IN LIVE CELL IMAGING

A. Altinok [1], *B. Sumengen* [2], *B. S. Manjunath* [2], *K. Rose* [2]

Dept. of Computer Science[1], Electrical and Computer Engineering[2]
University of California Santa Barbara, Santa Barbara, CA 93106

ABSTRACT

Fluorescence staining is the prevailing image acquisition technique in cell biology research. A problem of this method is the incidental fluorescence, that is the total fluorescence collected by a camera pixel originating from all sources in the sample except for the targeted point. As a result, typical images exhibit blurry regions at the periphery of fluorescence stained cell structures. Generic image processing algorithms address the problem indirectly and with limited success. We propose a technique directly targeting incidental fluorescence by optimizing a simple physical model. Our results indicate that incidental fluorescence can reliably be reduced, and often eliminated, without a significant loss in content. Resulting images are considerably better for human vision, while improvements are actually realized in automated processing. We demonstrate the performance of the technique on microtubule images that are known to suffer from incidental fluorescence.

1. INTRODUCTION

Fluorescence staining is the main live cell imaging method in biological research [1]. Developments in fluorescent dyes facilitated research on cellular processes through time lapse imaging [2]. Conceptually, the technique is based on staining bio molecules selectively with proteins carrying fluorescence dyes of known excitation and emission wavelengths. Then, sample is imaged with the excitation wavelength of the dye. Resulting image captures structures with stained molecules while all other structures remain as dark background.

Images of fluorescence stained molecules suffer from incidental fluorescence showing as blurry edges around stained cellular structures, Fig.1. Often, recorded intensity levels become very close between objects and background and standard contrast enhancement methods provide limited solutions. Fig.1a shows a region cropped after enhancing contrast. Effects of incidental fluorescence worsen in crowded regions, Fig.1b, and reliable segmentation becomes nearly impossible. Even in visibly isolated areas, Fig.1c, a smooth decrease in the intensity levels surrounding the object, due to fluorescing in the sample is evident.In general, researchers post-process images, yet the results are suboptimal for quantification and analysis purposes of biological phenomena. For instance, length measurements in Fig.1 would lack accuracy since the ends of the objects are blurry within a few pixels.

This study was funded by NSF-ITR 0331697.

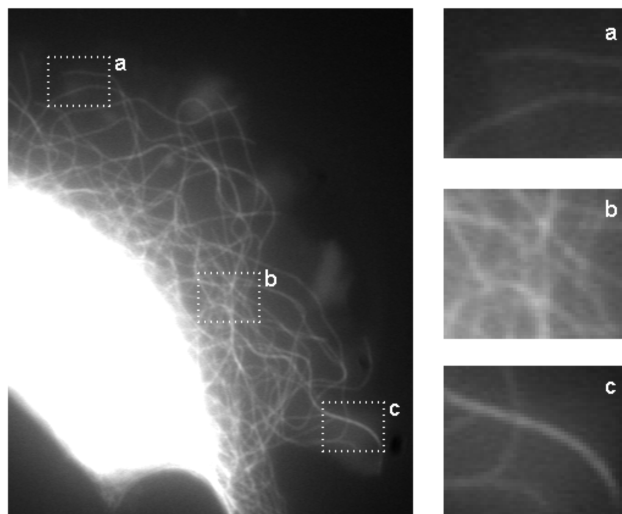


Fig. 1. Microtubule image with over exposed center. a) Object levels blending with background, b) indistinguishable objects, c) incidental fluorescence around a microtubule.

The main shortcoming of image processing algorithms is their generic nature, thereby addressing fluorescence related problems indirectly at best, typically within the context of random noise.

General image processing algorithms handle effects of fluorescence in the context of random noise, without considering underlying optics. Standard segmentation methods remain ineffective in reliable detection of fluorescing objects. Fig.6 top row shows binary images obtained from the first image at decreasing threshold levels. The effects of incidental fluorescence are evident as new microtubules become visible while others are lost when the surrounding levels are thresholded. Incidental fluorescence is evident between c and d as the microtubule in the center becomes thicker from c to d. Advanced techniques and content or model based statistical methods need prior knowledge about morphological properties of objects and often require heuristics or complex models for reliable operation.

Target user of enhanced images has been mainly humans, while statistical conclusions rely on larger sample sizes and automated analysis. Over the last decade, computer vision tools gained research momentum in biological image analysis [3]. A number of specialized imaging software [R] are commercially available. Main performance hit on vision systems is a direct consequence of difficulty in segmentation.

Most related previous work [4] focused on correction of the shading globally or in large areas of the images. In contrast, our focus is more local–around the micro tubules, which are only a few pixels thick. We propose an algorithm to remove incidental fluorescence based on computational modeling of underlying optics and reconstruction of the signal. Processing is demonstrated on microtubule frames taken from videos in [5].

Next, we briefly touch on the biological significance of the problem. In section 2, we present the details of the proposed method. We discuss implementation issues in section 3, followed by the results in section 4. We conclude with the discussion on application specific issues in section 5.

1.1. Fluorescence Imaging in Biology

Physical properties of fluorescence as well as its applications in biological imaging can be found in [6]. Careful sample preparation is an integral part of successful imaging. Results vary highly due to a number of factors like choice of fluorescent dye, appropriate imaging equipment, and operator skills. In practice, image acquisition is regarded as more of an art than science. For time lapse imaging, photobleaching, the natural decay of fluorescence over time, is a limiting factor. As a countermeasure, manual excitation adjustment during imaging results in disparate levels between frames generating difficult problems for standardizing recorded intensity levels. Studies that depend on intensity measurements require reliable estimation of expressed fluorescence. Incidental fluorescence contaminates this process. Proposed algorithm can be used to estimate actual fluorescence expression in the sample while enhancing fluorescing objects considerably.

A collection of biological and biophysical factors contribute to incidental fluorescence, such as neighboring cellular structures, free floating dye molecules, leakage from out of focus planes, however negligible, the natural fluorescence of bio-molecules, different refraction indices of media, and so on. The proposed method targets the collective effects of such factors as captured by the camera.

2. A COMPUTATIONAL MODEL OF INCIDENTAL FLUORESCENCE

Within the cell, we can think of each stained molecule as a light source releasing photons in each direction. The charge-coupled device (CCD) collects photons at each pixel. Key observation in our approach is that fluorescence captured at each pixel consists of photons from the corresponding stained point as well as from all other neighboring points in the sample, the latter we refer to as *incidental fluorescence*. Fig.2 is a graphical depiction of this observation. Shaded CCD pixel collects photons from all fluorescing points in the sample. Formally,

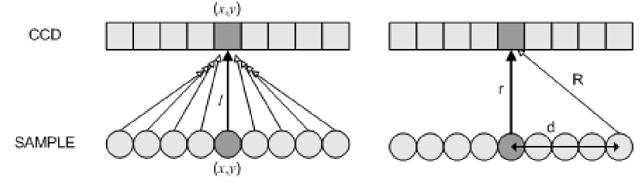


Fig. 2. Decomposition of recorded intensity at (x, y).

let (x, y) represent a fixed pixel in CCD and the corresponding point in the sample. Let $I'(x, y)$ refer to the total fluorescence captured at pixel (x, y), and $I(x, y)$ be the fluorescence due to sample point (x, y). Total incidental fluorescence at (x, y) emanating from all other points (x', y') is additive and given by $\sum F(x', y')$, where the sum is over (x', y'). In its basic form, we can write

$$I'(x, y) = I(x, y) + \sum_{(x',y')} F(x', y') \tag{1}$$

From optics we know that illumination is inversely proportional to squared distance. Thus, the *incidental* fluorescence at (x, y) can be written as

$$\sum_{(x',y')} F(x', y') = \sum_{(x',y')} \frac{I(x', y')}{r^2 + d((x, y), (x', y'))^2} \tag{2}$$

where r is the distance between the CCD and the sample and $d(\cdot)$ is distance between (x, y) and (x', y'). From (2) and (1), we arrive at a concise model of incidental fluorescence at each CCD element,

$$I'(x, y) = I(x, y) + \sum_{(x',y')} \frac{I(x', y')}{\gamma(r^2 + d((x, y), (x', y'))^2)} \tag{3}$$

(3) is a well defined system of equations capturing total incidental fluorescence at each pixel (x, y). The solution for I recovers actual fluorescence at each pixel given the observed intensity levels at neighboring pixels. Note that other formulations considering the angle between r and R are possible, here we assume $r \approx R$ and the angle is insignificant. In the next section, we discuss implementation issues.

3. IMPLEMENTATION

Vectorized distance transform matrices at each pixel make up the coefficient matrix in (4)

$$\begin{bmatrix} I'_{(1,1)} \\ I'_{(1,2)} \\ \vdots \\ I'_{(m,n)} \end{bmatrix} = \begin{bmatrix} c_{11} & \dots & c_{1mn} \\ c_{21} & \dots & c_{2mn} \\ \vdots & \ddots & \vdots \\ c_{mn1} & \dots & c_{mn^2} \end{bmatrix} \begin{bmatrix} I_{(1,1)} \\ I_{(1,2)} \\ \vdots \\ I_{(m,n)} \end{bmatrix} \quad (4)$$

or in compact form

$$I' = DI \quad (5)$$

where $c_{ij} = 1/(r^2 + d(\cdot)^2)$. Note that the size of the distance coefficient matrix D is very large. For instance, processing a 600×600 image would involve a 1.296×10^{11} element matrix!

Main computational bottleneck is solving (5) for I. It can be shown mathematically that significant part of incidental fluorescence is caused by close neighbors and a global solution is not needed. A reduction in computational complexity can be realized in several ways: i. setting the coefficients for distant neighbors of (x, y) to 0 and obtaining a highly sparse D, ii. local processing with overlap yielding smaller D, and iii. reducing the number of variables in (5) either by eliminating known background pixels or processing only high intensity regions. Essentially, the major distinction in potential reductions is that processing can be guided by the recorded intensity levels in the image or performed locally within a block of a predefined size, irrespective of image content. Other computational reductions exploiting the structural properties of D are possible.

Border handling is an important implementation choice since no intensity data exists beyond image borders. Among standard border handling methods, a realistic option is to process a centered subimage of $(n-k) \times (m-k)$ from an $n \times m$ image, using intensities in the border area.

A closer approximation to the global solution could be obtained by adding an ϵ to each denominator of c_{ij} in (4), representing all disregarded incidental fluorescence. Good estimates of ϵ can be derived as a function of pixel location and local intensities.

In this study, all results are computed by processing k-pixel neighborhoods ($k \in \{8, 16, 32\}$). For the borders of blocks, we used intensity values in overlapping areas to minimize the border effects. No further block fusion was performed. Value of r was determined empirically per image.

4. RESULTS

We present the results of the algorithm on microtubule frames revealing visual enhancement and benefits for further processing. Significant reduction in incidental fluorescence is visible in all images.

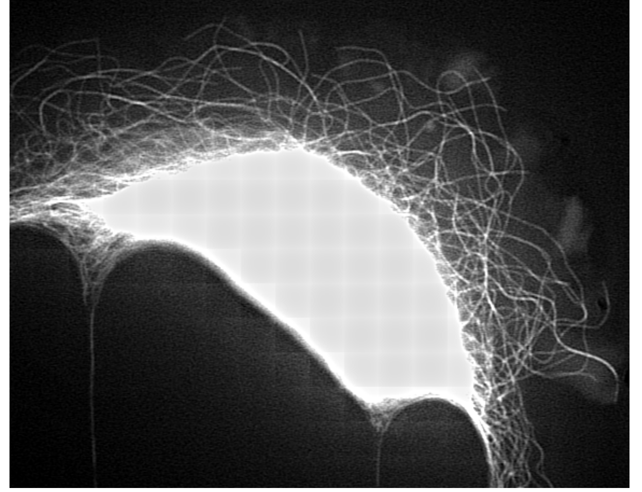
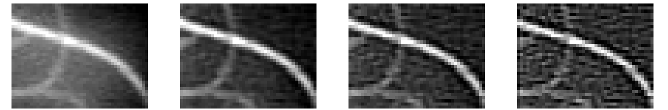


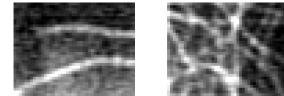
Fig. 3. Result of reducing incidental fluorescence on Fig.1.

Fig.3 shows processing of Fig.1 with 32×32 blocks with 25% overlap. Effects of local processing are visible mainly at overexposed center, which can be eliminated by using larger overlaps (Fig.7), or a sparse D globally. Note that border effects diminish at other regions. Fig.4 shows the effects of varying r on Fig.1c, confirming the physical sense of r. In all cases, incidental fluorescence around the brightest microtubule is effectively removed. Fig.5 shows results of processing Fig.1a and b.



(a) (b) (c) (d)

Fig. 4. (a) Original, (b) $r = 0.3$, (c) $r = 0.8$, (d) $r = 1.5$.



(a) (b)

Fig. 5. (a) Processing of Fig.1a, (b) Fig.1b, r = 0.4.

Fig.6 shows a comparison of thresholds before (upper row) and after (lower row) reducing incidental fluorescence. Improvements at all levels are substantial.

Fig.7a and b show original and processed versions of another microtubule frame. Improvements in peripheral regions are clear while detail within the center part validates the potential signal recovery. Fig.7c and d show details of a 128×128 region from Fig.4 with no significant degradation in stained structures while incidental fluorescence has been nearly eliminated. Note that relative intensity levels are preserved, distinctively visible at microtubule intersections.

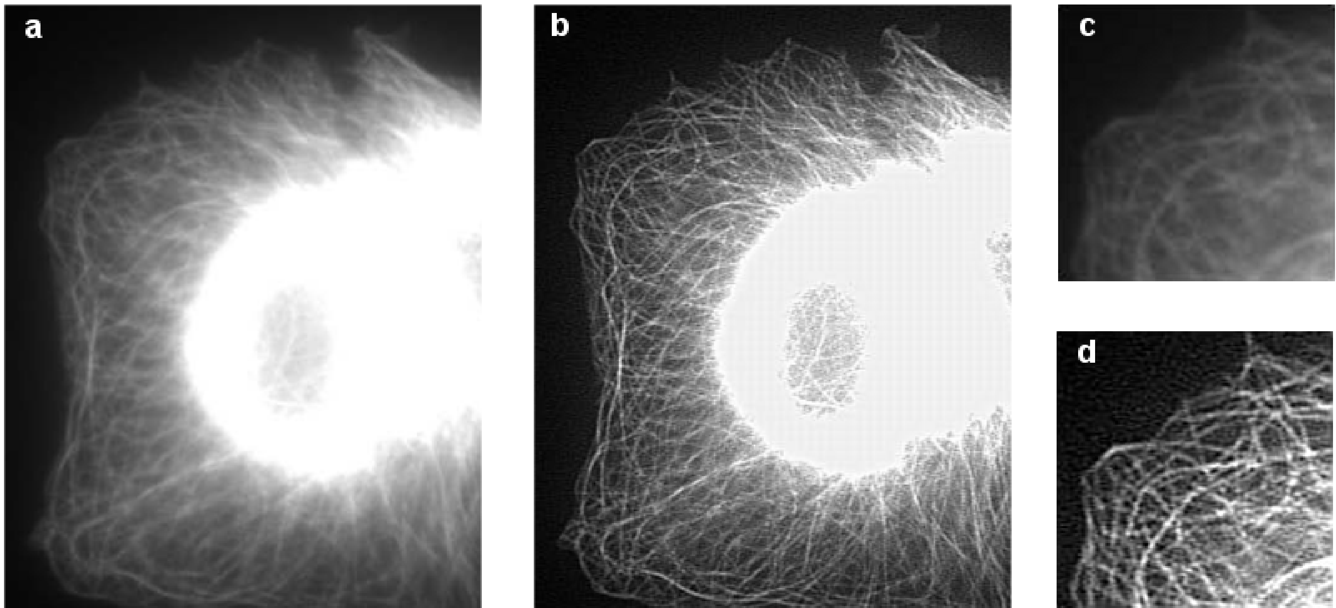


Fig. 7. Original (a) and processed (b) microtubule frames. Enlarged sections (c) and (d) shows that details and relative illumination are kept intact while incidental fluorescence was reduced considerably. 8x8 block with 50% overlap, $r = 0.6$.

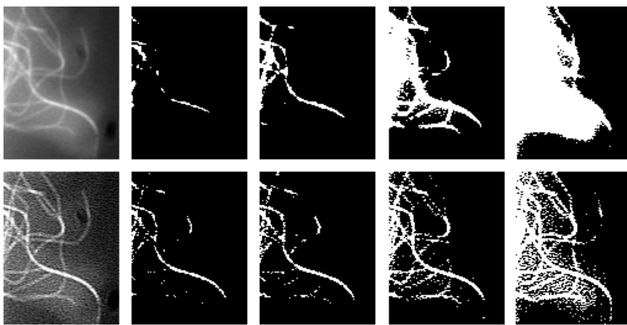


Fig. 6. Originals and thresholds at 0.8, 0.7, 0.5, and 0.4.

5. DISCUSSION AND CONCLUSIONS

Image processing algorithms are not designed specifically for fluorescence imaging. We present an algorithm targeting a problem specific to fluorescence imaging. In its basic form, the algorithm produces visible and measurable improvements in fluorescence images. Ideally, the model has no free parameters as the value of r should be available from the imaging system. In this paper, random noise is not considered as part of the model and sensitivity of the technique is under investigation. Furthermore, explicitly modeling fluorescence sources in the sample for their contributions to incidental fluorescence could improve application specific analysis.

Finally, confocal imaging partially addresses incidental fluorescence by optically filtering light from off-focus planes. Best images are acquired by laser scanning confocal microscopes, albeit considerably higher equipment costs. Even so, confocal systems operate at much slower speeds and are not preferred for high speed imaging of cellular activity. As parallel implementation of the algorithm is straightforward with minimum overhead, real time implementations can be incorporated in cameras with dedicated processing units at a lower cost.

6. REFERENCES

[1] D. M. Chudakov and K. A. Lukyanov, “Use of green fluorescent protin (gfp) and its homologs for in vivo protein motility studies,” *Biochemistry (Moscow)*, vol. 68, no. 9, pp. 952–957, Sep 2003.

[2] J. Lippincott-Schwartz and G. H. Patterson, “Development and use of fluorescent protein markers in living cells,” *Science*, vol. 300, no. 5616, pp. 87–91, Apr 2003.

[3] M. El-Saban et al., “Automated microtbule tracking and modelling,” in *submitted to ISBI06*, 2006, vol. -, pp. –.

[4] A. Entwistle, “A method for the blind correction,,” *Journal of Microscopy*, vol. 219, pp. 141–156, September 2005.

[5] K. Kamath et al., “βiii-tubulin induces paclitaxel resistance in association with reduced effects on microtubule dynamic instability,” *J. Biol. Chem.*, vol. 280, no. 13, pp. 12902–12907, Apr 2005.

[6] B. Matsumoto, *Cell Biological Applications of Confocal Microscopy, 2nd Ed.*, Elsevier Science, 2002.