31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Exploiting the multiplicative nature of fluoroscopic image stochastic noise to enhance calcium imaging recording quality

Federico Esposti, Maddalena Ripamonti, Maria G. Signorini

Abstract—**One of the main problems that affect fluoroscopic imaging is the difficulty in coupling the recorded activity with the morphological information.**

The comprehension of fluorescence events in relationship with the internal structure of the cell can be very difficult. At this purpose, we developed a new method able to maximize the fluoroscopic movie quality.

The method (*Maximum Intensity Enhancement*, MIE) works as follow: considering all the frames that compose the fluoroscopic movie, the algorithm extracts, for each pixel of the matrix, the maximal brightness value assumed along all the frames. Such values are collected in a *maximum intensity matrix*. Then, the method provides the projection of the target molecule oscillations which are present in the $\Delta F/F_0$ movie onto the *maximum intensity matrix*. This is done by creating a RGB movie and by assigning to the normalized ($\Delta F/F_0$) activity a single channel and by reproducing the maximum intensity matrix on all the frames by using the remaining color channels. The application of such a method to fluoroscopic calcium imaging of astrocyte cultures demonstrated a meaningful enhancement in the possibility to discern the internal and external structure of cells.

I. Introduction

FLUORESCENCE microscopy is one of the most basic tools used in biological sciences for the visualization of cells and tissues. The popularity of fluorescence microscopy for the examination of biological samples stems from its ability to target fluorescent probes to molecules at low concentrations with high selectivity and specificity [1]. The comprehension of the recorded activity critically relies on the possibility to understand the relationship between the cell activity-induced fluorescence and the fine morphology of the cell. As a matter of fact, the fluorescence deriving from the rising in concentration of a target molecule, e.g. Ca^{2+} in calcium imaging, appears as a bright spot on a dark background in the fluoroscopic movie. Up to now, this relationship is generally studied by merging the fluorescence movie with a post-fix immuno-labeled image. However, it is known that this procedure is not very precise because of the fixing procedure induces some conformational changes in the cells. The aim of this work is to propose a different approach to this problem.

Fluoroscopic images are affected by different kinds of noises, both deriving from the physics of the secondary emission process (i.e., the fluorescence emission) and from the electronics and optics of the acquisition system [1,2]. One of the main noise components is multiplicative noise, or common mode noise, which variance depends on the pixel mean brightness.

The presence of such a noise can be traced back principally to three origins: (i) The statistical intensity of the Poissonian process that describes the distribution of randomly emitted photons is proportional to the fluorescence signal intensity [1,3-4]. (ii) The employment of photo-multiplier tubes (PMT) or avalanche photodiodes introduces shot, leakage and flicker noises in the preprocessing of each pixel signal [3,5-6]. (iii) Random variations in the very-high gains of CCD camera transistors [3,5]. Taken together, these multiplicative noise sources shape the brightness distribution of each pixel in right-long-tailed bells, as Fig.1 shows.

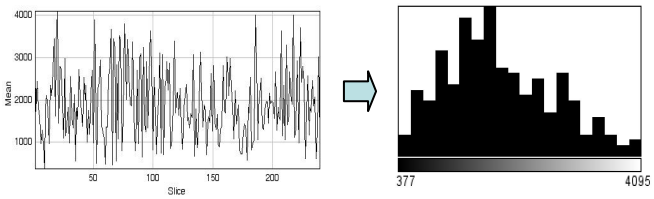

Fig.1: a) example time series representing a resting pixel (endowed with high basal brightness) fluorescence value over time (1 second per slice). b) Luminescence histogram of the series in a). It is worth noting the right-long-tailed distribution of b).

Hence, the higher the mean pixel brightness the longer is the right-tailed distribution of the noise. Therefore, by considering the multiplicative nature of fluoroscopic noise, while the histogram of silent (dark) pixels is endowed with a short right tail, the histogram of active (bright) pixels shows a pronounced right tail (i.e., a strong positive skewness).

Our intention is to exploit the presence of the multiplicative noise added to fluoroscopic images (before its standard reduction through $\Delta F/F_0$ normalization routines [5]) in order to enhance the representation of the cell morphology and to allow a reliable activity-and-morphology merging. In the following, we will consider as example of fluoroscopic recording, the widespread Ca^{2+} imaging.

F. Esposti is with the Politecnico di Milano, Dipartimento di Bioingegneria, Milano, Italy (corresponding author: phone: +39-02-2399-3328; fax: +39-02-2399-3360; e-mail: federico.esposti@polimi.it).

M. Ripamonti is with the Università Vita e Salute San Raffaele, Milano, Italy (e-mail: ripamonti.maddalena@hsr.it).

M. G. Signorini is with the Politecnico di Milano, Dipartimento di Bioingegneria, Milano, Italy (e-mail: mariagabriella.signorini@polimi.it).

II. Materials and Methods

A. Cell cultures and recording system

Calcium imaging movies from pure (or semi-pure) in-vitro cultures of primary astrocytes from P2 rat hippocampus were recorded at the Università Vita e Salute San Raffaele of Milan, Malgaroli's neurophysiologic laboratory. Cultures and recordings were achieved by M.R. Primary cultures of P2 rat hippocampal astrocytes were prepared as reported by others [7], with minimal modifications. The imaging was exploited through calcium fluoroscopy, by employing the Fluo-4 AM fluorescent dye. Fluo-4 presents excitation and emission peaks at 494nm and 516nm, respectively, where the fluorescence of Ca^{2+}-bound Fluo-4 is almost 100 times that of the Ca^{2+}-free form. Such characteristics make this dye particularly sensitive to changes in $[Ca^{2+}]$.

Cultures were incubated for 15 minutes at 37°C with 10μM Fluo-4 AM dissolved in Tyrode solution (Tyrode: NaCl 119mM, KCl 5mM, Epes 25 mM, CaCl2 2 mM, MgCl2 2mM and Glucose 6gr./liter), 0,0l% Pluronic acid f-127 was added to increase dye permeability. After incubation cells were washed out for 5 minutes RT with Tyrode solution. During recordings cells were maintained in Tyrode in 100% O2, 24°C.

Recordings were performed with a Zeiss LSM 510 confocal microscope and by employing a Zeiss FITC Narrow Band Laser with central excitation band 488nm (BP 505-530nm), nominal current 3.1A, used at the 25% of its nominal power. Acquisitions were performed by using a 40x Zeiss Objective, NA 0.8 in Water. The field of view of each recording was a square of 1302.7μm side, scanned by the laser light (pinhole 280μm) in 1.5sec with gain = 904 by obtaining a 512 x 512 output matrix at 12 bit. The total acquisition time was 360sec, for a grand total of 240 frames.

Images were analyzed and processed with ImageJ® and Matlab® software.

B. The method

By considering the multiplicative structure of noise, it is possible to enhance the contrast among silent and active cells, i.e., the contrast among silent zones and zones of the cell endowed with calcium dynamics. As a matter of fact, being active pixels brighter than silent pixels, the firsts are endowed with a broader noise distribution, as Fig.2 exemplifies.

Because of the ubiquity of Ca^{2+} signaling and the multiplicative nature of fluoroscopic noise, most of the cell structures, during a long recording (e.g., 360 seconds), are, at least once, the place of a $[Ca^{2+}]$ elevation.

Given that fluorescence emission is an incoherent process, i.e., each point presents independent random emissions, such "maximum brightness" instants are not synchronous. So, it is possible to collect in a single image all the maximum brightness points by projecting pixel-by-pixel the maxima of all pixel time series.

The *Maximum Intensity Enhancement* (MIE) method works as follow: (i) By considering all the frames that compose the fluoroscopic movie, the algorithm extracts for each pixel of the matrix the maximal brightness value assumed along all the frames. Such values are collected in a *maximum intensity matrix*.

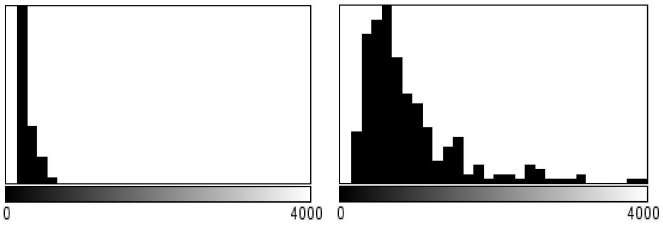

Fig.2: Example of fluorescence multiplicative noise. a) Fluorescence histogram of a resting pixel. b) Fluorescence histogram of an active pixel. It is noteworthy the long-tailed distribution of the active pixel.

(ii) Because of this procedure exacerbates the presence of noise, resulting in a number of high-saturated pixels, the matrix is filtered for Salt and Pepper noise with a 2D median filter (radius = 2 pixels) [8]. The output of this first step is exemplified in Fig.3 and Fig.4. As it can be seen in these figures, maximum intensity matrix is able to improve the visualization of both the cell internal structures (Fig.3) and of the overall cell morphology (Fig.4).

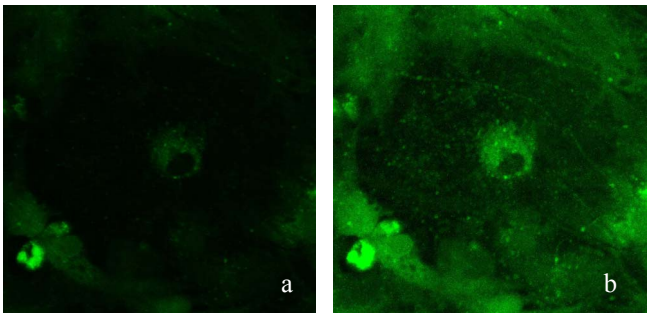

Fig.3: Example of *Maximum intensity matrix* calculation. a) First frame from a calcium imaging movie. The field of view frames a single astrocyte. In a) it is only advisable the density that enwraps the cellular nucleus (Endoplasmic reticulum). b) *Maximum intensity matrix* of a). In the latter is advisable the whole (calcium-active) internal structure of the cell.

(iii) The "true" calcium concentration variations are computed by $\Delta F/F_0$ normalization of the original movie [5], i.e. a normalization of fluorescence in respect to the resting condition fluorescence. This is done by averaging a small number of initial frames of the movie and by dividing the rest of the frames (pixel by pixel) by this average. This allows to create a new movie representing the fluorescence variations, providing a normalization of calcium activity in respect to the starting conditions. As a matter of fact, $\Delta F/F_0$ processing is able to eliminate the multiplicative or common mode noise, since it scales the brightness average and variance on the basis of the initial luminescence conditions [9]. This step can also incorporate any user desired image intensity processing.

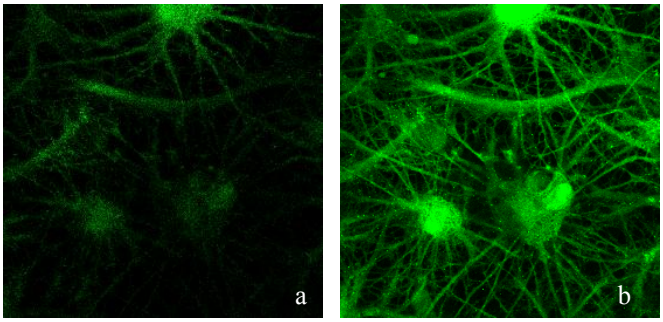

Fig.4: Example of *Maximum intensity matrix* calculation. a) First frame from a calcium imaging movie. b) Maximum intensity matrix of a).

(iv) In order to link the real calcium activity and the cell morphology, the method finally provides for the projection of calcium oscillations which are present in the $\Delta F/F_0$ movie onto the *maximum intensity matrix*. This is done by creating a RGB movie and by assigning to the $\Delta F/F_0$ activity a single color channel (e.g., Red in Fig.5) and by reproducing the *maximum intensity matrix* on all the frames by using the remaining color channels (e.g., Blue + Green in Fig.5).

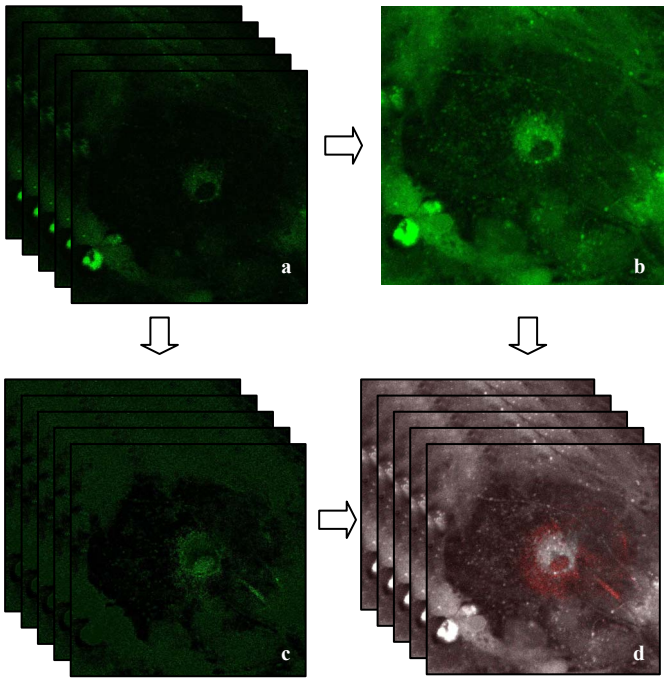

Fig.5: Summary of the *maximum intensity enhancement* (MIE) method procedure. a) The original calcium imaging movie. b) The *maximum intensity matrix* is calculated by creating a matrix showing the fluorescence maxima all pixel present in all frames and filtering it by a median filter. c) The original movie is processed according to the $\Delta F/F_0$ procedure. d) Final output. An RGB movie is creating by merging b) and c).

Fig.5 summarizes the complete algorithm. By considering, a), the original calcium imaging movie, b), the *maximum intensity matrix* is calculated and filtered and, c), the $\Delta F/F_0$ movie is computed. Then, d), the maximum intensity matrix and the $\Delta F/F_0$ movie are merged together in a unique RGB movie, representing both the activity and the morphology of the cell.

III. Discussion

MIE is a form of voxel projection method that performs pixel intensity projection along time instead of space. The cornerstone of the method is the exploitation of the multiplicative nature of fluoroscopic imaging noise, in order to enhance the contrast among active and silent pixels.

The quality enhancement depends on two factors: 1) the image mean brightness is raised by a value that corresponds to the weighed average of the right tail widths of all pixel histograms (Fig.6, left). 2) Most importantly, the contrast is increased by a value corresponding to the average difference between the short tails of silent pixel histograms and the long tails of active pixel histograms (Fig.6, right).

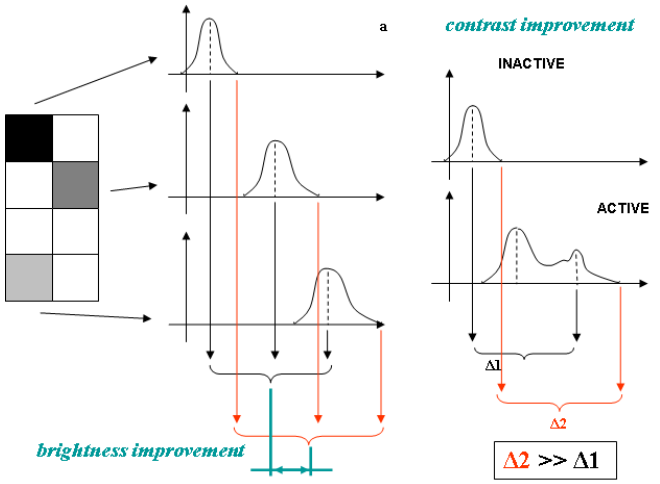

Fig.6: Graphic representation of the main advantages maximum intensity matrix methods offers. Right: the resulting image is characterized by a mean brightness higher than the original frame by a value that corresponds to the weighed average of the right tail widths of all pixel histograms. Left: the resulting image shows a great improvement in the contrast, corresponding to the average difference between the short tails of silent pixel histograms and the long tails of active pixel histograms.

The proposed image quality enhancement method is very effective with different kind of fluorescence microscopy set-ups (e.g., confocal or 2-photon microscopes) but presents two main *caveats*: (i) it should be used on images that do not present sample motion or cell migrations. (ii) It should not be applied to saturated images.

Finally, it should be noted that the intensity projection, i.e. the *maximum intensity matrix*, do not actually represents the overall cell morphology, but it is able to show all the structures that, reached by the fluorescence dye, present some fluoroscopic activity. Hence, MIE is able to represent all structures endowed with a target-molecule concentration increase but not completely silent structures.

IV. Conclusions

In this paper we propose a new method for the enhancing of fluorescence imaging recording quality. The method, called *Maximum Intensity Enhancement* (MIE), exploits the multiplicative nature of fluoroscopic noise to enhance the

contrast among silent and active zones of the cell. Active, in this context, means a cell zone which is endowed with a fluctuation in the concentration of the fluorophore target molecule. The improvement in the cell visualization, both in terms of internal structures and overall morphology, is a central requirement for the coupling of recorded activity and cell internal and external morphology.

ACKNOWLEDGMENT

We greatly thank A. Malgaroli, from the Università Vita e Salute San Raffaele, Milano, for the coordination of experimental activity and for the interesting discussions on this subject.

REFERENCES

[1] Q. Wu, F. Merchant, Kenneth Castleman, "Microscope Image Processing", Academic Press, pp. 576, 2008.

[2] C. Vonesch, F. Aguet, J.-L. Vonesch, M. Unser, "The colored revolution of Bioimaging", *IEEE Signal Processing Magazine*, 2006, pp. 20-31.

[3] K.A. Lidke, B. Rieger, D.S. Lidke, T.M. Jovin, "The role of photon statistics in fluorescence anisotropy imaging", *IEEE Trans. Im. Proc.*, **14**, 9, 2005, pp. 1237-1245.

[4] L.J. van Vliet, D. Sudar, I.T. Young, "Cell Biology", 2nd ed. New York: Academic, 1998, vol. III, pp. 109-120.

[5] J.B. Pawley, "Handbook of Biological Confocal Microscopy", Third ed., Plenum press, 1995.

[6] Y.P. Tan, I. Llano, "Fast Scanning and efficient photodetection in a simple two-photon microscope", *J. Neurosci. Meth.*, **92**, 1-2, 1999, 123-135.

[7] K.D. McCarthy, L.M. Partlow, "Preparation of pure neuronal and non-neuronal cultures from embryonic chick sympathetic ganglia: a new method based on both differential cell adhesiveness and the formation of homotypic neuronal aggregates", *Brain Res*., vol. **114**, pp. 391-414, 1976.

[8] A.K. Jain, "Digital Signal Processing", Prentice-Hall, 1989.

[9] A. Bullen, P. Saggau, "High-speed, random-access fluorescence microscopy: II Fast quantitative measurements with voltage-sensitive dyes", Biophys. J., **76**, 1999, pp. 2272-2287.