AN OVERVIEW OF IMAGE ANALYSIS IN MULTIDIMENSIONAL BIOLOGICAL MICROSCOPY

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ABSTRACT

Following an overview of image analysis applications in 2D and 3D dynamic biological microscopy, we present work developed in our laboratory dedicated to two central aspects of cell biology of infection, cell shape and motility analysis and particle tracking. We describe a fully automatic segmentation and tracking method designed to enable quantitative analyses of cellular shape and motion from 4D (3D+t) microscopy data. To get at a better understanding of pathogens/host cell interactions and to document infectious disease processes in living systems, it is necessary to characterise the dynamic properties of pathogens. We describe a method to detect and track multiple moving biological spot-like particles showing different kind of dynamics in image sequences acquired through multidimensional fluorescence microscopy.

1. INTRODUCTION

The advent of multidimensional microscopy (real-time optical sectioning and confocal, TIRF, FRET, FRAP, FLIM) has enabled biologists to visualize cells, tissues and organs in their intrinsic 3D and 3D+t geometry, in contrast to the limited 2D representations that were available until recently. These new technologies are already impacting biological research in such different areas as high-throughput image-base drug screening, cellular therapies, cell and developmental biology and gene expression studies, as they are putting at hand the imaging of the inner working of living cells in their natural context. Expectations are high for breakthroughs in areas such as cell response and motility modification by drugs, control of targeted sequence incorporation into the chromatin for cell therapy, spatial-temporal organization of the cell and its changes with time or under infection, assessment of pathogens routing into the cell, interaction between proteins, sanitary control of pathogen evolution, to name but a few. Deciphering the complex machinery of cell functions and dysfunction necessitates large-scale multidimensional image-based assays to cover the wide range of highly variable and intricate properties of biological material. However, understanding the wealth of data generated by multidimensional microscopy depends critically on decoding the visual information contained therein. Over the last few years, this area of research has fuelled a full array of challenging topics for the signal and image processing community, ranging from adaptive image acquisition [1], segmentation [2], automated analysis of complex protein patterns [3] to mathematical microscopy [4] and localisation at the nanoscale [5, 6].

Within the vast interdisciplinary field of biological imaging, this paper concentrates on two aspects central to cell biology, particle tracking and cell shape and motility analysis, which have many applications in the important field of infectious diseases. While giving a panorama of state-of-the art work in these topics, we put a particular emphasis on work developed in our laboratory.

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2. PARTICLE TRACKING

Molecular dynamics in living cells is a central topic in cell biology, as it opens the possibility to study with sub-micron resolution molecular diffusion, spatio-temporal regulation of gene expression and pathogen motility and interaction with host cells. For example, it is possible, after labelling with specific fluorochromes, to record the movement of organelles like phagosomes or endosomes in the cell [7], the movement of different mutants of bacteria or parasites [8] or the positioning of telomeres in nuclei [9]. In many cases, the biological fluorescent targets are visualised as moving bright spots that need to be localized and associated into tracks, from which the number, position, spatial distribution, movement phases and diffusion coefficients can be estimated and used to quantify the biological dynamics. When analysing moving biological spots the major difficulties are primarily due to the facts that, at least in 2D microscopy, the spots frequently go in and out of focus, and that their appearance can change, making detection very challenging. In addition, the fact that spots may aggregate and change their individual dynamics over time makes tracking a very difficult problem.

Several methods have been proposed to tackle the problem in 2D [10, 11, 12, 13, 14, 15]. Some of these methods are based on intensity thresholding [10], template matching [11]or local maxima extraction [12] for detecting the spots and on nearest neighbour association (NNA) [10] or constrained NNA [12] to perform the tracking. In [13], a method using the combination of four techniques, namely highly sensitive object detection, fuzzy logic-based dynamic object tracking, computer graphical visualisation, and measurement in time-space is proposed to track well separated cells. The method in [14] is based on concepts from operational research and graph theory, and it proceeds in four steps: particle detection, generation of candidate matches, i.e., a set of possible displacement vectors between successive frames; scoring of candidate matches and selection of the candidate subset with maximum global score and no topological ambiguity. The method in [15] does not use the traditional frame-by-frame approach but rather considers the whole sequence as a spatiotemporal volume where the tracks are defined as minimal paths in an image-dependent metric.

Tracking in 3D+t has been addressed only more recently, either in the case of single particles [16] or multiple particles [6, 17, 18]. The method in [16] uses a matched filter as a prefiltering step to a dynamic programming procedure that extracts the trajectory. The method in [6] uses a 3D PSF model for spot detection and associates

spots on the basis of the weighted minimal distance between them. In the following we describe the methods we have developed to perform the detection and the tracking of microscopic spots directly on four dimensional (3D+t) image data [17, 18]. They are able to detect with high accuracy multiple biological objects moving in three-dimensional space and incorporates the possibility to follow moving spots switching between different types of dynamics. Our methods decouple the detection and the tracking processes and are based on a two step procedure: first, the objects are detected in the image stacks thanks to a procedure based on a three-dimensional wavelet transform; then the tracking is performed within a Bayesian framework where each object is represented by a state vector evolving according to biologically realistic dynamic models.

2.1. Spot detection

In [17], we proposed a spot detection method based on a multiscale approach that uses a shift invariant discrete wavelet transform and on the selective filtering of wavelet coefficients. This scheme allows to separate and characterize objects of different sizes by selecting only a vicinity of detail images at scales adapted to the size of the spots. The extraction step consists in retaining the significant responses of the locally supported detail signal filter to the desired features, at the different scales of the wavelet representation. This is accomplished through a denoising technique using a threshold value which is image and scale dependent and which can be computed automatically from the data. The main advantage of wavelet-based detection is to be robust to the local variation of contrast and to efficiently segment fluorescent spots imbedded in noisy images such as those acquired in biological microscopy.

2.2. Bayesian multitarget tracking

Bayesian target tracking methods consist in filtering successive measurements coming from a detector and to compute the posterior distribution from the set of already detected measurements. When tracking one target with no clutter, there is no association problem and optimal Bayesian filters such as Kalman filter [19] and Grid Based Filter [20] or suboptimal Bayesian filters such as Extended Kalman Filter [19], and Particle Filter (PF) [20] are well suited to build the successive probability density functions of the actual target state. However, all the previous filters are bound to use just one dynamic model in their scheme which is problematic when the objects' dynamics vary with time as it is the case with biological objects. This is why in our applications we have used instead the Interacting Multiple Models (IMM) filter [21]. The IMM has been designed, first in the context of radar imaging, with the capability to have different models in parallel and to select and switch to the model which is more accurate to represent the movement during a given period. The IMM has the additional ability to rapidly self-adapt to transitions. This makes the IMM an ideal choice for tracking biological objects. In [18], we proposed to use three different models of dynamics adapted to biological object dynamics: random walk, first order linear extrapolation and second order linear extrapolation. They model respectively Brownian motion and directed movement with constant speed or acceleration, which are representative modes of motion encountered in biology [22]. We also made the additional realistic hypothesis that during movement, the biological objects can switch abruptly between the three models.

In presence of clutter and ill-performing detection, several algorithms exist to resolve the association problem [23]. The most efficient one is probably the Probabilistic Data Association Filter [19, 23]. In the case of several targets in clutter, the problem of association is of major importance. The most popular algorithms to solve the association problem are the Joint PDAF [23] and the Multi Hypothesis Tracking [24]. Both algorithms however present important limitations by making the two following assumptions: i) a target can generate at most one measurement per scan and ii) a measurement could have originated from at most one target. In theory, a *perfect* detector should give exactly one measurement per tracked object and enable a 2-D optimal assignment algorithm as [25] to find the association which maximizes the joint association event probability of the Bayesian filter. When tracking a high number of deformable sources in real world applications, the previous assumptions are often not met due to spurious detections, causing existing methods to fail. We therefore proposed in [26] an algorithm which allows to perform the tracking in cases when a single target generates several measurements or several targets generate a single measurement. The novel idea presented in that paper was the introduction of a set that we called *virtual measurement* set which supersedes and extends the set of measurements. This set is chosen to optimally fit the set of *predicted measurements* at each time step. This is done in two stages: 1. a set of feasible joint association events is built from *virtual measurements* that are created by successively splitting and merging *real measurements*; 2. the joint probability is maximized over all feasible joint association events. In contrast to other Bayesian approaches, our technique allows tracking to succeed in difficult cases where multiple objects are detected as a single object or, conversely, when a single object is detected as several sub-objects.

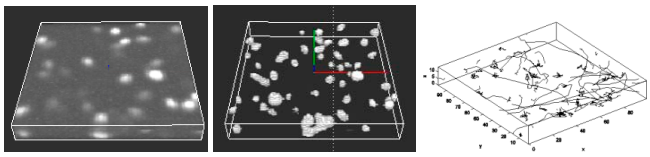

Fig. 1. Tracking of fluorescent objects in 3D+t. (a) original image stack. (b) result of detection. (c) view of the tracks.

3. CELL TRACKING WITH ACTIVE CONTOURS AND SURFACES

Another important project of our lab is motivated by the problem of cell motility. The ability of cells to move and change their shape is important in many important areas of biology, including cancer, development, infection and immunity. Our aim is to develop algorithms to segment and track moving cells in dynamic 2D or 3D microscopy. For this purpose, we have adopted the framework of active contours and deformable models that is widely employed in the computer vision community, e.g. [27, 28, 29, 30]. The segmentation proceeds by evolving the front according to evolution equations that minimize an energy functional (usually by gradient descent). This energy contains both data attachment terms and terms encoding prior information about the boundaries to be extracted, e.g. smoothness constraints. Tracking, i.e. linking segmented objects between time points, is simply achieved by initializing front evolutions using the segmentation result of the previous frame, under the assumption that inter-frame motions are modest. In the following, we describe some of our work on adapting these methods to the needs of cellular imaging in biological research.

3.1. Region based image energies

An important part of a deformable model method is the choice of the image-dependent energy terms. This choice obviously depends on

the type of imaging used. In fluorescence microscopy, the intensity of cells stained with cytoplasmic markers is on average larger than that of the unstained background, but the signal to noise ratio may be very low due to limitations on exposure time, especially in dynamic 3D imaging. Gradient based active contour methods such as [27], where the image energy is the integral of a locally computed edge map along the contour only, give poor results on such images, where boundaries are often fuzzy, and are very sensitive to initialization. After first attempts with gradient vector flow, where intensity gradients are allowed to diffuse throughout the image [28, 31], we turned to a region oriented approach, where the image energy is computed from surface integrals over the entire image domain. Specifically, we adopted the model of active contours without edges from [29], where image energy terms are the intensity variances inside and outside the contour [32, 33, 34]. This model provides strong robustness to noise and allows segmentation of cells with blurred edges. We confirmed this quantitatively on simulated images of cells image with a realistic diffraction limited point spread function causing blur, and at low signal to noise ratios [33].

Non fluorescent imaging techniques, such as phase contrast or differential interference contrast microscopy, are popular because they are inexpensive and allow observations over extended time periods. In such images, the average intensity level of cells is often similar to that of the background, but the cells generally appear as textured objects due to intracellular structures such as vesicles. We found that the local average intensity deviation is a simple but efficient filter that highlights the interior of cells and in effect transforming phase contrast images into fluorescence-like images, and allowing us to use the region energy of [29, 32, 34] to discriminate cells from the background.

Although region oriented models are far more robust than purely edge oriented models, using gradient information as an additional energy term [33] can still improve the segmentation in cases where cell edges are strong, for instance for fluorescently labeled cell membranes, or when region information is locally absent, for instance for vesicle-free pseudopodia imaged with non-fluorescent microscopy [31] as illustrated in Fig. 2.

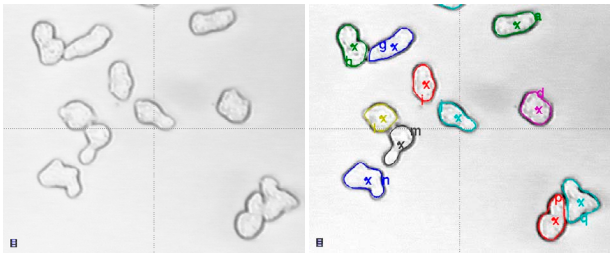

Fig. 2. Tracking of amoebae with parametric active contours. (a) amoeba on a glass slide imaged with phase contrast video microscopy. (b) computed contours.

3.2. Boundary representation, topology, and touching cells

Next to the energy, the other main technical ingredient of a deformable model is the mathematical representation of the boundary, which is generally done either explicitly, using parametric curves or meshes, e.g. [27, 28], or implicitly using the level set method [30, 29], where the front is defined as the zero level set of a scalar function defined over the image domain. Both approaches have distinct advantages and drawbacks for cellular imaging, and we currently employ both of them in diverse applications. The main differences concern the handling of topology and computational speed. It is well known that level set methods are well suited to handle topological changes of the evolving front. This in turn allows automatic detection of spatially isolated cells at the onset of processing [32], of dividing cells [33], and is particularly suited to handle 3D data [33], in contrast to parametric models. Despite these advantages, we still employ parametric active contour methods for large 2D image sequences [31], essentially because level set methods suffer from comparatively large computational cost.

Another important point for cell tracking concerns the handling of touching cells: if previously isolated cells move together and touch, most level set methods will merge the associated objects, because they allow no constraints on object topology [32, 33]. We have however overcome this limitation by employing multiple level set functions coupled by a non-overlap constraint [32, 33]. Parametric contours by construction maintain the topology of already identified objects. However independently evolving parametric active contours will also lead to incorrect segmentations (each contour will tend to swallow all touching cells). To prevent this, we have described a method that couples the active contours via a single multiple-contour energy that includes non-overlap constraints [34].

Non-overlap constraints are sufficient to keep track of cell identity, but cannot guarantee correct segmentations when boundary information at the cell-cell interface is weak. In the worst cases, one contour or surface may encompass all objets while the other models collapse. To avoid this, we introduced an additional energy term that penalizes changes of cell volume (in 3D images). This is justified by the biophysical property of cell volume homeostasis [33].

4. CONCLUSION

We have presented a short review of methods that have been developed in the area of 3D and 3D+t image analysis tools for multidimensional biological microscopy. These methods have been adapted to the increased dimensionality of the data and are able in a number of cases to perform the automated processing and analysis of multichannel temporal 3D sequences. Many more developments are however needed to tackle the challenges posed by biological imaging: huge amounts of data, high variety of imaging protocols, high variability of the biological objects, huge amounts of cellular players. Many of the techniques needed in this field of imaging are still challenging topics of research in applied mathematics and image processing and have not been considered in the context of multidimensional microscopy. A major interdisciplinary effort is therefore required to provide the new tools that are critically needed for the advancement of molecular medicine and biotechnology-based health care strategies.

5. REFERENCES

[1] T.E. Merrymann and J. Kovacevic, "An Adaptive Multirate Algorithm for Acquisition of Fluorescence Microscopy Data Sets," *IEEE Trans. Image Processing*, vol. 14, no. 9, pp. 1246–1253, 2005.

[2] C. Ortiz-de-Solorzano, R. Malladi, S. A. Lelièvre, and S. J. Lockett, "Segmentation of Nuclei and Cells using Membrane Related Protein Markers," *J. Microscopy*, vol. 201, no. 3, pp. 404–415, March 2001.

[3] T. Zhao, M. Velliste, M.V. Boland, and R.F. Murphy, "Object Type Recognition for Automated Analysis of Protein Subcellular Location," *IEEE Trans. Image Processing*, vol. 14, no. 9, pp. 1351–1359, 2005.

[4] M.G. Gustafsson, "Nonlinear Structured-Illumination Microscopy: Wide-Field Fluorescence Imaging with Theoretically Unlimited Resolution," *PNAS*, vol. 102, no. 37, pp. 13081–13086, 2005.

[5] J. Gelles, B. J. Schnapp, and M.P. Sheetz, "Tracking kinesin-driven movements with nanometre-scale precision," *Nature*, vol. 331, pp. 450–453, 1988.

[6] D. Thomann, D.R. Rines, P.K. Sorger, and G. Danuser, "Automatic Fluorescent Tag Detection in 3D with Super-Resolution: Application to the Analysis of Chromosome Movement," *J. Microsc.*, vol. 208, pp. 49–64, October 2002.

[7] C. Murphy, R. Saffrich, J.-C. Olivo-Marin, A. Giner, W. Ansorge, T. Fotsis, and M. Zerial, "Dual function of rhod in vesicular movement and cell motility.," *Eur. Journal of Cell Biology*, vol. 80, no. 6, pp. 391–398, 2001.

[8] F. Frischknecht, P. Baldacci, B. Martin, C. Zimmer, S. Thiberge, J.-C. Olivo-Marin, S. L. Shorte, and R. Menard, "Imaging Movement of Malaria Parasites during Transmission by Anopheles Mosquitoes.," *Cell Microbiol.*, vol. 6, no. 7, pp. 687–94, Jul 2004.

[9] V. Galy, J.-C. Olivo-Marin, H. Scherthan, V. Doyle, N. Rascalou, and U. Nerhbass, "Nuclear Pore Complexes in the Organization of Silent Telomeric Chromatin," *Nature*, vol. 403, pp. 108–112, 2000.

[10] J. Apgar, Y. Tseng, E. Federov, M.B. Herwig, S.C. Almo, and D. Wirtz, "Multiple-particle tracking measurements of heterogeneities in solutions of actin filaments and actin bundles," *Biophys. J.*, vol. 79, pp. 1095–1106, 2000.

[11] F. Briquet-Laugier, C. Boulin, and J.-C. Olivo-Marin, "Analysis of moving biological objects in video microscopy sequences.," *Proc. of SPIE*, vol. 3642, pp. 1–11, 1998.

[12] J.C. Crocker and D.G. Grier, "Methods of digital video microscopy for colloidal studies," *J. Colloid Interface Sci.*, vol. 179, pp. 298–310, 1996.

[13] W. Tvarusko, M. Bentele, T. Misteli, R. Rudolf, C. Kaether, D. L. Spector, H. H. Gerdes, and R. Eils, "Time-resolved analysis and visualization of dynamic process in living cells.," *PNAS*, vol. 96, pp. 7950–7955, 1999.

[14] P. Vallotton, A. Ponti, C.M. Waterman-Storer, E.D. Salmon, and G. Danuser, "Recovery, visualization, and analysis of actin and tubulin polymer flow in live cells: a fluorescent speckle microscopy study," *Biophys. J.*, vol. 85, pp. 1289–1306, 2003.

[15] S. Bonneau, M. Dahan, and L.D. Cohen, "Single quantum dot tracking based on perceptual grouping using minimal paths in a spatiotemporal volume," *IEEE Trans. Image Processing*, vol. 14, no. 9, pp. 1384– 1395, 2005.

[16] D. Sage, F.R. Neumann, F. Hediger, S. Gasser, and M. Unser, "Automatic tracking of individual fluorescence particles: Application to the study of chromosome dynamics," *IEEE Trans. Image Processing*, vol. 14, no. 9, pp. 1372– 1383, 2005.

[17] A. Genovesio, B. Zhang, and J.-C. Olivo-Marin, "Tracking of multiple fluorescent biological objects in three dimensional video microscopy," *IEEE International Conference on Image Processing ICIP 2003*, vol. I, pp. 1105–1108, September 2003, Barcelona, Spain.

[18] A. Genovesio and J.-C. Olivo Marin, "Interracting multiple model based method to track moving fluorescent biological spots," *IEEE International Symposium on Biomedical Imaging, ISBI'04*, pp. 1239–1242, April 2004, Arlington, USA.

[19] Y. Bar-Shalom and T.E. Fortmann, *Tracking and Data Association*, Academic Press, New-York, 1988.

[20] M.S. Arulampalam, S. Maskell, N. Gordon, and T. Clapp, "A tutorial on particle filters for online nonlinear/non-gaussian bayesian tracking," *IEEE Transaction on Signal Processing*, vol. 50, no. 2, pp. 174–188, 2002.

[21] H. A. P. Blom and Y. Bar-Shalom, "The interacting multiple model algorithm for systems with markovian switching coefficients," *IEEE Transaction on Automatic Control*, vol. 33, no. 8, pp. 780–783, August 1988.

[22] M. Lakadamyali, M.J. Rust, H.P. Babcock, and X. Zhuang, "Visualizing infection of individual influenza viruses," *PNAS*, vol. 100, pp. 9280–9285, 2003.

[23] Y. Bar-Shalom and W. Dale Blair, *Multitarget-Multisensor Tracking Applications and Advances*, vol. III, Artech House, Oct 2000.

[24] D. B. Reid, "An algorithm for tracking multiple targets," *IEEE Transaction on Automatic Control*, vol. AC-24, pp. 843–854, December 1979.

[25] R. Jonker and A. Volgenant, "A shortest augmenting path algorithm for dense and sparse linear assignment problems.," *Computing*, vol. 38, pp. 325–340, 1987.

[26] A. Genovesio and J.-C. Olivo Marin, "Split and merge data association filter for dense multi-target tracking," *ICPR 2004 (International Conference on Pattern recognition)*, August 2004, Cambridge (UK).

[27] M. Kass, A. Witkin, and D. Terzopoulos, "Snakes: Active Contour Models," *Int'l J. Computer Vision*, vol. 1, pp. 321–331, 1988.

[28] C. Xu and J. L. Prince, "Snakes, Shapes, and Gradient Vector Flow," *IEEE Trans. Image Processing*, vol. 7, no. 3, pp. 359–369, 1998.

[29] T.F. Chan and L.A. Vese, "Active Contours Without Edges," *IEEE Trans. Image Processing*, vol. 10, no. 2, pp. 266–277, 2001.

[30] J.A. Sethian, *Level Set Methods and Fast Marching Methods*, Cambridge University Press, New York, 1999.

[31] C. Zimmer, E. Labruyère, V. Meas-Yedid, N. Guillén, and J.-C. Olivo-Marin, "Segmentation and Tracking of Migrating Cells in Videomicroscopy with Parametric Active Contours," *IEEE Trans. Medical Imaging*, vol. 21, pp. 1212–1221, 2002.

[32] B. Zhang, C. Zimmer, and J-C. Olivo-Marin, "Tracking Fluorescent Cells With Coupled Geometric Active Contours," *Proc. IEEE Int'l Symp. Biomedical Imaging (ISBI 2004)*, pp. 476–479, 2004.

[33] A. Dufour, V. Shinin, S. Tajbakhsh, N. Guillén, J.-C. Olivo-Marin, and C. Zimmer, "Segmenting and Tracking Fluorescent Cells in dynamic 3-D Microscopy with Coupled Active Surfaces," *IEEE Trans. Image Processing*, vol. 14, no. 9, pp. 1396–1410, September 2005.

[34] C. Zimmer and J.-C. Olivo-Marin, "Coupled Parametric Active Contours," *IEEE Trans. Pattern Analysis and Machine Intelligence*, vol. 27, no. 11, pp. 1838–1842, 2005.