Medical Image Analysis 20 (2015) 34–51

Contents lists available at ScienceDirect

Medical Image Analysis

journal homepage: www.elsevier.com/locate/media

Automated segmentation and tracking of non-rigid objects in time-lapse microscopy videos of polymorphonuclear neutrophils

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ARTICLE INFO

Article history:
Received 23 January 2014
Received in revised form 28 September 2014
Accepted 11 October 2014
Available online 8 November 2014

Keywords:
Tracking of non-rigid objects
Segmentation
Time-lapse microscopy
Polymorphonuclear neutrophils

ABSTRACT

Time-lapse microscopy is an important technique to study the dynamics of various biological processes. The labor-intensive manual analysis of microscopy videos is increasingly replaced by automated segmentation and tracking methods. These methods are often limited to certain cell morphologies and/or cell stainings. In this paper, we present an automated segmentation and tracking framework that does not have these restrictions. In particular, our framework handles highly variable cell shapes and does not rely on any cell stainings. Our segmentation approach is based on a combination of spatial and temporal image variations to detect moving cells in microscopy videos. This method yields a sensitivity of 99% and a precision of 95% in object detection. The tracking of cells consists of different steps, starting from single-cell tracking based on a nearest-neighbor-approach, detection of cell–cell interactions and splitting of cell clusters, and finally combining tracklets using methods from graph theory. The segmentation and tracking framework was applied to synthetic as well as experimental datasets with varying cell densities implying different numbers of cell–cell interactions. We established a validation framework to measure the performance of our tracking technique. The cell tracking accuracy was found to be >99% for all datasets indicating a high accuracy for connecting the detected cells between different time points.

1. Introduction

Cell migration plays an important role in many physiological processes, including embryonic morphogenesis, tissue repair, mitosis, and immune response, as well as pathological processes, such as carcinogenesis, tumor growth, and metastasis (Ridley et al., 2003). Specific cues, such as chemokines and growth factors, can induce the direction of migration (chemotaxis) and/or the migration activity (chemokinesis). It is important to identify key regulatory mechanisms and to understand the molecular basis underlying cell migration. This is a challenging problem but may eventually reveal potential targets for pharmacological interventions in diseases involving cell migration.

Image-based systems biology includes live cell microscopy as a powerful approach to study the behavior of cells in biological systems. Imaging of biological systems combined with the automated analysis for functional, dynamical, and morphological aspects is required to increase our understanding of complex processes. In the context of cell migration, this typically includes tracking and quantification of large numbers of cells and events. To date, manual cell tracking is still a common method of choice for analyzing cells within these time-lapse movies. However, in addition to being very labor-intensive and time consuming, results of manual cell tracking can be subjective. User-dependent estimations of cell positions (usually cell centroids) and the selection of subsets of representative cells has a high impact on the tracking outcome and the interpretation of cell migration patterns (Huth et al., 2010). Additionally, complex features, such as cell morphology and contour features, can hardly be captured and quantified by manual measurements. Therefore, automated segmentation and tracking algorithms are urgently needed to overcome the limitations of manual cell tracking and to enable high-throughput analysis of microscopy time-lapse images. These algorithms face several challenges including low signal-to-noise ratios, dynamic cell morphologies, high cell densities, cluster formation, or cells

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http://dx.doi.org/10.1016/j.media.2014.10.002
1361-8415/

moving in and out of the frame or focal plane. These issues require sophisticated segmentation and tracking methods.

Here, we present a fully automated multi-target segmentation and tracking framework for non-rigid objects, which is applied for migration studies of unstained polymorphonuclear neutrophils (PMNs). PMNs have a central role in innate immunity and represent 50–70% of circulating leukocytes in humans. They are essential for defending the host against invading microorganisms. Several mechanisms enable PMNs to eliminate pathogens intra- and extracellularly including phagocytosis, degranulation, release of antimicrobial substances, or formation of neutrophil extracellular traps (NETs) (Kolaczkowska and Kubes, 2013; Nathan, 2006; Brinkmann et al., 2004; Véronique et al., 2000).

The segmentation and tracking framework involves the detection of unstained cells with the peculiar feature to exhibit a highly variable morphology. The non-rigidity of cells is challenging for accurate tracking techniques, since shape or size features cannot be used to correctly identify cells between consecutive frames. We developed a segmentation technique that detects moving objects based on local and temporal gray level variations of the images. This approach allows detection of cells independent of their morphology and image illumination. Our tracking method first creates segments of cell trajectories (tracklets) for time frames where no interactions with other cells occur. Here, we assume a random motion model to extract cell migration patterns in an objective fashion. Then, cell–cell interactions in form of touchings and cluster formation are analyzed and the position of individual cells are estimated to subsequently extend the cell tracks. Finally, tracklets are globally combined allowing for gaps between them to handle segmentation and classification errors.

To evaluate the performance of the presented automated segmentation and tracking algorithm we constructed a framework of performance measures. Beside standard features, like the number of false positive and false negative tracks, we also evaluate track fragmentations and tracklet merging errors. We test and evaluate the segmentation and tracking technique on synthetic and experimental data, where the latter are compared to the results of a manual analysis.

2. Related work

This section aims to give an overview about common segmentation and tracking techniques for microscopy images and videos. We outline advantages and disadvantages for the discussed image processing approaches. Cell segmentation methods for microscopy and medical images are reviewed by Meijering (2012), Pham et al. (2000), and Rittscher (2010), while cell tracking methods are reviewed by Chenouard et al. (2009b), Meijering et al. (2006), Meijering et al. (2009), Meijering et al. (2012), Rittscher (2010), Yilmaz et al. (2006). Chenouard et al. (2014) and Maška et al. (2014) provide benchmark datasets and a comparison of different tracking algorithms for fluorescence microscopy data.

2.1. Segmentation

A large variety of image processing techniques exists for the segmentation of individual non-stained cells in microscopy images. The presumably simplest kind of approaches are background subtraction, using a mean or median image computed from the whole set of images, and global thresholding. These techniques produce satisfactory results on images with cells that have significantly different intensity values compared to the background. However, they are very sensitive to noise and to spatial as well as temporal illumination changes. More enhanced techniques provided by adaptive thresholding (local thresholding) or by Otsu's method (Otsu, 1975) increase the segmentation accuracy, but these methods suffer from shortcomings as far as relationships among neighboring pixels are not included.

Edge detection methods based on the gradient of the image (first order derivative), *e.g.*, Sobel, Scharr, *etc.*, are used to detect cell boundaries. Methods involving the second derivative (LoG, DoH, DoG) are used to detect blobs and the advantage of these linear filtering techniques is that they are fast and easy to compute. However, the disadvantage of these methods is that they typically require additional post-processing steps. Edge thinning and linking is for example done with the Canny edge detector (Canny, 1986). Non-linear filtering utilized in morphological operations strongly relies on cell morphology and shape and requires knowledge about these features as prior assumptions. Its application potential for non-rigid objects is therefore very limited.

Deformable models for image segmentation are based on parametric curves in 2D (active contours, snakes) or surfaces in 3D (active meshes) that minimize a predefined energy functional, which includes image-based and shape-based energy terms (Kass et al., 1988). This method requires knowledge about the image content and object shapes to taylor the energy-terms to the specific segmentation problem. Additionally, the positioning of active contours required for initialization is often done manually and the number of deformable models has to be pre-estimated from the number of objects in the image.

Machine learning approaches for cell segmentation can be categorized into two groups: supervised and unsupervised methods. Supervised methods use features of a learning dataset with known labels to generate inter-class discriminators (*e.g.*, *k*-nearest-neighbor classifier, Parzen Window, Support Vector Machines, decision trees, *etc.*). Nunez-Iglesias et al. (2013) demonstrate hierarchical agglomerative clustering from superpixels in neural tissue. Zaritsky et al. (2011) use a cascade of support vector machines (SVMs) including textural features in the context of wound healing and scatter assays. Unsupervised learning methods usually cluster data dependent on pre-defined features recursively and recompute parameters for each class until stopping criteria are reached. This includes *k*-means, fuzzy *c*-means algorithm, expectation maximization (EM), *etc.* Permuter et al. (2006) demonstrate image segmentation with a Gaussian mixture model (GMM) learned from colored texture and structure features. Liang et al. (2010) use a GMM trained with image intensities to detect particles in fluorescence images. For the performance of learning methods and classification the selection of appropriate image and object features is crucial.

2.2. Tracking

Tools for automated tracking of cells and particles have been developed since the early 1980s (Meijering et al., 2006). The development of robust tracking algorithms faces new challenges by ongoing advances in microscopy and imaging technologies. To date, most biological applications require their own sophisticated tracking techniques that differ, for example, with regard to the assumptions about the underlying motion model or noise parameters.

Automated cell tracking techniques can be divided into two major groups: (1) deterministic approaches and (2) probabilistic approaches. Deterministic approaches can be further divided into (1.1) independent segmentation followed by frame-to-frame association and (1.2) time-dependent evolution of models.

Deterministic approaches that segment images first and build associations between the data points of consecutive frames in a second step require sophisticated approaches for object linking. The nearest-neighbor-association (NNA) approach links objects dependent on a distance measure that is *e.g.*, based on distances between cell centroids. Including other cell features, such as color