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Improving reliability of live/dead cell counting through automated image mosaicing

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ABSTRACT

Cell counting is one of the basic needs of most biological experiments. Numerous methods and systems have been studied to improve the reliability of counting. However, at present, manual cell counting performed with a hemocytometer still represents the gold standard, despite several problems limiting reproducibility and repeatability of the counts and, at the end, jeopardizing their reliability in general. We present our own approach based on image processing techniques to improve counting reliability. It works in two stages: first building a high-resolution image of the hemocytometer's grid, then counting the live and dead cells by tagging the image with flags of different colours. In particular, we introduce *GridMos* (http://sourceforge.net/p/gridmos), a fully-automated mosaicing method to obtain a *mosaic* representing the *whole* hemocytometer's grid. In addition to offering more significant statistics, the mosaic "freezes" the culture status, thus permitting analysis by more than one operator. Finally, the mosaic achieved can thus be tagged by using an image editor, thus markedly improving counting reliability. The experiments performed confirm the improvements brought about by the proposed counting approach in terms of both reproducibility and repeatability, also suggesting the use of a mosaic of an entire hemocytometer's grid, then labelled trough an image editor, as the best likely candidate for the new gold standard method in cell counting.

1. Introduction

Since the invention of the hemocytometer (attributed to Louis-Charles Malassez, 1842–1909), cell counting has become a common procedure performed routinely in thousands of laboratories all over the world. Furthermore, at the beginning of the 20th century, with the introduction of the cell staining in standard analyses, *e.g.* Trypan Blue to assess cell viability [1], live and dead cell counting became one of the most common biological procedures [2]. Nowadays, obtaining accurate counts of cells still is a crucial issue for many applications in different fields [3], such as industrial manufacturing [4], botany [5], biology [6], veterinary medicine [7], and human

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medicine [8]. In oncology, for example, live and dead cell counting is typically performed to monitor the viability of cancer cells after different chemical [9] or radiobiological treatments [10]. Count accuracy can strongly influence the choice of treatment [11] and also lead to adjustments in the chemotherapeutic protocol [12] and in the drugs dosage administered to the patient [13].

At present, a wide number of methods and systems are available for cell counting [14] ranging from fully automated commercial instruments [15] and flow cytometry systems [16], to image processing methods [17] also embedded in software provided in bundle with automated microscope or image acquisition systems [18]. New approaches are constantly being developed [19]. However, the original method of manual cell counting using a hemocytometer and a manual tally counter together with a light widefield microscope, the most widely used approach over the last three decades [4], still represents the gold standard [20] as well as the predominant method [21]. A recent study reports that more than 70% of operators use a hemocytometer each day for counting cells [22]. Nonetheless, the use of a hemocytometer and a tally counter leads to subjectivity [21] and, if more operators are used to improve accuracy, overall reliability may be jeopardized by cell conditions changing between counts.

In this work we present a computer vision-based approach to improve reproducibility (inter-rater reliability) and repeatability (intra-rater reliability) of the gold standard counting method. In particular, we propose a non-invasive method to "freeze" the status of the cells in the hemocytometer that can then be used for repeated counting by one or more operators, independently of each other. This is achieved through *GridMos* (http://sourceforge.net/p/gridmos), a multi-image based method written in MATLAB (©, The MathWorks, Inc., MA, USA), that yields one high resolution mosaic [23] from several images depicting overlapping parts of the whole hemocytometer's grid. The procedure of "fusing" several images into only one has various names, *e.g.* image registration, aligning, or, more generically, mosaicing [24]. In particular, the mosaicing methods permit to overcome the trade-off between pixel resolution and width of sensor's Field Of View (FOV, area of the sample represented into the image [25]) obtaining one final large image. Furthermore, counting cells using a single mosaic rather than a set of images, provides a more robust approach to solve the problem of establishing *a priori* a representative field of the whole sample [26] and preventing double counts, for instance in case of same cells repeated between images or at the edge of the different FOVs [27].

Images in histology and cytology are normally obtained by using a light widefield microscope [28], which is not typically endowed with motorized stage [29]. Accordingly, several mosaicing software tools are typically used to obtain a mosaic of a set of overlapping images manually acquired. The first solution is offered by tools needing a manual outline of the images layout to achieve the final mosaic, such as *MosaicJ* [30]. Alternatively, in other tools such as *Hugin* panorama stitching software (http://sourceforge.net/p/hugin), the registration scheme is defined by selecting one of the available models, that however are not suitable for the hemocytometer's grid. Finally, several fully-automatic solutions have been then proposed to obtain mosaics without requiring any prior information [31,32]. However, these tools typically fail when used to registered images with highly repeated patterns only, such as the squared blocks of an empty hemocytometer's grid. In general, very few stitching tools can be used with effectiveness also to obtain a mosaic of microscopy images [33], *Microsoft Image Composite Editor* (http://research.microsoft.com/en-us/um/redmond/groups/ivm/ICE/) and *Autostich* [34] among them, originally proposed for outdoor image mosaics. Nevertheless, registration and image blending are not fully described in *Microsoft Image Composite Editor* [35] and *Autostich* forces on adopting a homographic registration model, often degenerating in a distorted mosaic when used with hemocytometer's images. Furthermore, some of these methods fail to define the correct shift between the images to be stitched when reference features are not marked, as in the case of images characterized by a very low contrast (*e.g.*, brightfield images of cells [36]).

GridMos has been designed specifically to automatically obtain mosaics of hemocytometer's images acquired by manually moving the sample holder of the microscope. In practice, it is an extension of the mosaicing method presented in one of our previous studies [31] as regards the spatial registration strategy that we implemented from scratch to correctly stitch images with repeated patterns. In particular, to determine where the images must be stitched, the overlapping region between each pair of subsequent images is analyzed to detect meaningful correspondences and, consequently, the right spatial shift considering the first image acquired as the reference one. In practice, in order to accurately register the images of the hemocytometer's grid, we devised and implemented a proper frequency domain approach based on the analysis of the peaks of phase correlation. Furthermore, we developed an effective "blending" technique to make the final mosaic seamless and consistent so as to fix the problem of visible seams in the stitching zones (*i.e.*, colours inhomogeneity and vignetting [37]), which could lead to incorrect cell counting, above all in automatic methods [38]. As a matter of fact, manual counting is a very tedious task and often operators when possible try exploiting, in the very early stage, automatic software to assist them in tagging what, at a first sight, appear to be single cells.

In our counting approach, once achieved the mosaic of the whole hemocytometer's grid (obtained with *GridMos* or, in principle, with other software tools considered accurate enough for one's own purposes), an image editor software is used to count the cells by manually adding flags of different colours [39]. The idea to count the cells by tagging an image is not new [40]. Many works report comparisons of automatic image processing methods with or the manual cell counting performed by looking to the microscope's oculars [41], or by tagging an image [42]. It is worth mentioning the work of Drury *et al.* [43], where counts performed by using labelled images where compared to counts obtained by looking at the same images and counting visually trough a manual tally counter (*i.e.*, without adding flags). Nevertheless, two operators only were enrolled to count, and they did not even analyzed live and dead cells separately. Recently, Young *et al.* [44] improved the statistics by asking four operators to count, but they did not report results regarding either inter-observer variability or dead cells. In conclusion, no statistical analyses have been