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Notes & Tips

Automatic cell counting with ImageJ

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ABSTRACT

Cell counting is an important routine procedure. However, to date there is no comprehensive, easy to use, and inexpensive solution for routine cell counting, and this procedure usually needs to be performed manually. Here, we report a complete solution for automatic cell counting in which a conventional light microscope is equipped with a web camera to obtain images of a suspension of mammalian cells in a hemocytometer assembly. Based on the ImageJ toolbox, we devised two algorithms to automatically count these cells. This approach is approximately 10 times faster and yields more reliable and consistent results compared with manual counting.

Counting mammalian cells before transfer into plates or dishes is an important task in cell biology. A hemocytometer is most frequently used to perform this task, and cells need to be manually counted on eight 1 × 1-mm areas on the two panels of the hemocytometer. The number of cells per panel usually averages 100 to 300; thus, on average, one needs to count approximately 800 to 2400 cells with a manual counter (“clicker”) while looking into the eyepiece of the microscope. A commercial cell counter provides considerable improvement, but the costs of the instrument and consumables are high (see Table S1 in online Supplementary material).

If an image of the cells spread in one layer on a known area is available (see “Attaching a Web Camera to a Conventional Microscope” in Supplementary material), software solutions are applicable. There are a few powerful software suites that allow for automatic counting of mammalian cells (Cell Profiler) [1], fluorescently labeled bacterial cells (CellC) [2], mammalian colonies (CellCounter) [3], bacterial colonies (OpenCFU) [4], and other circular objects. There are also several manual standalone cell counting assistants, plug-ins, and guides for ImageJ [5,6] that facilitate cell counting by replacing the manual clicker with multiple digital counters [5,7,8] or placing a semi-transparent grid over the image to help with focus [9]; however, every cell still needs to be visually registered and manually accounted for.

The common drawbacks of the existing solutions include a lack of focus on routine mammalian cell counting for cell manipulation, complex interfaces, narrow application ranges or extreme versatility (directly proportional to complexity), manual data input and lack of batch processing, manual adjustment of counting parameters for every image, use of separate standalone software solely for counting, and a requirement for expensive hardware for cell imaging.

We used ImageJ as the platform of choice for development of an image processing algorithm in the form of ImageJ macros. ImageJ has several useful tools to approach the problem. One option, the “Find Maxima ...” method, determines and counts the local intensity maxima in an image. For the first algorithm to find maxima properly, the image needs to be blurred. This filter replaces each pixel with the average of its 3 × 3 neighborhood, thereby eliminating small imperfections of high intensity that contribute to false positives. Next, the appropriate noise threshold was empirically estimated and applied (see “Macro 1” in Supplementary material).

Alternatively, the “Threshold ...” command can separate background from the particles of interest, which can then be counted by the “Analyze Particles ...” command. The RGB image is converted to grayscale (8 or 16 bits), and the threshold is found – the intensity value that allows everything below and above this value to be colored white and black, respectively. The ImageJ analysis algorithm can then be applied to count all of the black particles of a particular shape and size. We set a cutoff for all particles with an area less than 100 pixels (noise) but did not impose any restrictions on shape because of the tendency of cells to coalesce at higher concentrations; thus, in this case the software would at least detect the aggregate, although only as one particle (see “Macro 2” in Supplementary material).

Finally, we integrated the core functionality into the interactive envelope that prompts for the folder containing the image files to be analyzed, parses it for jpeg images, applies either core analytical algorithm, shows and saves the results, and copies them into the

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system clipboard. Plug-in execution in ImageJ can be assigned to a keyboard shortcut ("Plugins>Shortcuts>Create Shortcut ..."); thus, cell counting turns into a fast procedure initiated with a press of a button.

To test the algorithms, cells were prepared and counted manually by three investigators with considerable experience in mammalian cell culture. Images of each of the eight areas on two panels of the hemocytometer were taken (Fig. 1A), and the cells were counted automatically (Fig. 2A). Because the camera captured the area of a hemocytometer that is two times larger than the 1×1-mm area designated for manual counting, for the purpose of fair comparison the original images were cropped accordingly. The images were processed using Macro 1, employing blurring of the image (Fig. 1C) and counting of the intensity maxima (Fig. 1D), and Macro 2, involving estimation of the intensity threshold and subsequently making the image binary (Fig. 1B). There are some cells that were not accounted for by either algorithm due to the cells' tendency to coalesce at higher density; however, the rate of these events normally does not exceed 10% and may be somewhat offset by the inevitable inclusion of a few false positives.

The two algorithms showed remarkable consistency with each other and with the manual cell count (Fig. 2A) throughout the cell concentration range from very high to very low despite the issue with cells coalescing at higher concentrations. Importantly, it took from 3.5 to 14 min to count the cells manually depending on the dilution factor (i.e., cell concentration; see Fig. 2B), but it took only 1.5 min to carefully take eight images of the areas of interest. The additional time needed to create a folder, copy the images into this folder, and run either macro was less than 10 s.

We found that when manual cell count data were normalized via multiplication by the corresponding dilution factor, the values obtained differed significantly from one another (see Fig. S1A in Supplementary material), whereas they clearly should be the same. Thus, there is a well-defined trend for a significant decrease of the measured cell count as the dilution of the sample increases. We hypothesized that this discrepancy may arise due to uneven distribution of cells in the hemocytometer assembly, and the probability of such a phenomenon increases with a decrease in the cell concentration.

In contrast, when cells are counted automatically, there is no limitation to a particular area of the hemocytometer with the exception of densely gridded vertical and horizontal medians (intersections of lines heavily contribute to false positives). Moreover, the area captured by the camera (2 mm^2) is two times larger than the suggested areas for manual cell counting, thereby encompassing a larger cell population. Therefore, we carefully examined all of the areas of interest, took images of the portions that seemed most representative and homogeneous, and analyzed these areas using our algorithms (Fig. S1B). The average total cell count was consistent between samples of different dilutions (for both methods) and was higher than that for the highest manual count (220 ± 17 cells per 1×1-mm area for both automatic methods vs. 185 ± 5 cells for the manual count). This result demonstrates a key advantage of automatic cell counting of consistency, as well as simplicity, accuracy, and speed, and poses an important question with regard to the reliability of manual cell counting at low cell concentrations.

Lastly, the automated approach to cell counting leaves "a digital trace." Macros 1 and 2 save files with the results, and Macro 2 also saves the processed images in a separate directory within the one being analyzed. Both algorithms copy the results into the clipboard at the end of the run; hence, all that is left for the user is to paste the data into any spreadsheet template.

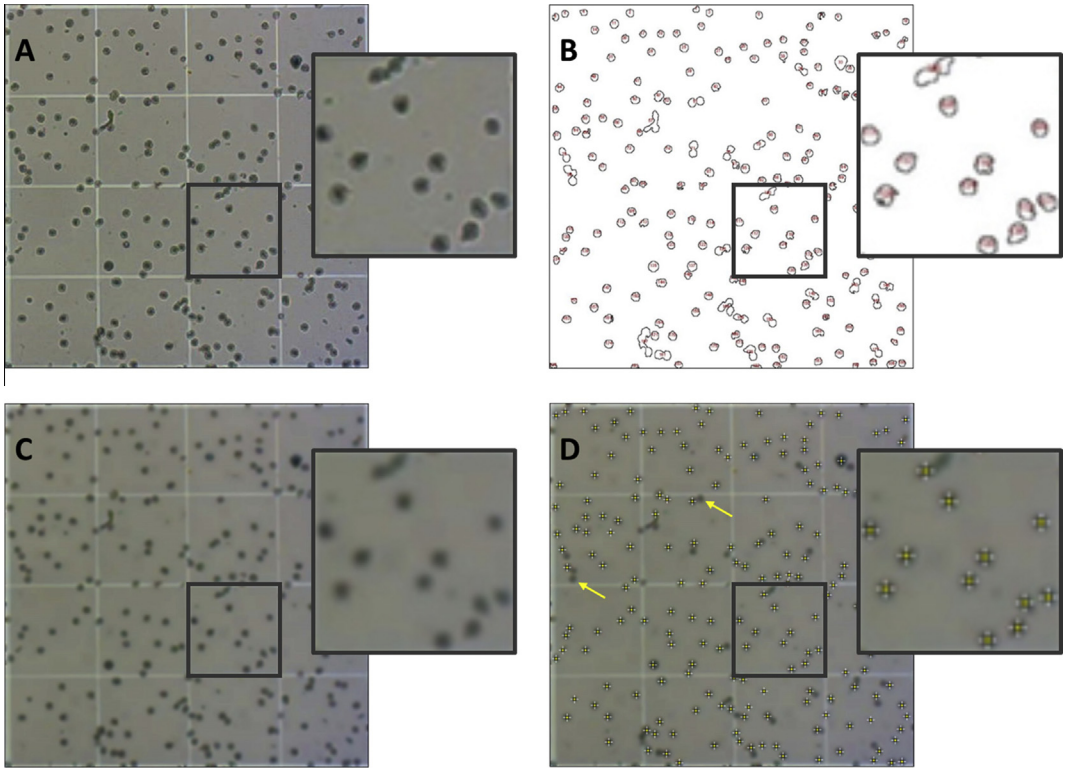

Fig.1. Application of the automatic cell counting methods. Cells in suspensions prepared by serial dilutions were deposited into a hemocytometer, and images of each of the eight areas on the two panels were taken and cropped to 1×1 mm. A representative image of one area at $1\times$ dilution is shown in panel A. The image was processed using the "Threshold ..." method (B). Alternatively, the image in panel A was blurred to give the image in panel C, and then the maxima were counted and all cells accounted for were laid over the original image as a mask of yellow crosshairs to give the image in panel D. Yellow arrows indicate representative cells that were not counted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

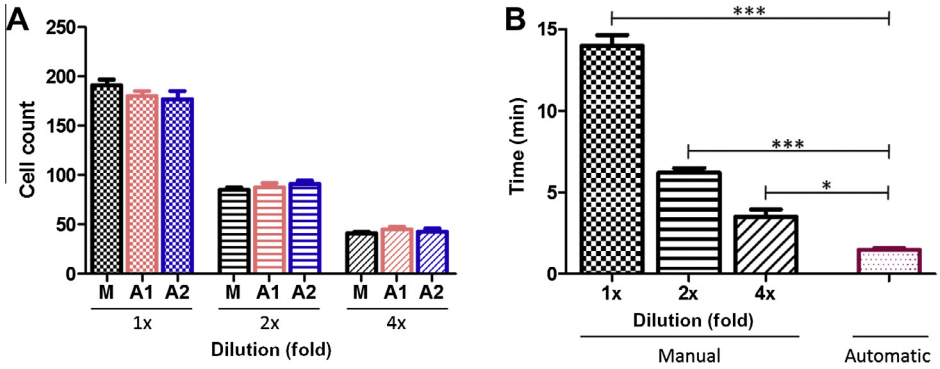

Fig.2. (A) Results of manual and automatic cell counting averaged over eight 1 × 1-mm areas. Cell count values obtained manually (M) and automatically by Macro 1 (A1) and Macro 2 (A2) are not significantly different from one another at a given dilution. (B) Total time in minutes required to perform manual cell counting (Manual) and to take corresponding pictures of cells in a hemocytometer (Automatic). Error bars are ± standard errors. $^{*}P < 0.05$; $^{***}P < 0.001$ (t test).

In conclusion, we have developed a robust approach to automatically count mammalian cells in images of a cell suspension in the hemocytometer assembly. The approach takes 2 to 10 times less time than manual counting, leaves a digital trace, yields the same results as manual counting for the same area but allows for selection of the most representative areas, and does not impose a penalty on the number of areas processed, thereby yielding consistent and more reliable results that are independent of the sample dilution. A comparison of some parameters for commercially available cell counters with our approach is presented in Table S2 of the Supplementary material. Instructions for downloading and using the macros, along with the code for each macro, are provided in the Supplementary material as well as at https://github.com/grishagin/CellCounting.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2014.12.007.

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