High-throughput image based single cell isolation

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Summary

The flow cytometry-based fluorescence-activated cell sorter (FACS) is a widespread method to separate cells on a molecular basis. However, in case of sensitive cell types or low cell numbers the isolation of single cells is challenging. Recent protocols still apply manual single cell isolation when intact cells are needed for further investigations, e.g., RNA or DNA sequencing. Previously, we have demonstrated that both fluorescent and unlabeled live cells in a Petri dish observed with a microscope can be automatically recognized by computer vision and picked up by a computer-controlled micropipette. The method could be routinely applied as a FACS down to the single cell level with a very high selectivity. Now we show that the application of a sCMOS camera in our setup allows the high resolution and fast imaging of live cells before gentle sorting. Exposition and therefore the scanning time could be decreased due to the low noise of the camera. Additionally, the large sensor size helped to minimize the number of images captured which were necessary to cover the region of interest. We conclude that a high definition sCMOS camera can greatly improve the performance of image-based single cell sorting. We believe that our approach will help to recognize and isolate live cells similarly to sophisticated face recognition algorithms. Sorting of sensitive cells will be feasible on the basis of both long term time-lapse videos and short term dynamic cellular processes.

Introduction

Next-generation DNA sequencing methods [1] made genome sequencing, transcriptome profiling, investigation of DNA-protein interaction, and epigenome research fast, accurate and relatively cost effective. Recent technologies are so sensitive that deep sequencing of a single cell has become available and spreading rapidly to establish a new branch of molecular biology.

The heterogeneity of cellular DNA and RNA is significant in cancerous tissues providing a lethal strategy for the tumour. Even if a very small portion of cells can escape the therapy due to the genetic diversity of cancer, the disease can recur. Single cell sequencing offers a chance to gain a better understanding of cancer evolution [2]. During embryogenesis stem cells are differentiating towards specific cell types to build up the whole animal. Transcriptome analysis of single cells helps to explore this process and also the regeneration of our tissues. Of equal importance is the fact that most immune cell types consist of several subpopulations; the diversity of which can be investigated by selecting individual cells for subsequent analysis.

Dendritic cells (DCs) play a key role in immunology. They are the cell type that initiate and drive the activation and differentiation of T lymphocytes. Co-stimulatory molecules – like CD86 – on the surface of dendritic cells are indispensable for proper T cell activation. The combination and density of co-stimulatory molecules expressed on DCs contribute greatly to T cell differentiation and therefore are important in shaping adaptive immunity. A micropipette allows the sorting of single live cells based on uniquely defined characteristics. For instance, from a mixed DC-T cell culture, cells in contact with each other can be separated from individual cells using the micropipette, which is impossible by employing classical flow cytometry or magnetic sorting.

Cells have to be isolated one by one to perform single cell DNA or RNA deep sequencing. However, single cell isolation is still a major bottleneck of the field. In most experiments, researchers isolate cells manually. Although fluorescence-activated cell sorting (FACS) based on flow cytometry has a very high-throughput, it is difficult to apply in several studies, e.g. in case of low cell numbers or sensitive cell types. Additionally, the fluorescent image of a cell contains far more information than its total fluorescent signal measured in FACS or in microfluidic devices. In the many cases when fluorescent labelling is problematic, cell sorting on the basis of label-free imaging is greatly beneficial.

We applied a straightforward imaging based technique to recognize and isolate single human DCs. A digital camera in concert with the motorized stage equipped onto an inverted fluorescent microscope scans in the region of interest of the Petri dish [3, 4, 5]. After successfully locating a cell, the CellSorter computer-controlled micropipette picks up selected individual cells, and deposits them in a submicroliter volume of culture medium into PCR tubes. Our method can also be applied on unlabelled cell populations in combination with high content label-free image analysis.

Methods

DC preparation and labelling

Monocytes were isolated from buffy coats (a fraction pf blood) obtained from healthy donors by magnetic separation using CD14 magnetic beads (Miltenyi) and differentiated toward monocyte-derived dendritic cells (MDCs) in 6 days by GM-CSF plus IL-4. MDCs were labelled with CD86-FITC monoclonal antibody strengthened by a secondary anti-mouse Alexa488. CD11b on MDCs was labelled by anti-CD11b-PE mouse monoclonal antibody. (Staining of CD11b allows the identification of myeloid cells in a mixed culture.)

75,000 MDCs were placed into a 35 mm glass Petri dish (Greiner) coated previously with 1mg/ml PLL-g-PEG (SuSoS) for 30 min to decrease the strength of cell adhesion. After that, cells were incubated at 37 C in 5% CO2 atmosphere for 30 min and then washed several times to remove floating cells from the Petri dish [4].

Computer-controlled micropipette

We used a CellSorter computer-controlled micropipette [3, 4, 5, 6] on a Zeiss Axio Observer A1 microscope with an Andor Zyla 5.5 sCMOS camera mounted on its side port (Fig. 1, 2). Fluorescent images were captured in two channels using FITC and TRITC filter cubes (Chroma) and a metal halide light source (Zeiss HXP 120 C). The microscope was automated by a motorized stage and fine focus (Marzhauser). The micropipette was moved up and down by a micromanipulator (Marzhauser HS6/3).

The CellSorter microfluidic system allows high-throughput single cell sorting directly from the Petri dish employing a motorized micropipette (Fig. 2-3). The device facilitates the isolation of a subpopulation of viable adherent cells expressing fluorescent or luminescent markers or labelled by fluorescent molecular probes highlighting specific cellular activities. It collects single cells for further cultivation, cloning, RNA or protein preparation. Any cell type can be sorted after minimal preparation for appropriate surface attachment. An average sorting process takes 10-20 minutes.

10 PCR strips containing 80 tubes can be filled in a cycle with single cells. Sample holder insert admits also a glass cover slip for testing cell deposition *in situ*. In a minute 3-4 cells, each in less than 1 µl medium can be deposited into different PCR tubes. When collecting multiple cells, sorting speed is 1 cell/second. Number of cells picked up in a single run is 1-1000.

Both unlabelled (Fig. 4) and fluorescent cells are recognized by computer vision. Cell isolation can be controlled by high content label-free image analysis. Multichannel detection is carried out using the fluorescent filter setup of the microscope. The path of the micropipette is computed using a traveling salesman algorithm (Fig. 5). All the cells along the path will be visited by the micropipette to pick them up.

Image processing

The schematic in Fig. 2 was generated with Solid Edge CAD software. Mosaic images in Fig. 4-6 were generated by the CellSorter software. Images presented in Fig. 6 were processed using the open source gimp and imageJ software. The brightness and contrast of single channel fluorescent images were adjusted and loaded into the red and green channels of the final RGB image.

Results and discussion

Using a Zeiss 40x EC Plan Neofluar objective lens and the Andor Zyla 5.5 camera we could readily detect the weak fluorescent signal of live MDC-s, which was almost invisible through the ocular lens. The large sensor size of the camera helped to minimize the number of images captured which were necessary to cover the region of interest (ROI). We scanned the ROI capturing fluorescent images in each of the two channels. The CellSorter software recognized the fluorescent cells in both channels and generated a mosaic image of the ROI. Cells could be selected on the basis of the ratio of fluorescent intensities measured in the two channels, which corresponded to those cells which expressed the two markers with a prescribed relative level.

We found that the CD86 markers were aggregated into pronounced patches rather than co-localizing with the more diffuse CD11b molecules (Fig. 7).

Single cell picking and deposition into the PCR tubes could be carried out with a speed of 3-4 cells per minute, which was limited by the motorized stage and micromanipulator. We are planning to use specialized multi-well microplates instead of PCR tubes to further improve throughput. We measured the efficiency of sorting using a similar cell type, (human monocytes) in our previous study [4] and found a rate of 54 ±4%.

Single cells were picked up from the Petri dish and deposited into PCR strips or onto glass cover slips inside tiny, ~0.5 µl drops. The system could be flexibly programmed to pick up more cells one-by-one in a cycle and deposit them into the same tube.

Using a high definition sCMOS camera it has been shown that image-based single cell detection, recognition, and isolation can be enhanced by the CellSorter computer-controlled micropipette, even in the case of weakly fluorescent live immune cells prepared from human blood.

Figure captions

Figure 1. CellSorter automated micropipette installed on an inverted fluorescent microscope equipped with the Andor Zyla 5.5 sCMOS camera.

Figure 2. Drawing of the CellSorter micropipette positioned above the objective lens. A large area of the Petri dish containing live cells can be scanned in several fluorescent channels using the motorized stage, focus and digital camera in concert. Cells are recognized by the CellSorter software (or selected manually by the operator) in the mosaic images (Fig. 4). Selected cells will be picked up by the automated micropipette and deposited into the PCR tubes. Micropipette is moved up and down by a micromanipulator. Insert shows a zoomed in image of the micropipette and Petri dish.

Figure 3. CellSorter micropipette with blue fluorescent excitation.

Figure 4. Mosaic image merged from adjacent microscopic fields captured in phase contrast mode of 3T3 cells on PDMS surface 1 h after seeding. Automatically detected cells are indicated by yellow or red frames. Cells with a neighbour closer than the sorting resolution of 60 µm (red frames) were excluded from the selection for sorting. Scale bar: 1 mm.

Figure 5. Path of the micropipette computed using a traveling salesman algorithm. All the cells along the path will be visited by the micropipette to pick them up.

Figure 6. Image of the surface after sorting showing that most cells that were selected for sorting were removed, while cells not selected remained unaffected by the sorting process.

Figure 7. Fluorescent image of MDCs captured in the FITC and TRITC channels, indicating the CD86 and CD11b proteins, respectively. Two channels were combined in an RGB image. Weakly fluorescent cells could be readily detected by the camera and recognized by the software (blue frames). Cells could be selected on the basis of their relative fluorescence in the two channels. Insert in the upper left corner highlights the spotted pattern of CD86 marker proteins.

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