Method for high throughput phagocytic assay

**Object**: this document describes a method for assessing the efficiency of internalization of an object, hereafter named the target, by a living unicellular organism capable of phagocytosis.

**Context**: currently, phagocytic assays are either analyzed by a human eye counting the number of internalized targets and the number of phagocytic cells, or by rudimentary automated analysis giving as a unique output the number of targets internalized and phagocytic cells. However, the growing development of targeted cancer cell therapies, whose purpose is to use the phagocytic abilities of one’s macrophages to destroy one’s cancer cells, leads to a dire need to evaluate precisely the efficiency of phagocytosis of specific targets, in specific conditions.

Advances in the quality of acquired images, either by confocal microscopy as used in [DeLoid2009, Steinberg2007], epifluorescence microscopy [Yeo2013, Beletskii2005] or cytometry [Ackerman2011], and advances in segmentation algorithms enables to program automatic detection of the cells and the targets. Automatic detection of the phagocytosis poses three main challenges: segmentation of the cells, segmentation of the targets, and sorting the internalized targets from them. Any deficiency in the two first steps will lead to wrong sorting during the third step. Several methods for the automatic sorting and counting of phagocytosis assays have been published through the last decade, all with similar processes. Cells are segmented using a nuclear and/or cytoplasmic staining, while targets are segmented using either bright field images or fluorescent staining. The sorting is usually solved with a staining step after the phagocytosis, during which only non-ingested targets are stained, thus allowing to sort between doubled stained targets and single stained ingested targets. Alternatively, a quenching agent disables the fluorescence of external targets. The number of internalized targets and the number of cells gives a phagocytic index, quantity that roughly indicates the efficiency of internalization in given conditions. However, at the last step of the process, computation of the phagocytic index, most of the information that could have been acquired and stored is discarded. The phagocytic index was easy to obtain when the data was slowly generated by the eye of the experimenter, but automatic analysis allows to collect, store and interrogate much more complex data, especially through relational databases that are the foundation of current data science.

**The method**: the novelty of the method is the combination of three existing tools for the purpose of detecting phagocytic efficiency: fluorescence confocal microscopy, automated image analysis and SQL database. The method has two important benefits: it can rapidly analyze a large sample of cells, on the order of a thousand cells per hour, and through the use of a database it gives access to an unprecedented level of quantitative and qualitative data. Though high-throughput analysis has already been presented by [Steinberg2007,DeLoid2009,Ackermann2011 and Yeo2013], all are focused on counting cells and targets in order to obtain the phagocytic index. Here our purpose is not only to count, but to label each object, cell or target, and relate it with the other objects on screen. As such, our detection strategy differs for previous methods. When only the number of cell on screen is important, counting the nuclei is easier and sufficient. Here, we want to know which cell has internalized which target, implying that we need to know the cell boundaries, to test whether a specified target is within a particular cell. Labeling the cytoplasm or cell membrane, and the nucleus is then mandatory. Moreover, fluorescent proteins moved on the liquid droplets that were our targets, meaning that detection could not rely on fluorescence to locate the objects. We thus developed an algorithm that could detect droplets on bright field images. Our imaging strategy, both for cells and targets, had to be adapted to fit our data collection objectives.

1st step: Phagocytic assay

The method can test the difference in internalization efficiency of different cell lines, of different mutations, exposition of the cell to drugs, physical (size, shape, surface tension, rheology), chemical (nature of lipid or solid surface) or biochemical (opsonization, functionalization) properties of the targets.

Required:

* An adherent cell line able to internalize the targets. In our assay, RAW 264.7 murine macrophages.
* Targets to be tested. In our assay, lipid droplets, but could also be solid beads, zymosan particles, Sheep Red Blood Cells.

Different types of target are brought in contact with different types of competent cells. It is to be noted that if the different targets or different competent cells can be distinguished on image, they can be mixed in one experiment without compromising the assay. For example, cells expressing or not a fluorescent plasmid can be presented with targets of different sizes. During image processing, expressing and non expressing cells can be sorted via measurement of plasmid fluorescence, while targets can be sorted by size, allowing to measure the influence of each parameter.

After contact, remaining targets are rinsed and cells fixed for observation.

2d step: Imaging

Required:

* Fluorescent marker for cell cytoplasm
* Fluorescent marker for cell nucleus
* Confocal or spinning disk microscope

The cells’ cytoplasm and nucleus must be marked with two different fluorescent probes, according to manufacturer’s instruction. Here we used actin probes such as phalloidin and SiRactin or membrane probe such as Wheat Germ Agglutinin as a cytoplasmic marker, and DAPI or Hoechst as a nuclear marker.

Using a fluorescent marker on targets is not mandatory, but can be of help if edge detection is made difficult by a small difference in refractive index with water.

The magnification should be enough to clearly distinguish the cell borders and targets.

Two images should be taken for each field: one with the focus of the basal level of the cell, i.e. in contact with the substrate, the other at this level + typical radius of the targets. The first image will define the contour of the cell, the second will be the best focus of internalized targets. The difference between the two images will help to locate targets’ borders.

3rd step: Automated Image analysis

Any programmable image analysis software can do this step. Here it was done using Cell Profiler, a tool providing matlab blocks of image processing code.

The first step is to locate the targets on the images, either with fluorescence of the targets, either by finding the edges of the target of bright field images. Here the edges were found by analyzing the difference between the two images taken at a different focus. A filtering by size, shape or color may be needed after this step to avoid false targets.

The second step is to locate the cells’ nuclei, using fluorescence, and then propagate this location on the fluorescent image of the cytoplasm to find the cells’ edges. A filter by size, shape or color can be used to avoid false detection.

The third step combines the localization of the cells and of the targets to test whether targets are internalized. Cytoplasm localization is translated into a binary image; the same is done for target localization. Multiplication of the two images gives as a result a binary where 1 is a pixel where a target is localized inside a cell, 0 is everywhere else. The mean value of this binary on the area of a given target is the percentage of its area that is inside a cytoplasm. A filtering by this parameter can be completed by other criteria, such as the absence of cytoplasmic fluorescence at the location of the target, for the algorithm to decide whether the target it internalized.

4th step: SQL Database storage

All measurements performed during step 3 should be recorded in a relational database. One table stores all the data from cell measurements such as area, shape indicators, fluorescence intensity, with each cell identified by a unique primary key. One table stores all the data from the targets. A third table stores all the relationships between cells and targets, especially which cell has engulfed which target. Using those three tables, complex questions about the links between cells’ and targets’ parameters can be answered, such as: have bigger cells internalized more targets? Are big targets internalized preferentially by big cells?

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