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Macropinosome quantitation assay

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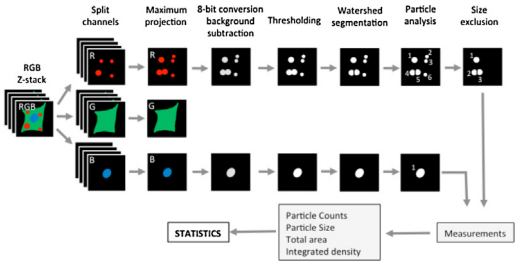
GRAPHICAL ABSTRACT



ABSTRACT

In contrast to phagocytosis, macropinocytosis is not directly initiated by interactions between cell surface receptors and cargo ligands, but is a result of constitutive membrane ruffling driven by dynamic remodelling of cortical actin cytoskeleton in response to stimulation of growth factor receptors. Wang et al. (2010) [13] developed a reliable assay that allows quantitative assessment of the efficiency and kinetics of macropinosome biogenesis and/or maturation in cells where the function of a targeted protein has been perturbed by pharmacological inhibitors or by knock-down or knock-out approaches. In this manuscript we describe a modified quantitative protocol to measure the rate and volume of fluid phase uptake in adherent cells. This assay:

- uses fluorescent dextran, microscopy and semi-automated image analysis;
- allows quantitation of macropinosomes within large numbers of individual cells;
- can be applied also to non-homogenous cell populations including transiently transfected cell monolayers. We present the background necessary to consider when customising this protocol for application to new cell types or experimental variations.

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Development of macropinosome quantitation *assay*

Introduction

Although initially distinguishable by their large size – relative to other endocytic organelles – macropinosomes undergo homotypic fusion and fission [1] and may also converge with other endocytic pathways becoming visually intractable within minutes post internalization. In an attempt to quantify the rate of fluid uptake into macropinosomes several studies introduced horseradish peroxidase (HRP) [2,3] or Lucifer Yellow [4,5] as fluid-phase markers. In these assays the enzyme activity or the fluorescence associated with the marker was measured in the lysate of the cells via spectrophotometry. Such methods however, often neglect the substantial contribution of other endocytic pathways to the total amount of fluid-phase marker internalized by the cells, which in turn compromises the specificity of the assay. Since the rate of macropinosome formation, macropinosome numbers and their size are all characteristics that are suitable for investigation via microscopy, we focussed on development of a protocol for quantitative analysis of macropinocytosis using fluorescence imaging and semi-automated image analysis.

Model cell line and timing of the fluid-phase uptake

Macropinocytosis has been studied extensively in antigen-presenting cells such as macrophages and dendritic cells, but many other cell types including epithelial cells and fibroblasts also exhibit macropinocytosis. With the intention to establish a model system which can be used as a platform to investigate the effect of candidate proteins or compounds on macropinocytosis in mammalian epithelial cells of non-cancer origin, we developed a quantitative image-based assay to analyze macropinocytosis in Human Embryonic Kidney cells (HEK293). We have previously shown that within the first 3–5 min post formation at the cell periphery, 80–90% of macropinosomes in HEK293 cells are positive for Rab5 and SNX5 but only 5% are positive for Rab7 suggesting that fusion with late endosomes and acquisition of endolysosomal markers begins from about 5 min post macropinosome formation [6]. Therefore, macropinosomes in HEK293 cells should be examined within this relatively short period to exclude those that may have converged with endolysosomal compartments. In the protocol we describe, cells are pulsed with a fluorescent fluid-phase marker only for 5 min and after a brief wash to remove non-internalized dextran and reduce the extracellular fluorescence, the cells are fixed to ensure that only early macropinosomes will be detected in the cells. Optionally, to further prevent macropinocytic uptake during the washing step, cells can be washed in cold PBS before fixation as macropinocytosis has been shown to be inhibited at 16 °C [7].

Selection of a fluid-phase marker

One of the most convenient fluid-phase markers to study endocytosis by pulse-chase experiments is dextran, which has been also used as a fluid-phase marker for macropinosomes [8]. Dextran is a hydrophilic, non-digestible carbohydrate commercially available in a wide range of molecular sizes and photostable fluorophore conjugates with well differentiated excitation/emission spectra suitable for fluorescence microscopy. Our assay has been designed to quantify macropinosomes in HEK293 cells expressing GFP either as a reporter of shRNA-mediated knock-down or, as a tag of a protein of interest. For straightforward emission spectra separation we therefore use 10,000 MW dextran conjugated to tetramethylrhodamine (dextran–TMR) although dextran conjugated with any other suitable fluorophore can be used. Fluorescently conjugated dextrans are supplied as fractions of specific molecular weight commonly from 10 to 2000 kDa which corresponds to molecular