Using Immunofluorescent Cell Marker Quantification as an Identification Method for M1 and M2 Macrophage Phenotypes

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Abstract

Macrophages as antigen presenting cell (APC) play a vital role in orchestrating immune responses against foreign materials. The activation status of macrophages could be determined by tracking the expression of various cell markers that can be a signal for their immune activity behaviour following cellular stimulation either towards healing or inflammation. Previously numerous immunofluorescent cell markers have been used for distinguishing between pro-inflammatory macrophage phenotype (M1) and anti-inflammatory macrophage phenotype (M2) qualitatively, although most of those fluorescent cell markers express in both phenotypes. We have developed a new strategy to identify M1 and M2 phenotype quantitatively by using immunofluorescent cell markers. This approach enables the identification of different macrophage functional phenotypes quantitatively, and their degree of polarisation. Macrophages were polarised to M1 and M2 phenotypes by GM-CSF+IFN-γ and M-CSF+IL-4, respectively. Control cells were un-polarised (naïve) macrophages or monocytes were considered as macrophage progeny. For assessing cell polarisation all cell types were stained for nucleus. Also, their surface markers were stained with calprotectin for M1 cells and mannose receptor (MR) for M2 cells, followed by fluorescent microscopy examination. Cell images were analysed using CellProfiler software in order to measure the fluorescent signal intensity of the cell markers, and create a specific profile for each cell type. These profiles formed the basis for M1 and M2 phenotype identification. By using such fluorescent signal parameters we were able to identify M1 and M2 phenotypes effectively and distinguish them from naïve macrophages and monocytes.

*Keywords***:** Macrophages; M1and M2 phenotypes; CellProfiller; macrophage identification.

1.Introduction

Macrophages as a constituent of the innate immunity and as APC, play a crucial role in defence against foreign pathogen invaders as well as in human body haemostasis. They contribute to phagocytosing dead cells and microbes, recruiting immune cells to the micro-environment, presenting antigens and providing essential activation signals for T cells (Goerdt & Orfanos, 1999, Martinez & Gordon, 2014, Kratky *et al.*, 2011).

Various macrophage subsets have been identified, each subset with distinct functional characteristics (Sutterwala *et al.*, 1997). For example, M1 (classically activated) macrophages with pro-inflammatory and anti-tumour functions (Sutterwala *et al.*, 1997) secrete large amount of pro-inflammatory interleukin 12 (IL-12) and (IL-23) (Mantovani *et al.*, 2004). M1 phenotype is induced by interferon gamma (IFN-γ) secreted mainly from T helper 1 (T_H1) cells, natural killer (NK) cells or CD8$^+$ cytotoxic T cells (CTLs) in the presence of bacterial cell wall components such as lipopolysaccharide (LPS) (Mosser & Edwards, 2008). However, M2 (alternatively activated) macrophages with anti-inflammatory and pro-wound healing activities (Sutterwala *et al.*, 1997), secrete high levels of cytokine IL-10 (Fleming & Mosser, 2011). They can be induced by cytokines IL-4 and/or IL-13, secreted mainly by T_H2 cells (Mosser & Edwards, 2008) or mast cells (Bradding *et al.*, 1992).

In vitro, M1 phenotype can be polarised from monocytes by IFN-γ (Garcia *et al.*, 2014) and LPS (Mills *et al.*, 2000). In addition, granulocyte macrophage colony-stimulating factor (GM-CSF) has been used as a macrophage priming signal (Hamilton, 2002, 2008) which enhance the pro-inflammatory properties of polarised cells (Verreck *et al.*, 2004, Garcia *et al.*, 2014). By contrast, monocyte can be polarised toward M2 phenotype by adding IL-4 (Garcia *et al.*, 2014). M2 macrophage anti-inflammatory function can be enhanced with macrophage colony-stimulating factor (M-CSF) (Verreck *et al.*, 2004, Garcia *et al.*, 2014).

Cluster of Differentiation 68 (CD68) marker expresses in macrophages intracellularly and this marker is often used for macrophage identification (Sindrilaru *et al.*, 2011). In order to follow up macrophages activation status, a panel of: cell markers, secreted cytokines, transcription factors or metabolites are employed. For instance, production of high leveles of pro-inflammatory cytokines such as IL-1β, IL-6, tumour necrosis factor alpha (TNF-α) (Hofkens *et al.*, 2011, Hao *et al.*, 2012) IL-12 and IL-23 (Mantovani *et al.*, 2004) are considered as main characteristics of M1 macrophages subset. Furthermore, M1 macrophages have been shown to perform high level expressions of calprotectin (27E10 antigen) (Bartneck *et al.*, 2010), nitric oxide synthase 2 (NOS2) (Edin *et al.*, 2012), chemokine (C-C motif) receptor 7 (CCR7) (Agrawal, 2012), and CCR2 (Willenborg *et al.*, 2012). On other hand, M2 macrophages

are identified by the secretion of high amounts of IL-10 cytokine (Mantovani, 2006), and transforming growth factor β (TGF-β) (Hao *et al.*, 2012). Moreover, M2 phenotype express high levels of mannose receptor (MR, CD206) (Agrawal, 2012, Mantovani, 2006) , the scavenger receptor CD163 (Edin *et al.*, 2012) (Mantovani, 2006), and IL-1 receptor antagonist (IL-1RA) (Baitsch *et al.*, 2011).

With regard to gene expression and transcription factor phosphorylation, human M1 macrophages are identified by the expression of high levels of IL23a (IL23p19) and prostaglandin-endoperoxide synthase 2 (Ptgs2 or Cox2) gene, and phosphorylation of signal transducer and activator of transcription 1 (STAT1) and/or STAT3. In contrast, the main characteristics of human M2 macrophages, is the expression of high levels of chitinase 3-like 2 (Chi3l2 or Ykl39) and Kruppel-like factor 4 (Klf4) gene and phosphorylation of STAT6 (Murray & Wynn, 2011).

M1 and M2 macrophage activation results in murine that can be identified by distinct cell marker profile, while there is some overlap in the cell marker expression between both phenotype activation (status in human macrophages. For instance, Arginase-1 (Arg-1), known as murine M2 marker, has been expressed in both M1 and M2 macrophage phenotypes in human (El Kasmi *et al.*, 2008). In addition, M2 markers, chemokine (C-C motif) ligand 18 (CCL18) and MR can also be expressed on monocytes stimulated   with LPS and GM-CSF or IFN-γ, respectively (Porcheray *et al.*, 2005). Accordingly, it seems that there are quantitative divergences in cell marker expression between human M1 and M2 macrophage phenotypes, rather than qualitative differences (Davis *et al.*, 2013).

The complexity of characterisation in M1/M2 human macrophages by surface cell markers has encouraged investigation in an alternative approach that would be less resource-intensive and simpler.

Studies that have reported morphological differences between macrophage phenotypes (Porcheray *et al.*, 2005, Chinetti-Gbaguidi *et al.*, 2011, Leitinger & Schulman, 2013, Pelegrin & Surprenant, 2009, Lee *et al.*, 2013, McWhorter *et al.*, 2013, Vereyken *et al.*, 2011, Rostam *et al.*, 2017) has been categorised for Image based Machine Learning for identification of macrophage subsets (Rostam *et al.*, 2017). This new approach has led us to hypothesise that cell surface marker signal intensity could be quantified and used as an indicator of activation status in macrophages.

The aim of the present study was to quantify the cell surface markers signal intensity, calprotectin (M1 cell marker) and MR (M2 cell marker) in M1 and M2 macrophage subtypes respectively. Data were collected and used to build a threshold to identify different macrophages status. Monocytes were stimulated *in vitro* for 6 days with M1 (GM-CSF+IFN-ᵧ) or M2 (M-CSF+IL-4)-inducing cytokines.

1.   Materials and Methods

1.1   Monocyte isolation and culture

Buffy coats were obtained from the National Blood Service following Ethics committee approval (National Blood Services, Sheffield, UK; 2009/D055). Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats by Histopaque-1077 (Sigma-Aldrich) density gradient centrifugation. Monocytes were isolated from PBMCs using the MACS magnetic cell separation system (positive selection with CD14 MicroBeads and LS columns, Miltenyi Biotec) (Rostam *et al.*, 2017, Rostam *et al.*, 2016, Singh *et al.*, 2017). RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin and 100 µg/ml streptomycin (all from Sigma-Aldrich) (henceforth referred to as "complete RPMI medium") used for monocytes were suspension with the cell density of 1×10^6 cells/ml. 1 ml of the suspension (=1 x 10^6 monocytes) was seeded on round coverslip 12mm in each well of 24 tissue culture well plate, then incubated at 37°C, 5% CO_2 in a humidified incubator for six days.

1.2   Immunofluorescent staining

On day 6 all adherent cells on round coverslips were fixed by using 4% paraformaldehyde (EMS Diasum) in phosphate buffer saline (PBS), then followed by blocking with 1% (w/v) Glycine (Fisher Scientific) 3% (w/v) bovine serum and albumin (BSA) (Sigma-Aldrich) in PBS. Another blocking step with 5% (w/v) goat serum (Sigma) in PBS was done. Then, attached cells were stained with 1 µg/ml CD206 (MR) rabbit anti human primary Atibody (Abcam) and 2 µg/ml calprotectin anti-human mouse IgG1 Antibody (Thermo Scientific), followed by incubation at room temperature for one houre. After that washed with PBS, cells were stained with goat anti-mouse Rhodamin-x IgG (H+L) secondary antibody (8 µg/ml) (Invitrogen), and goat anti-rabbit Alexa flour-488 goat anti-rabbit IgG (H+L) secondary antibody (8 µg/ml)(Invitrogen) at room temperature for another hour. Also, DAPI (4',6-Diamidino-2-Phenylindole) was used for staining nuclei (250 ng/ml) (Invitrogen). FluorSave™ anti-fade medium (Calbiochem) and Fluoromount™ (Sigma-Aldrich) were used for saving fluorescent signals. Fluorescence microscope (IMSTAR) was used to image the seeded cells on the slides. Then CellProfiler image analyser software (http://www.cellprofiler.org/) used to count positively MR cell number and calprotectin positive stained cells.

Cells of different activation states were produced using cytokine addition monocytes seeded on normal glass slides: for polarisation to M1 a mixture of 20 ng/ml IFN-γ (R&D Systems) and 50 ng/ml GM-CSF (Miltenyi Biotec) was added to a total volume of 1ml; for M2 differentiation 20 ng/ml of IL-4 (Miltenyi Biotec) and 50 ng/ml M-CSF (Miltenyi Biotec) were added to the well volume of 1 ml. The cells were incubated at

$37^{\circ}C$, 5% CO_2 in a humidified incubator for 6 days. On day 3 of incubation, 500 µl of the medium was replaced with fresh complete RPMI medium containing the same concentration and mix of cytokines that were used for cell stimulation at the beginning of culture. After six day of incubation M1 and M2 macrophages were stained with calprotectin (M1 marker) and MR (M2 marker). Images of both phenotypes were taken with an automated fluorescent microscope. Automated image analysis software (CellProfiler) was used to measure and record the maximum fluorophore intensity per image for nine different images. This was repeated for two different samples for the same biological donor and the average values of calprotectin in M2 and MR in M1 were calculated. These values were used as threshold intensity values of calprotectin and MR in order to categorise cells not exposed to cytokines, with each cell exhibiting fluorescence intensity above these calprotectin and MR thresholds categorised as M1 and M2 respectively.

2.3   Polymer Surfaces Synthesis

Polymer surface were been synthesized by using methods described previously (Anderson *et al.*, 2004, Hook *et al.*, 2012). Briefly, Each polymerisation solution was composed of monomer (50%, v/v) in dimethylformamide with photoinitiator 2,2-dimethoxy-2-phenyl acetophenone (1%, w/v). Polymers were purchased from Aldrich, Scientific Polymers and Polysciences and coated onto epoxy-coated slides (Xenopore) dip-coated with poly(2-hydroxyethyl methacrylate) pHEMA (4% w/v, Sigma) in ethanol (95% v/v in water). Coated surfaces were sterilised by exposure to UV light for 15 minutes. The hits materials were scaled up as polymer coupons formed by pipetting polymerization solution (6µL) onto a pHEMA coated slide and irradiating for 10 mins at $O_2 < 1300$ ppm with a long wavelength UV source. Once formed, volatile components were removed from the polymers at <50 mTorr for 7 days. Polymers were characterized by water contact angle measurements and time-of-flight secondary ion mass spectrometry as described previously (Taylor *et al.*, 2007, Urquhart *et al.*, 2007).

2. Results

2.1. Determining macrophage pro or anti-inflammatory phenotype using fluorescent microscopy

The expression of cell surface marker use as a method to identified macrophage. Automated microscopy with high quality image analyser was used to perform high throughput scanning for the adhered cells on glass slide surface (Murray *et al.*, 2014, Xue *et al.*, 2014). High throughput method was used for the glass slide screening by the automated fluorescent microscopy (IMSTAR) to determine the level of calprotectin (M1 marker) expression in M1 pro-inflammatory macrophages population, and the level of MR (M2 marker) in M2 anti-inflammatory macrophage population (Rostam *et al.*, 2016) (Figure.1). To examine macrophage polarisation under the effect of polymers, we

examined the expression of markers in both populations (cytokine polarised, M1 and M2) on glass slides. The mean of fluorescent intensity pixel for calprotectin and MR value was calculated in M2, M1 cytokine polarised cell, respectively. That was used to categorised the phenotypes of seeded macrophages on the polymers surfaces as M2 or M1 when they their marker fluorescent expression above the average levels in M1 or M2 respectively.

2.2. Macrophage polarisation

Using fluorescence microscopy the number of MR$^+$ and calprotectin$^+$ cells was quantified for each homo-polymer using the M1 and M2 identification criteria developed on cytokine differentiated naïve macrophages. Homo-polymer number (decyl methacrylate) was the most effective at polarising the cells towards the M2 phenotype, with 2.9 times more cells expressing MR (68±28 cells) compared to calprotectin (24±21cells) as seen in Figure 2. A high degree of cell attachment (88±23 cells) was also observed on this homo-polymer. Other materials polarising cells towards the M2 phenotype included homo-polymer numbers (hexyl acrylate) with ratios of MR$^+$ to calprotectin$^+$ cells of 2.6 The homo-polymers(hydroxypropyl acrylate) were the most effective at polarising cells towards the M1 phenotype whilst still supporting the attachment of more than 50 cells , with 3 times more calprotectin$^+$ cells than MR$^+$ cells (**Figure 2**).
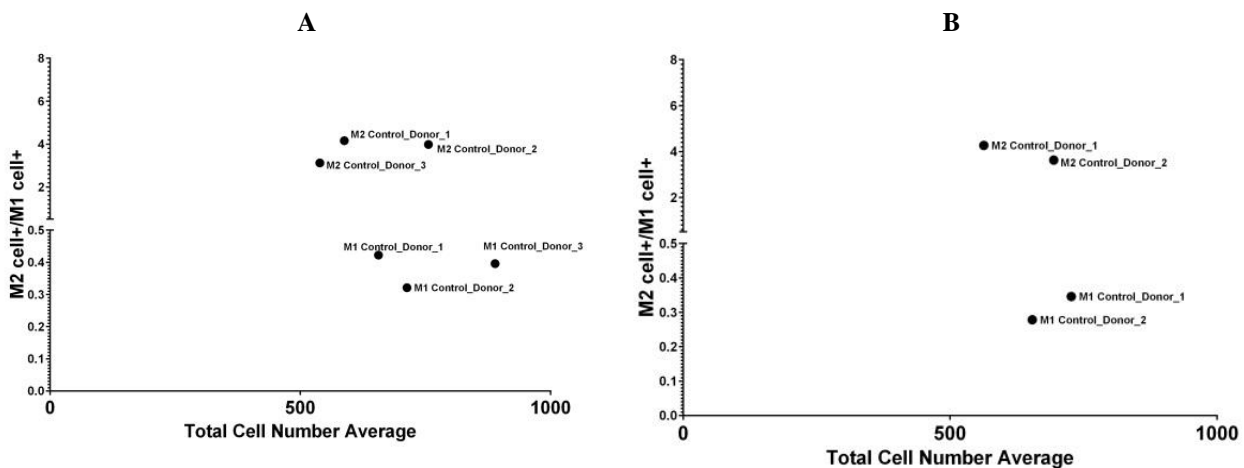
Figure 2: Determining macrophage pro or anti-inflammatory phenotype using fluorescence microscopy (A,B) Scatter plot for number of M2 /M1polarised cells with cytokines on glass slide, X-axis average total cell number of the adherent cells ,Y-axis is number of cells expressed MR$^+$(M2- phenotype)/ number of cell expressed calprotectin (M1-phenotype)on glass slide . *n*=3(D, homo-polymer arrays experiment) and 2 (E, co-polymer arrays experiment) of different samples (M1 and M2) for each sample with 2 replicates.
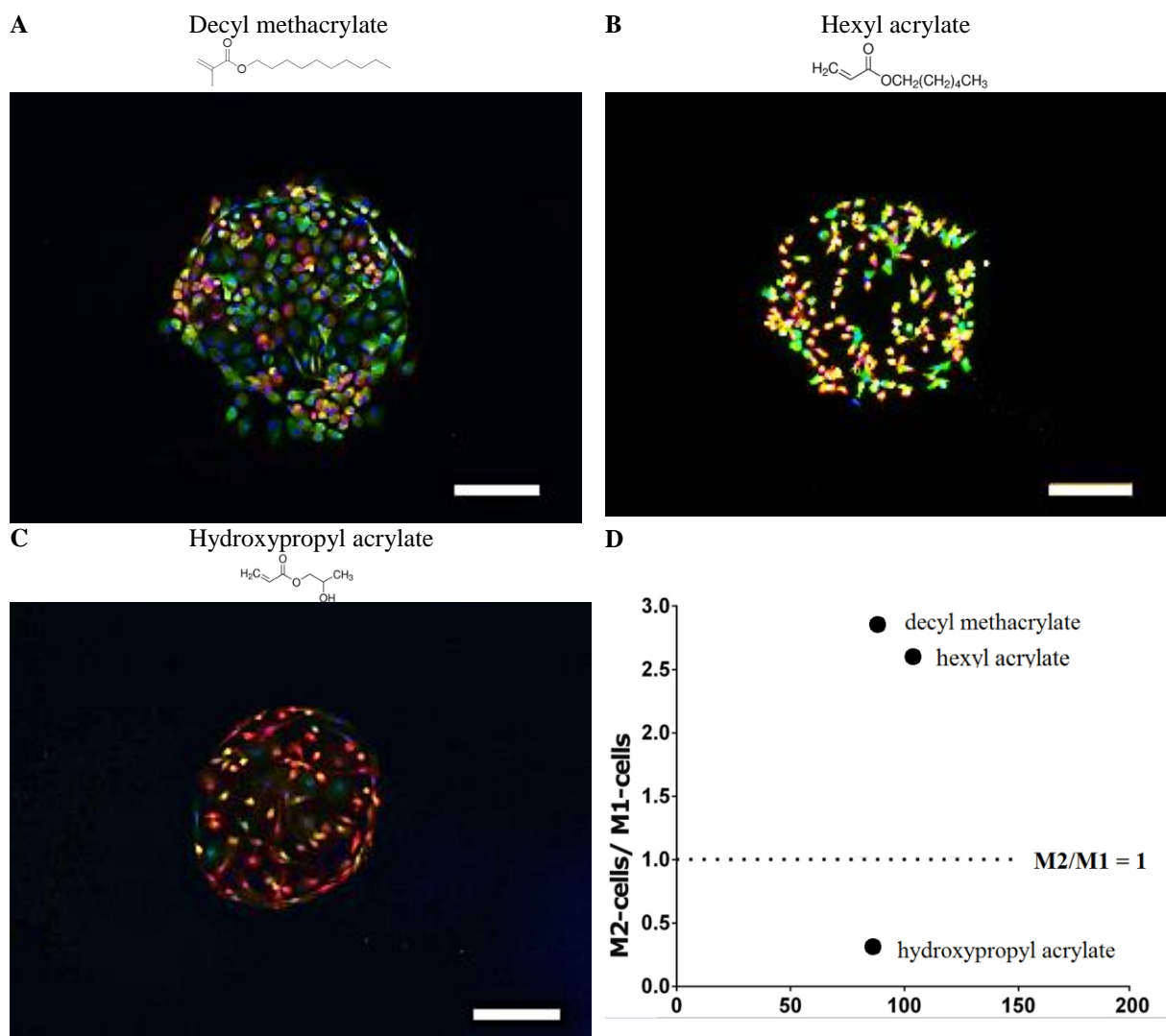
Figure 2: Effect of homo-polymers on macrophage polarisation and cell adherence. (**A**) Effective homo-polymers; (decyl methacrylate); (**B**) (Hexyl acrylate); and **(C)** 98.(hydroxypropyl acrylate) with their chemical structure, Fluorescent images of cells stained for calprotectin (27E10 antigen, red), and mannose receptor (MR, green) and nucleus (DAPI blue) on most effective homo-polymers, Scale bar = 200 µm.(**D**) Scatter plot for **number of** M2 cell/M1 cell on homo-polymers, x-axis average total cell number on homo-polymer ,Y-axis is number of cells expressed MR⁺(M2-like phenotype)/ number of cell expressed calprotectin (M1-like phenotype)on homoplolymer. Dotted line is cells with M2-like cells (MR⁺ cells)/M2-like cells (Calprotectin⁺ cells) =1. Data below SNR threshold (mean/SD≥2) were excluded, *n*=3 of different donors each donor with average of three replicates.

3. Discussion

In this work, for first time MR and calprotectin has been quantified in M1 and M2 macrophage subsets, and used as threshold for M1 and M2 identification.

M2 macrophages expressing high amount of MR can be induced *in vitro* by IL-4 and IL-13 (De Paoli *et al.*, 2014). MR in macrophages observed to operate control of innate immunity and it is also believed to be involved in regulating antigen presentation and lymphocytes trafficking to lymph nodes. In addition, MR as a scavenger receptor has preference for collagens and for glycosylated proteins (Hagert *et al.*, 2018). However, M2 macrophages may express low amount of calprotectin (Hsu *et al.*, 2009) which has

been considered as M1 cell marker (Bartneck *et al.*, 2010, Rostam *et al.*, 2017, Rostam *et al.*, 2016).

M1 macrophages which can be stimulated by Lipopolysaccharide (LPS) and interferon-γ (IFN-γ) (Huang *et al.*, 2017) can be characterised by expression of a high level of calprotectin (Bartneck *et al.*, 2010, Rostam *et al.*, 2017, Rostam *et al.*, 2016) which could induce pro-inflammatory cytokine production properties of the macrophages. However, calprotectin may express in monocytes and M2 macrophages (Xia *et al.*, 2018).

From visual inspection of cell surface marker expression of macrophages, immediate differences in their respective signals has been noticed, these differences has been quantified by CellProfiller software which can detect signal intensity of each cell markers and their distributions across the cell surface (Rostam *et al.*, 2017). The mean of maximum expression of calprotectin in M2 phenotype cells can be used as a save threshold for M1 phenotypes. In addition, M2 can be identified when the mean of MR signals of any cell exceeded the mean of maximum MR intensity signals of M1 phenotypes. Later, this way of cell identification successfully has been used to identify the macrophage polarisation to word M1 and M2 under the impact of surface chemistry modulation (Rostam *et al.*, 2016).

Biomaterial surface chemistry has previously been shown to modulate macrophage adhesion and function (Rostam *et al.*, 2015). In this study a high throughput screening strategy has been used to collect image data from incubated cells, the M1 and M2 phenotypes controls, and from macrophages seeded on different homo-polymers. This new approach used to investigate the effect of different homo-polymers surface chemistries on human monocyte differentiation. By using the method M1 and M2 biased homo-polymers has been identified depending on data analysis by the new method effectively.

4. Conclusion

Using immunofluorescent cell marker quantification, as a new effective identification method for M1 and M2 macrophage phenotypes in mixed macrophage population could pave the way for further investigations in this area. This method is capable of achieving high degrees of accuracies, in contrary to macrophage phenotype heterogeneity that can affect the cell marker signal expression. However, presented data provide strong indications for ability of this method to perform M1 and M2 subtype identification with less resource intensive and fast way of identification, still it is be too early to suggest this approach as an alternative for conventional cell phenotyping in wide scale cell identification.

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