Kinetic phenotyping of monocyte-to-macrophage differentiation using quantitative phase imaging ptychography

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Background

The phenotype of a cell results from the integration of multiple signalling pathways activated in response to internal and/or external cues. When exposed to a drug, a cell can initiate several cellular processes that are molecularly complex but manifest as readily observable and quantifiable traits or behaviors, such as morphology and motility. Visual cell phenotyping involves identifying and quantifying these observable characteristics from cellular images.[1]

Here, we utilised the Phasefocus Livecyte system to kinetically profile the visual phenotype of monocytes during differentiation into macrophages to inform on the activity of a test compound. To complement these studies, we assessed the potential of the test compound to modulate the protein expression of CD markers associated with macrophage differentiation.

Phasefocus Livecyte System

The Phasefocus Livecyte system utilises ptychography, a form of Quantitative Phase Imaging (QPI) that uses a digital Virtual Lens™ to translate diffraction patterns formed by a sample into high-contrast quantitative phase images.

Unlike traditional phase contrast images, videos produced by Livecyte from timelapse imaging are well-suited for downstream analysis, as cells appear as bright features against a dark background. QPI-based phenotypic assay readouts are comparable to fluorescent image capture, but without the phototoxic side effects that can result from repeated exposure to fluorescent light (**Fig. 1**).

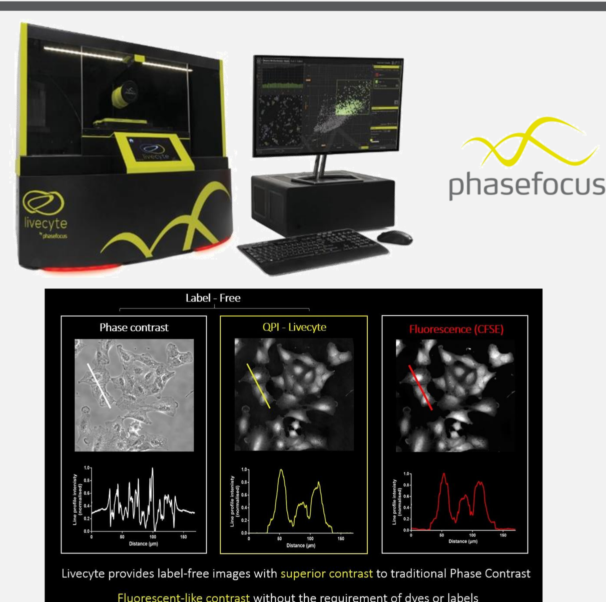

Fig. 1: Comparison of Phasefocus Livecyte QPI imaging vs traditional brightfield or fluorescence imaging.

Monocyte-to-Macrophage Differentiation

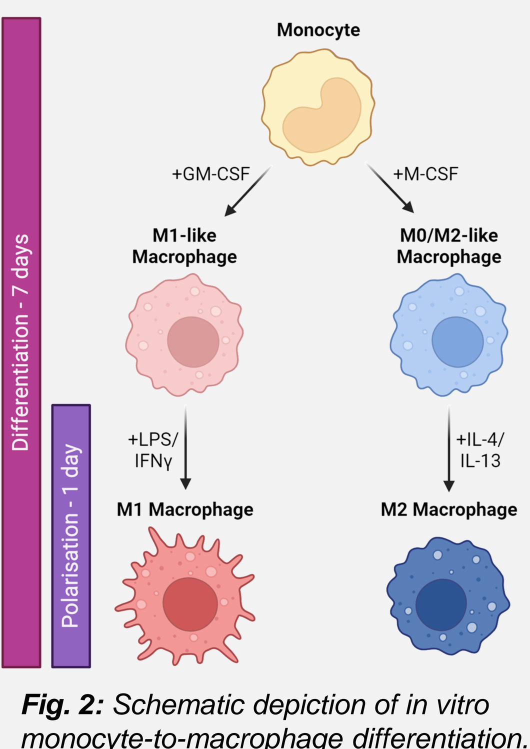

Fig. 2: Schematic depiction of in vitro monocyte-to-macrophage differentiation.

Macrophages are immune cells with critical roles in various diseases, both beneficial and harmful, including infection, inflammation and autoimmunity, cancer, and tissue repair and fibrosis.[2]

Tissue-resident macrophages differentiate from blood-derived monocytes and exhibit functional heterogeneity dependent upon the cytokines encountered during differentiation and activation. The two main types of macrophages are:

- **M1 macrophages (classically activated)**: M1 macrophages are activated by pro-inflammatory signals. They are highly effective at killing pathogens.
- **M2 macrophages (alternatively activated)**: M2 macrophages are activated by anti-inflammatory signals, such as IL-4 and IL-13. They promote tissue repair, wound healing, and resolve inflammation.

Methodology & Resources

Cell Culture: Human peripheral blood mononuclear cells were isolated by density gradient centrifugation. CD14⁺-monocytes were purified by positive selection using an immunomagnetic isolation kit and seeded in 96-well plates at 50,000 cells/well and incubated at 37°C/5% CO_2. Granulocyte macrophage-colony stimulating factor (GM-CSF) or macrophage-colony stimulating factor (M-CSF) was added to induce differentiation into M1-like and M0 (unpolarized, resting)/M2-like macrophages after 7 days of culture, respectively. Full polarisation into M1 or M2 macrophages was induced by addition of lipopolysaccharide (LPS)/interferon gamma (IFNγ) or interleukin-4 (IL-4)/IL-13 at day 6 of the differentiation protocol, respectively. The *in vitro* differentiation protocol is depicted schematically in **Fig. 2**.

Livecyte Assays: Monocytes were treated with DMSO vehicle (0.1% final assay concentration) or 10 µM of a test compound. Timelapse image was then performed using the Livecyte system featuring a humidified chamber set to 37°C/5% CO_2. Images were obtained in 15-minute capture intervals from Day 0 → Day 3 and Day 3 → Day 6, separated by a culture media change.

To display the Livecyte results in a qualitative format, the Livecyte Cell Analysis Toolbox identifies peaks in pixel intensity to assign a seedpoint to each cell within each image (**Fig. 3**). Cell segmentation is based on pixel intensity thresholds specific to the ptychographic QPI. The Livecyte Adaptive Correction algorithm combines this with temporal (frame-to-frame) information which produces downstream readouts displayed in the system Dashboards.

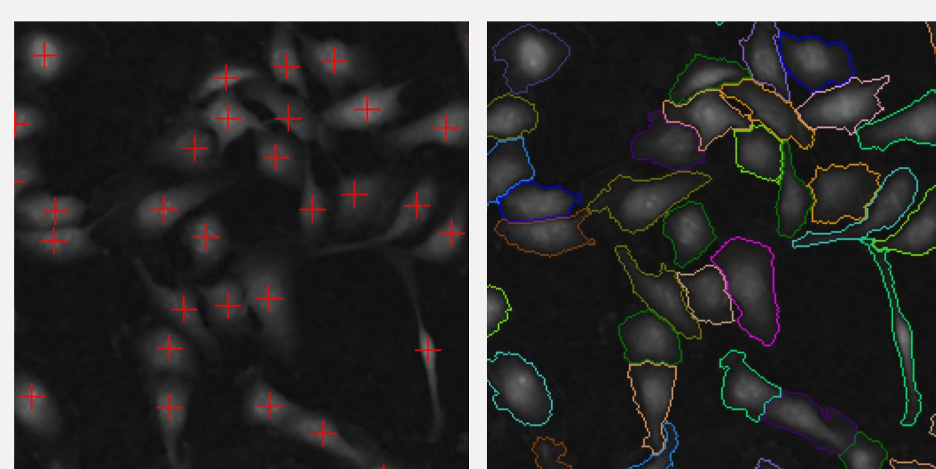

Fig. 3: (Left) Seedpoint assignment to each cell based on peak pixel intensities. (Right) Livecyte cell segmentation by algorithm.

Immunofluorescence (IF) Staining: Macrophages were pre-treated with phosphate buffered saline containing 10% foetal bovine serum and an Fc receptor blocker, then incubated with a fluorophore-tagged antibody at 1:50 dilution and 4 µM Hoechst 33342. Stained macrophages were washed with Tris-buffered saline with Tween-20 prior to fixation with 4% formaldehyde. Fluorescent membrane marker staining was assessed on the Molecular Devices ImageXpress Pico Automated Cell Imaging System.

Image Analysis: Cell segmentation of immunofluorescence images was performed using the Cellpose algorithm[3] and incorporated into a Fiji (Fiji is just ImageJ) analysis pipeline to determine the number of multi-nucleated cells based on Hoechst 33342 stained nuclei.

Results

Compound treatment caused a deceleration in the rate of change of morphological parameters associated with monocyte-to-macrophage differentiation

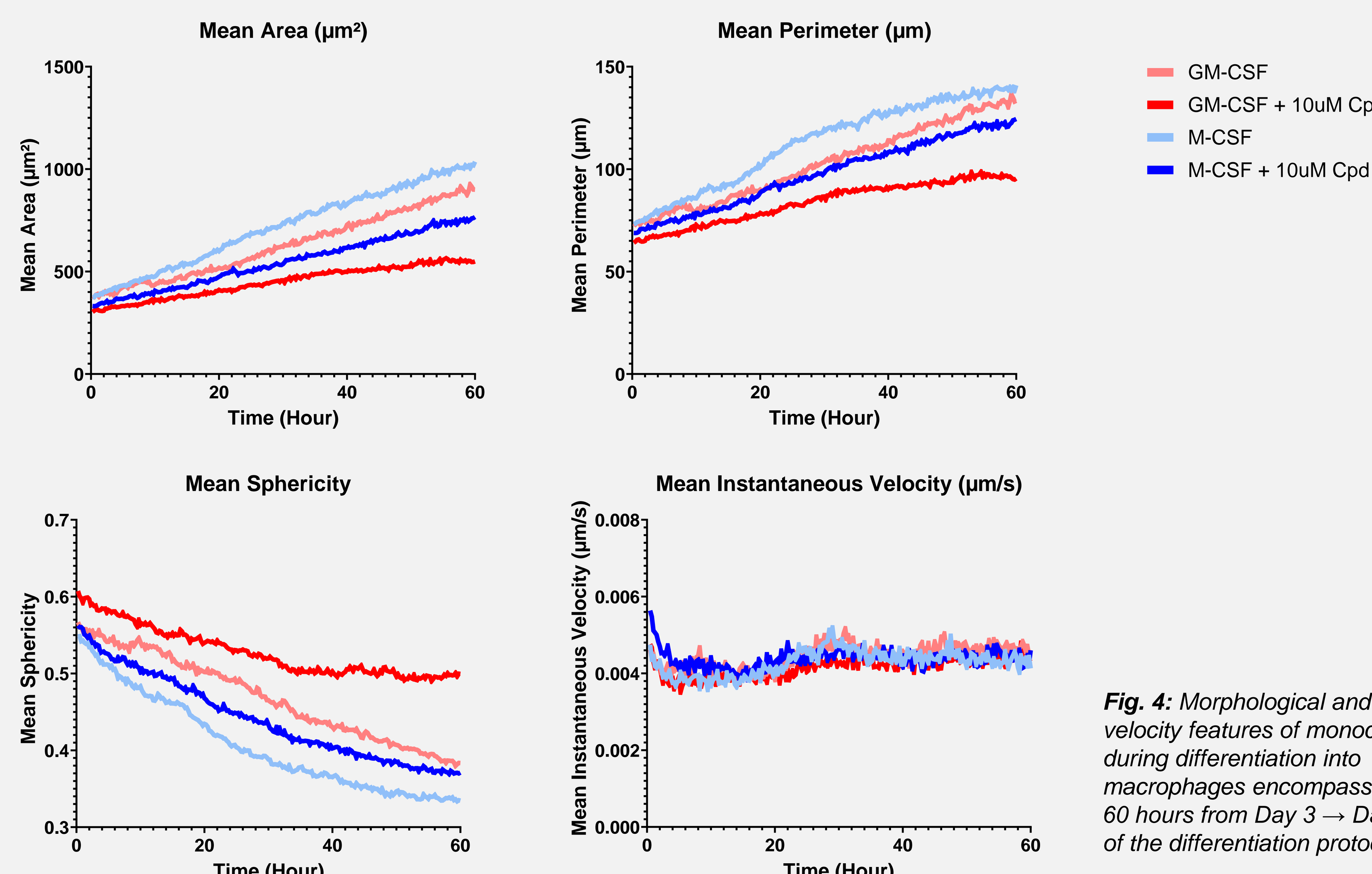

Fig. 4: Morphological and velocity features of monocytes during differentiation into macrophages encompassing 60 hours from Day 3 → Day 6 of the differentiation protocol.

Monocytes were induced to differentiate into M1-like or M0/M2-like macrophages in the presence or absence of a test compound. Livecyte data from Day 3 to Day 6 revealed that, in the presence of DMSO vehicle, the mean area and mean perimeter of monocytes increased during differentiation into both M1-like or M0/M2-like macrophages. In contrast, mean sphericity decreased during the time-course (**Fig. 4**). Comparison of monocyte and macrophage morphology demonstrated a more common occurrence of cellular protrusions/extensions in the latter that may be indicative of increased cell-to-cell communication (**Fig. 5**). Treatment with test compound reduced the rate of change of these morphological traits (**Fig. 4**), indicating a deceleration of processes associated with monocyte-to-macrophage differentiation.

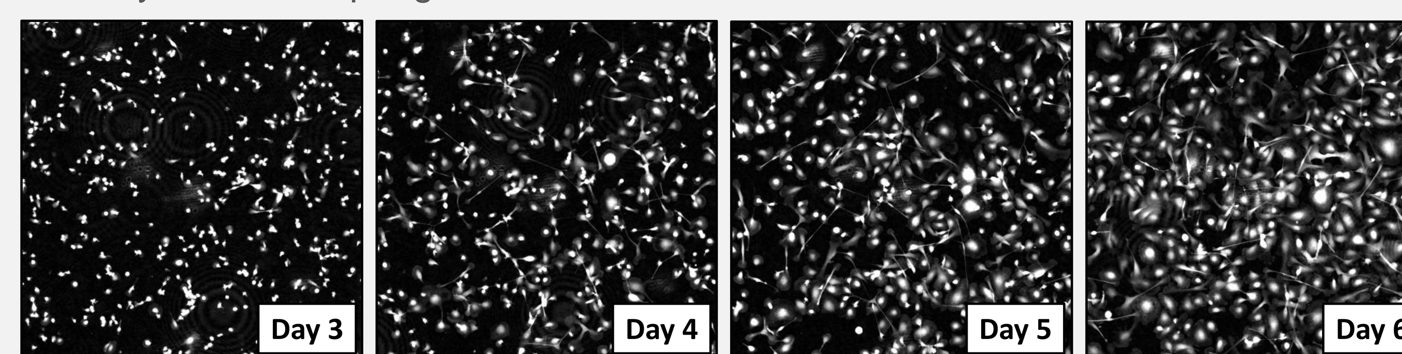

Fig. 5: Livecyte QPI images of M-CSF-differentiated macrophages taken 24 hours apart.

Compound treatment suppressed CD40 expression during monocyte-to-macrophage differentiation, an effect that was overridden upon polarisation

We next examined the expression of M1- and M2-associated membrane markers, CD40 and CD206.[4] IF imaging indicated that continuous treatment with the test compound over the 7-day differentiation period inhibited CD40 expression in M1-like macrophages (**Fig. 6**). Interestingly, stimulation of M1 polarisation by the addition of LPS/IFNγ overcame this inhibition (data not shown). In contrast, CD206 expression was not significantly affected by compound treatment in M-CSF stimulated monocytes (**Fig. 6**) as well as in IL-4/IL-13-polarised M2 macrophages (data not shown).

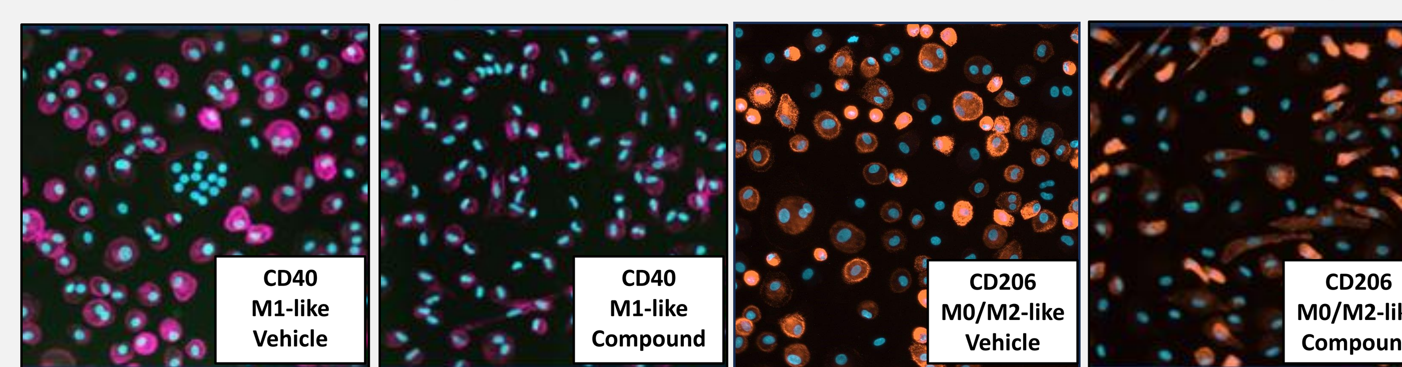

Fig. 6: IF staining for CD40 (purple) and CD206 (orange) in macrophages at day 7 of differentiation.

Compound treatment reduced the frequency of multi-nucleated cells in M-CSF-differentiated macrophages

In these studies, we observed macrophages that appeared to fuse into multi-nucleated cells, a strategy used predominantly by M2 macrophages to handle large, persistent threats, enhance phagocytosis, and manage tissue repair and remodeling.[5] Such fused cells were able to be tracked, characterised by a notable increase in area (**Fig. 7**). Further analysis indicated that compound treatment induced a reduction in cells containing 2 or more nuclei (**Fig. 7**).

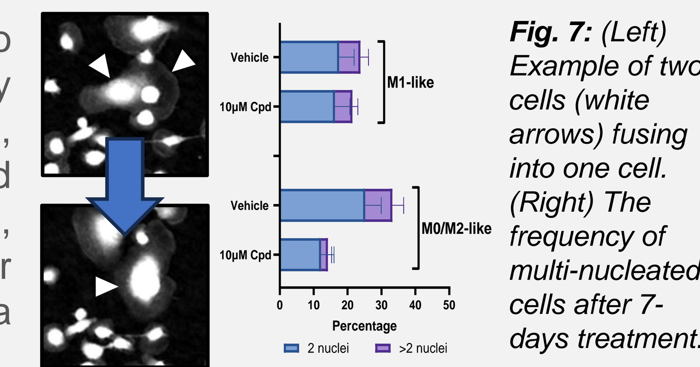

Fig. 7: (Left) Example of two cells (white arrows) fusing into one cell. (Right) The frequency of multi-nucleated cells after 7-days treatment.

Summary

By kinetic imaging, we captured phenotypic data on subtle morphological changes in response to compound treatment in a monocyte-to-macrophage differentiation assay:

- Monocytes increased in size (mean area and perimeter) and featured more cellular protrusions/extensions (reduced sphericity) during differentiation into macrophages. Treatment with the test compound decelerated the rate of change of these morphological changes.
- IF analysis revealed a compound induced inhibition of CD40 expression in GM-CSF differentiated macrophages, as well as a reduced frequency of multi-nucleated cells in M-CSF differentiated macrophages.

These results demonstrate the growing capabilities of novel imaging systems in *in vitro* drug discovery compound screening and mechanism of action studies. Moreover, the conclusions from these data outline the importance of capturing detailed kinetic experimental data with multiparameter readouts to aid the profiling of live cell phenotypic responses compared with traditional platforms.

References:

[1] Kasprowicz R., Suman R., & O'Toole P. Characterising live cell behaviour: Traditional label-free and quantitative phase imaging approaches. *Int J Biochem Cell Biol.* 2017 Mar:84:89-95. doi: 10.1016/j.biocel.2017.01.004. PMID: 28111333.
[2] Park M., Silvin A., Ginhoux F., & Merad M. Macrophages in health and disease. *Cell.* 2022 Nov 10;185(23):4259-4279. doi: 10.1016/j.cell.2022.10.007. PMID: 36368305.
[3] Stringer, C., Wang, T., Michaelos, M., & Pachitariu, M. Cellpose: a generalist algorithm for cellular segmentation. *Nature methods.* 2021 Jan;18(1):100-106. doi: 10.1038/s41592-020-01018-x. PMID: 33318659.
[4] Lis-López L., Bauset C., Seco-Cervera M. & Cosín-Roger J. Is the macrophage phenotype determinant for fibrosis development? *Biomedicines.* 2021 Nov 23;9(12):1747. doi: 10.3390/biomedicines9121747. PMID: 34944564.
[5] Ahmadzadeh K. *et al.* Multinucleation resets human macrophages for specialized functions at the expense of their identity. *EMBO Reports.* 2023 Jan 4;24(3):e56310. doi: 10.15252/embr.202256310. PMID: 36597777.