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High content analysis of phagocytic activity and cell morphology with PuntoMorph

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Abstract

Background—Phagocytosis is essential for maintenance of normal homeostasis and healthy tissue. As such, it is a therapeutic target for a wide range of clinical applications. The development of phenotypic screens targeting phagocytosis has lagged behind, however, due to the difficulties associated with image-based quantification of phagocytic activity.

New Method—We present a robust algorithm and cell-based assay system for high content analysis of phagocytic activity. The method utilizes fluorescently labeled beads as a phagocytic substrate with defined physical properties. The algorithm employs statistical modeling to determine the mean fluorescence of individual beads within each image, and uses the information to conduct an accurate count of phagocytosed beads. In addition, the algorithm conducts detailed and sophisticated analysis of cellular morphology, making it a standalone tool for high content screening.

Results—We tested our assay system using microglial cultures. Our results recapitulated previous findings on the effects of microglial stimulation on cell morphology and phagocytic activity. Moreover, our cell-level analysis revealed that the two phenotypes associated with microglial activation, specifically cell body hypertrophy and increased phagocytic activity, are not

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Conflict of Interest

The authors declare no competing financial interests.

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highly correlated. This novel finding suggests the two phenotypes may be under the control of distinct signaling pathways.

Comparison with Existing Methods—We demonstrate that our assay system outperforms preexisting methods for quantifying phagocytic activity in multiple dimensions including speed, accuracy, and resolution.

Conclusions—We provide a framework to facilitate the development of high content assays suitable for drug screening. For convenience, we implemented our algorithm in a standalone software package, PuntoMorph.

Keywords

Phagocytosis; high content analysis; image processing; phenotypic screening; microglia; drug discovery; morphometry

Introduction

Phenotypic screening is a powerful approach for discovering agents with disease-modifying activity (Reaume, 2011; Swinney, 2011; Eder, 2014). Modern day phenotypic screens typically rely on high content analysis (HCA) of cellular assays, taking advantage of the combined application of digital microscopy, robotics, and computational science (Al-Ali, 2016). By screening compounds directly on biological systems as opposed to purified gene products, their effects on complex cellular functions can be readily observed. As a consequence, high content assays have been developed to screen for agents that modulate a wide variety of disease-relevant cell functions, including morphology, viability, differentiation, growth, process extension, and subcellular localization (Buchser, 2004; Chaudry et al., 2009; Al-Ali, 2013; Moffat, 2014; Lim et al., 2015).

Phagocytosis of cell debris and pathogens is an essential homeostatic activity whose dysregulation contributes to the pathophysiology of numerous diseases (Blander, 2017; Lim et al., 2017). In systemic lupus erythematosus, for example, disease etiology has been linked to a failure in the phagocytic clearance of apoptotic cells, which become the source of pro-inflammatory mediators as well as autoimmunity-inducing self-antigens (Herrmann et al., 1998). In models of Alzheimer's disease, impaired clearance of amyloid beta deposits by microglia and blood-borne monocytes has been associated with worsening of the clinical phenotype (Simard et al., 2006; Butovsky et al., 2007), pointing to dysregulated phagocytosis as a key determinant of the human pathology (Zuroff et al., 2017).

Although prototypic phagocytic cells are monocyte/macrophages in the immune system and microglial cells in the central nervous system (CNS), numerous other cells possess phagocytic activity. Examples include astrocytes, dendritic cells, and neutrophils (Iyoda et al., 2002; Nordenfelt and Tapper, 2011; Morizawa et al., 2017). Phagocytosis by microglia is a feature of virtually all neurodegenerative diseases, where microglia serve as the CNS resident macrophages, and act as primary effectors of the innate immune response (Guilliams et al., 2014; Prinz et al., 2014).

Despite the ubiquity and high disease relevance of phagocytosis, developing high content screens utilizing a phagocytic readout has lagged behind. This is in part due to the difficulty of quantifying phagocytosis in 2D images of fluorescently labeled cell cultures, and the requirement for extensive normalization and correction across images from different cultures. As such, methods that screen for changes in phagocytic activity are typically based on flow cytometric quantification (Fricker et al., 2012) or ELISA detection. However, these techniques require cell detachment from the culture plates, or whole lysis of the culture, which preclude the simultaneous evaluation of cell morphology associated with phagocytosis.

To overcome these limitations, we developed an image analysis algorithm that can accurately count phagocytosed beads on 2D cell culture images. The algorithm utilizes statistical learning to automatically determine analysis parameters for each individual image, therefore requiring minimal user input. We validated it experimentally using a microglial assay system. For convenience, we implemented the algorithm in an easy-to-use software package called PuntoMorph. In addition to bead counting, PuntoMorph calculates a suite of commonly used HCA parameters, making it a standalone option for HCA users. PuntoMorph accurately captured these features, and demonstrated a superior performance compared to other analytical methods. Remarkably, our analysis also revealed that the two activated microglial phenotypes (increased cell body size and phagocytic activity) may not be correlated at the level of individual cells. PuntoMorph is available free of charge to the scientific community and can be downloaded at <https://github.com/HA-Lab/PuntoMorph>. We also provide a detailed description of the algorithm and source code, which can be easily customized or incorporated into preexisting analytical pipelines.

Materials and Methods

Primary mouse microglia cultures

Cultures were prepared as previously described (Gao et al., 2017). Briefly, cortices from postnatal day 3–5 C57BL/6 pups were harvested in cold HBSS and enzymatically dissociated into single cell suspensions with a papain-based Neural Tissue Dissociation Kit (Miltenyi) followed by passage through a 70 µm cell strainer. Microglial cells were isolated by positive selection with anti-CD11b magnetic MicroBeads in combination with LS columns (Miltenyi), according to manufacturer's protocols. Microglia were seeded in poly-L-ornithine-coated 24-well plates (50,000 cells/well) in DMEM/F12 medium supplemented with 20% fetal bovine serum (FBS), 1% MEM Non-Essential Amino Acids, 1% penicillin/streptomycin, 1% GlutaMAX, and 1% Sodium-Pyruvate (all from Gibco-Life Technologies™). Culture plates were maintained at 37°C in 5% CO₂ atmosphere. After 3 days, the medium was changed and the FBS concentration was reduced to 10%.

Phagocytosis assay

Microglial phagocytosis was assayed as previously described (Gao et al., 2017). Briefly, after one week in culture, microglia were exposed to RO0480500-002 (RO48, 5 µM in 0.05% DMSO) or its corresponding vehicle (0.05% DMSO), and LPS (100 ng/ml) or its corresponding vehicle (medium alone) for 24 hr. Following treatments, green fluorescent

beads (FluoSpheres® Carboxylate-Modified Microspheres (505/515), ThermoFisher) were added (1.5 µl of a 1:10 dilution), and the cells were incubated for 2 hr at 37°C in 5% CO₂ atmosphere. Cells were washed three times with ice-cold culture medium to arrest bead uptake, and immediately fixed with 4% PFA at room temperature for 15 min. Fixed cultures were then permeabilized with 0.4% Triton X-100 in PBS (15 min, room temperature), blocked with 5% normal goat serum (1 hr, room temperature), and incubated with anti-Iba1 primary antibody (1 hr, room temperature; rabbit, 1:500, Wako), followed by anti-rabbit AlexaFluor 594 secondary antibody (1 hr, room temperature; 1:750, Invitrogen). Nuclei were stained with DAPI for 10 min at room temperature. Cells were stored in PBS at 4°C until image acquisition.

Image acquisition

Images were taken at a 10× magnification with a ZEISS Axiovert 200M fluorescence microscope. From each well, three non-overlapping images from the top, middle and bottom areas were randomly taken. From each picture, the number of beads per cell was obtained by manual counting using Image J, or by automated counting using PuntoMorph and Cellomics™ (Thermo Fisher Scientific) software.

PuntoMorph workflow

Pre-processing, background correction, and contrast enhancement—All processing is performed with the MATLAB's Image Processing Toolbox v2016b (MathWorks Inc.). Images are partitioned into their three separate channels containing intensity values for cell bodies, nuclei, and intracellular objects (beads). Cell body images are background-corrected using the contrast-limited adaptive histogram equalization (CLAHE) method (Zuiderveld, 1994). Prior to this, pixels with intensity values smaller than 5% of maximum intensity are deleted to decrease artifacts from debris present in the culture. A Rayleigh distribution is used as the transformation function in CLAHE, with a 0.3 clip limit and an alpha of 0.7. A global threshold is computed with the Otsu's method (Otsu, 1975), and is then used to convert the image into binary. Holes within cell objects are filled automatically, and small breaks are connected using a Euclidean distance transform. Finally, cell object contours are smoothed by morphological opening using a 2×2 pixel structuring element. Nuclear images are processed similarly, but without the CLAHE transformation step, as nuclear staining tends to be significantly more robust and less artifact-prone than cytoplasmic/surface staining.

Identification of nuclear centers—Nuclei touching the image borders are discarded in the binary image. The sizes of the remaining nuclei are then calculated and fitted to a two Gaussian mixture model, whereby the smaller mean corresponds to the average size of a single nucleus. The mean radius is calculated by approximating a circular shape for individual nuclei, and is used to perform a Hough transform circle detection (Young, 2016) to identify individual nuclei. This process locates nuclear centers with sufficient precision to significantly improve segmentation of clustered cells with multiple adjacent nuclei.

Identification of valid cells—Nuclei of viable cells are identified by determining whether their computed centers exist within the boundaries of the binary cell body image.

These centers are used to impose regional intensity minima in the corresponding cell body image (as processed above). The watershed transform algorithm (Meyer, 1994) is then used to demarcate cell boundaries. Cells that are in contact with image borders, lack a nucleus, or have a size smaller than 1.5 times the mean nuclear size are discarded from subsequent analysis. Finally, the remaining cell sizes and intensities are fitted to normal distributions, and objects with areas or intensities falling outside the 99% confidence interval are discarded to minimize interference from artifacts.

Analysis of morphological features—MATLAB's regionprops tool is used to measure various properties of the identified cells including area, convex area, eccentricity, major axis length, minor axis length, and perimeter. Membrane irregularity is calculated according to the formula:

$$\text{Membrane Irregularity} = \frac{\text{perimeter} - \text{convex perimeter}}{\text{convex perimeter}} \times \frac{\text{minor axis length}}{\text{major axis length}}$$

Counting of beads—The image in the inclusion body channel is thresholded and converted to binary to identify the location of the bead objects (composed of one or more beads clustered together) within the imaged field. The total intensities of all the objects are then acquired from the original image and fitted to a two Gaussian mixture model, whereby the smaller mean corresponds to the average total intensity of a single bead. The total intensity of each object is then divided by the mean total intensity of a single bead and rounded to the nearest whole number to approximate the total number of beads within each object. Finally, objects are assigned to individual cells based on their location in the cell body channel, and the total number of objects within each cell are calculated.

Statistical analysis

Data are represented as mean $\pm$ SEM. For multiple comparisons, data were analyzed by one-way ANOVA followed by Tukey's post-hoc test. Two-tailed Student's t test was applied in single comparisons. Correlation analyses were conducted with the Pearson's correlation method. P values equal to or less than 0.05 were considered statistically significant. Statistical analyses were performed with Prism 6 software.

Results

PuntoMorph workflow

PuntoMorph analytical flow (Figure 1) comprises of four stages: 1) nuclear detection; 2) cell body demarcation; 3) phagocytized bead quantification; 4) determination of morphological parameters. After pointing PuntoMorph to the directory containing the image files to be analyzed, the software detects each picture within all subfolders and automatically initiates sequential analysis. For each file, PuntoMorph splits the composite image into its constituent RGB channels. In our setting, the blue channel is reserved for DAPI nuclear stain, the red channel for the anti-Iba1 microglial marker, and the green channel for FITC-conjugated bead fluorescence. The first stage of the analysis identifies the individual nuclei within the nuclear channel. A two Gaussian distribution mixture model is fitted to the histogram of nuclear object areas to estimate the mean area of an individual nucleus. The average nuclear area is

then used to estimate the average radius, assuming a mostly circular shape. The radius is then used to perform a Hough transform circle detection (Young, 2016) to identify single nuclei and estimate the location of their center of gravity. The second stage of the analysis identifies and demarcates cell bodies. After the cellular marker image is processed and converted to binary, the watershed transform is applied to segment the cell bodies using the centers of gravity of their enclosed nuclei. This makes it possible to accurately segment clustered cells whose nuclei are touching or in very close proximity. The third stage of the analysis identifies and quantifies the number of beads within each cell. This is accomplished by fitting a two Gaussian distribution mixture model to the histogram of bead object intensities to estimate the mean intensity of an individual bead. The total intensity of each bead object is then divided by the mean bead intensity to calculate the number of beads within each object. This algorithm allows accurate quantification of the number of beads even when compacted in clusters, because the total intensity of the cluster scales linearly with the number of beads it contains (unlike the 2D area projection of the cluster onto the image). Finally, morphological parameters are computed for each individual cell, and the total number of beads within its borders are calculated accordingly.

For each image in the folder, PuntoMorph automatically generates an overlay of the analysis to the original image, with each cell labeled numerally. This allows users to track and check results cell by cell and apply filters to exclude artifacts from analyses. A table summarizing the results for all images in the analysis folder is generated and saved within the starting directory.

PuntoMorph accurately quantifies microglial phagocytosis

In a recent study, we performed manual counting of phagocytized fluorescent beads to demonstrate that LPS stimulation enhances microglial phagocytosis (Gao et al., 2017). Here, we performed a similar analysis with PuntoMorph, and compared the results to those obtained with two other quantification methods: 1) manual counting aided by Image J software, and 2) automated counting using the Neuronal Profiler BioApplication V4 (Cellomics®). On a sample of fifty randomly selected cells, the number of phagocytosed beads counted with PuntoMorph showed excellent correlation with the number obtained through manual counting under both non-stimulated and LPS-stimulated conditions (Figure 2A, B), as indicated by Pearson's correlation coefficients of 0.9494 and 0.9263, respectively. On the contrary, Cellomics analysis showed poor correlation with manual counting (Figure 2A, B), with coefficients of 0.2972 in non-stimulated and 0.4719 in LPS-treated cells. Notably, PuntoMorph maintained high accuracy even when analyzing LPS-stimulated microglia, where a high load of incorporated beads can lead to an underestimation using the manual counting method (Figure 2B, C). Both PuntoMorph and manual counting detected an increase in phagocytic activity in microglia stimulated with LPS (Figure 2D), recapitulating the findings of our previous study (Gao et al., 2017). We performed additional validation of our software by testing the phagocytosis modulating capacity of RO48, a small-molecule kinase inhibitor of ROCK and other kinases, which we previously showed to promote neurite outgrowth in vitro and axon sprouting in vivo (Al-Ali et al., 2015). This choice was based on recent evidence demonstrating that Y-27632, another inhibitor of ROCK and other kinases, interferes with microglial phagocytosis (Scheiblich and Bicker,

2017), In line with those findings, we demonstrated both by PuntoMorph analysis and manual counting that RO48 significantly reduced phagocytosis in LPS-stimulated microglia (Figure 2D).

PuntoMorph tracks changes in cell morphology

Cell morphology is a key indicator of cell function and, in the case of glial cells, is highly correlated with their activation state under pathological inflammatory conditions (e.g. microgliosis, astrogliosis) (Giaume et al., 2007; Anderson et al., 2014). With the development of the PuntoMorph software, our goal was not only to assess the presence of phagocytosed bodies, but also to measure cell morphology in order to better define the cell activation state. PuntoMorph enables the automated quantitative assessment of cell morphology by measuring various parameters, which include cell body area (Figure 3A, green area), convex area (Figure 3A, gray area overlapping the cell body region), and cell perimeter (Figure 3A, blue line). PuntoMorph can also discriminate cells by their jagged appearance, which is measured as Membrane Irregularity (formula for calculating irregularity is described in the Methods) (Figure 3A). This phenotype cannot always be reliably captured by the conventional Convex Area to Area ratio adopted by other morphometric software, where an increased ratio is presumed to be characteristic of cells with higher irregularity (also referred to as less solidity). This is due to the fact that cells with elongated morphology can have a high Convex Area to Area ratio (Figure 3A), and thus be mistakenly assessed as being more irregular (Figure 3A). PuntoMorph minimizes such false positives by correcting for high ellipticity in the assessment of irregularity.

Morphological parameters were evaluated in non-stimulated and LPS-stimulated microglia, in the absence or presence of RO48. Cell membrane irregularity was significantly increased after LPS stimulation, and RO48 exposure potentiated this effect (Figure 3B). Notably, RO48 increased membrane irregularity also in non-stimulated microglia (Figure 3B). With respect to cell perimeter, we found a significant increase after LPS stimulation (Figure 3C), which was further enhanced by RO48 treatment. RO48 significantly increased perimeter length also in non-stimulated conditions. Cell body area was augmented following LPS stimulation (Figure 3D), with a small but significant increase in the presence of RO48. The changes in morphological parameters measured after stimulation are consistent with the typical rearrangements observed during microglial activation (microgliosis), whereby cells assume amoeboid morphology and become hypertrophic (increased cell size).

PuntoMorph reveals lack of correlation between microglial activation phenotypes

Activated microglial cultures were previously shown to exhibit increased phagocytic activity along with enlarged cell body size. To determine whether the two phenomena are coupled at the individual cell level, we performed a correlation analysis of the phenotypes. While LPS does indeed increase both parameters in the population, the two phenotypes do not track together at the level of individual cells (Figure 4A). This suggests they may be under the control of different pathways, making it possible to decouple them by applying stimuli targeting each pathway independently. Interestingly, this appears to be the case for RO48, which leads to increased cell body size but a decrease in phagocytic activity in microglia (Figure 4B).

Discussion

Here we report the development of PuntoMorph, an image analysis algorithm that can accurately quantify phagocytosis by counting incorporated beads within 2D cell culture images, while simultaneously assessing corresponding cell morphometric parameters. This provides a high content analysis framework that can be developed to screen for agents that target these crucial cellular processes (Lim et al., 2017).

Despite phagocytosis being an essential homeostatic function both in health and disease, the development of high content phenotypic screens to quantify it has been problematic. Previous HCA attempts to assess phagocytosis have typically used a binary cut-off to classify cells as positive or negative for the phagocytosed material, and then quantified apoptotic activity or other cell parameters at the population level (Neher et al., 2011; Rinaldi et al., 2016). This approach permits the simultaneous acquisition of additional morphological features that may be relevant to the disease process. However, the binary assignment precludes detailed understanding of the relationship between phagocytosis and such parameters. We previously developed an assay to assess phagocytosis in individual microglia using fluorescently labeled beads (Gao et al., 2017). By counting the number of beads taken up by the cells, a discrete measure of phagocytic activity can be obtained at the level of individual cells. This permits a more nuanced assessment of phagocytic activity, making it possible to investigate correlations between phagocytosis and the other parameters. Existing HCA algorithms for intracellular object detection perform poorly at counting intracellular beads. This necessitates manual counting of phagocytosed objects, which is time consuming and susceptible to operator error. Furthermore, intracellular crowding can lead to superimposition and, in some cases, to active aggregation of inclusion objects. This makes it difficult to quantify phagocytosed beads in 2D images with area-based methods. To circumvent these issues, we developed the PuntoMorph algorithm to automatically determine the mean fluorescence intensity of individual beads within each image, and then use this information to accurately count the number of beads within each cell in that image. The program excludes from analysis beads that are not phagocytosed, without any requirement for defining a region of interest. This is due to the fact that phagocytic analysis in our program is predicated on cellular detection. Only beads that are located within the boundaries of an identified cell object are counted as phagocytosed.

We experimentally validated PuntoMorph using a microglial assay system. When activated by specific stimuli (e.g. pro-inflammatory mediators, pathogens), microglia rapidly change morphology from a small and often elongated cell body to a much larger and rounder cell body (Nimmerjahn et al., 2005). Our studies on microglial cultures recapitulated these findings at the level of the cell population, demonstrating that PuntoMorph not only accurately captured these behaviors, but also outperformed pre-existing object-detection algorithms as well as manual counting.

Interestingly, examining the data at the single cell level revealed that the two cell activation phenotypes (increased phagocytosis and increased cell size) are not coupled, suggesting that functionally distinct cell populations might exist within the microglial culture. This also raises the possibility that the two phenotypes might be under the control of separate

pathways, which may be modulated by distinct signals. Indeed, this is what we observe with the RO48 compound, which decreases mean phagocytic activity within the population while simultaneously increasing mean cell body area. Whether the differential effects of RO48 on microglial activation phenotypes contribute to its *in vivo* efficacy in promoting axon regeneration is presently not clear. Nevertheless, this certainly poses an interesting biological question which warrants further investigation.

Even though our choice for PuntoMorph validation was to use microglial cultures, this analysis is applicable to any cell type grown in a conventional 2D culture system where assessment of phagocytic activity is relevant to understanding biological functions or disease processes. Hence, astrocytes, macrophages and fibroblasts, to name a few, are cell populations to which PuntoMorph analysis could be readily extended. It is important to point out that while our software performs extremely well with 2D cultures, it cannot be directly applied to the analysis of slice cultures. This is due to the difficulty of segmenting cells in non-dissociated 3D tissue, which tends to be significantly more complex than segmenting cells in 2D. Moreover, the 3D nature of the slice culture makes it difficult to count beads based on mean fluorescence intensity, since beads outside the plane of focus could interfere with the analysis.

An additional benefit of PuntoMorph is that it gives users full control in assigning color channels, which allows for greater flexibility in assay design. The only requirement is that the three channels be colorized as RGB. Overall, PuntoMorph software provides a one-stop solution for automated high content analysis of cell culture images, whether for investigational biology or phenotypic screening exercises, providing a valuable tool for translational studies and drug discovery.

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Highlights

- We describe the development of a new algorithm for accurate and automated counting of phagocytosed beads in 2D images
- The algorithm is implemented in a free software package, PuntoMorph
- PuntoMorph simultaneously analyzes cell morphology on the same images
- PuntoMorph consistently outperforms automated and manual counting methods
- PuntoMorph includes additional modules for high content analysis, making it a standalone option for phenotypic screening
- The combination of the assay system and analytical algorithm is a powerful tool for drug discovery

Figure 1. PuntoMorph workflow

Phagocytosed fluorescent beads are visualized in the FITC channel (green). Microglial cells are identified by immunocytochemistry with an anti-Iba1 antibody (red) in combination with DAPI (blue) for nuclei counterstaining.

Figure 2. Automated quantification of microglial phagocytosis with PuntoMorph

(A, B) To assess the accuracy of PuntoMorph, fifty microglial cells were randomly picked from each treatment group. Intracellular beads were manually counted with Image J and, in parallel, automatically counted with PuntoMorph and Cellomics software in naïve condition (A), or after LPS stimulation (B). Each automated counting method was compared to manual counting. Pearson's correlation coefficients (r) are shown. (C) Schematic detailing how PuntoMorph may outperform other automated or manual bead counting methods. (D) Quantification of phagocytic activity of naïve versus LPS-stimulated microglia treated with RO48 or corresponding vehicle control. The assessment is conducted by manual counting and PuntoMorph automated counting. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA, Tukey post hoc test.

Figure 3. Automated analysis of cell morphometry with PuntoMorph

(A) PuntoMorph quantitatively captures microglial morphological changes by measuring multiple parameters, such as membrane irregularity, perimeter and cell area. (B) Quantification of cell irregularity in naïve versus LPS-stimulated microglia treated with RO48 or corresponding vehicle; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA, Tukey test; $\wedge p < 0.05$ vs DMSO/non-stim, Student's t test. (C) Quantification of cell perimeter length in naïve versus LPS-stimulated microglia treated with RO48 or corresponding vehicle; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA, Tukey post hoc test; $\wedge p < 0.05$ vs DMSO/LPS and $p < 0.01$ vs NT/LPS; $\wedge\wedge p < 0.01$ vs NT/non-stim, Student's t test. (D) Quantification of cell body area in naïve versus LPS-stimulated microglia treated with RO48 or corresponding vehicle control; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA, Tukey test; $\wedge p < 0.05$ vs DMSO/LPS and $p < 0.01$ vs NT/LPS, Student's t test. NT = non-treated.

Figure 4. Single cell level analysis with PuntoMorph

Correlation analysis between the number of beads incorporated by individual cells and their corresponding cell area following exposure to LPS or corresponding vehicle (control) (A), and RO48 or corresponding vehicle (control) (B). Pearson's correlation coefficients (r) are shown.