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Imaging Flow Cytometry to Evaluate Altered Endocytic CD1d Trafficking
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TITLE:

Imaging Flow Cytometry to Evaluate Altered Endocytic CD1d Trafficking

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SUMMARY:

Imaging flow cytometry provides an ideal approach to detect the morphological and functional alteration of cells at individual and populational levels. Disrupted endocytic function for lipid antigen presentation in pollutant-exposed human dendritic cells was demonstrated with a combined transcriptomic profiling of gene expression and morphological demonstration of protein trafficking.

ABSTRACT:

Populational analyses of the morphological and functional alteration of endocytic proteins are challenging due to the demand of image capture at a single cell level and statistical image analysis at a populational level. To overcome this difficulty, we used imaging flow cytometry and transcriptomic profiling to determine altered subcellular localization of the cluster of differentiation 1d protein (CD1d) associated with impaired endocytic gene expression in human dendritic cells (DCs), which were exposed to the common lipophilic air pollutant benzo[a]pyrene. The colocalization of CD1d and endocytic marker Lamp1 proteins from thousands of cell images captured with imaging flow cytometry was analyzed using IDEAS and ImageJ-Fiji programs. Numerous cellular images with co-stained CD1d and Lamp1 proteins were visualized after gating on CD1d$^+$Lamp1$^+$ DCs using IDEAS. The enhanced CD1d and Lamp1 colocalization upon BaP exposure was further demonstrated using thresholded scatterplots, tested with Mander's coefficients for co-localized intensity, and plotted based on the percentage of co-localized areas using ImageJ-Fiji. Our data provide an advantageous instrumental and bioinformatic approach to measure protein colocalization at both single and

45 populational cellular levels, supporting an impaired functional outcome of transcriptomic
46 alteration in pollutant-exposed human DCs.
47
48 **INTRODUCTION:**
49 Antigen presentation typically involves intracellular protein trafficking, which has been often
50 investigated using morphological characterization and phenotypic profiling of antigen
51 presenting cells[1-3]. To integrate the advantages of imaging and phenotyping methods, we
52 describe an imaging analysis platform at both single cell and population levels to demonstrate
53 an altered protein colocalization in human dendritic cells (DCs). In peptide antigen
54 presentation, major histocompatibility complex (MHC) class I molecules bind a short peptide (8-
55 10 residues) in the endoplasmic reticulum to activate conventional CD8^{+} T cells, while MHC
56 class II molecules bind a relatively longer peptide (~20 residues) in endocytic compartments to
57 activate conventional CD4^{+} T cells[1,4]. In contrast, lipid-specific T cells are activated by CD1
58 proteins with lipid antigens loaded mainly in endocytic compartments[5,6]. Lipid antigen
59 presentation requires the supply of lipid metabolites produced in lipid metabolism[7-10] and the
60 loading of functional lipid metabolites to CD1 proteins in endocytic compartments[5,6]. In this
61 context, various cellular factors modulating lipid antigen presentation, especially in
62 environmental exposure of lipophilic pollutants and immune disorders, are critical to be
63 defined. In this study, we used transcriptomic analysis, image profiling, and cellular and
64 populational imaging analysis of human monocyte-derived DCs to determine the endocytic
65 protein trafficking contributing to lipid antigen presentation in pollutant exposure. Certainly,
66 this combined platform can be applied to studying subcellular protein trafficking and
67 colocalization in different biological processes.
68
69 Technically, subcellular protein localization was usually demonstrated using confocal
70 microscopy and statistically analyzed in a limited number of detected cells[1-3,11]. Moreover, flow
71 cytometry has been broadly applied to gate cell populations with co-stained signals of multiple
72 proteins at a cellular level[12]; however, this lacks a detailed visualization of subcellular protein
73 colocalization. To achieve comprehensive and statistical analyses of percentage protein
74 colocalization at both cellular and population levels, we incorporate imaging profiling and
75 analysis approaches to determine the features of protein colocalization with biological
76 relevance. Specifically, we use imaging flow cytometry to detect the colocalization of CD1d and
77 lysosomal-associated membrane protein 1 (Lamp1) proteins in this study. Quantitative analysis
78 of colocalized molecules was previously difficult to be performed at a populational scale. In this
79 study, we adapted the ImageJ-Fiji program to examine the percentage of protein colocalization
80 with a large number of co-stained cells at both populational and individual cellular levels.
81 Specifically, we measured co-localized areas, intensity, and populational size to support the
82 conclusion that the CD1d protein was largely retained in late endocytic compartments of
83 human DCs in exposure to a lipophilic pollutant benzo[a]pyrene (BaP)[13]. This combined cellular
84 and population imaging analysis provided highly reproducible, comprehensive, and statistically
85 significant results of CD1d-Lamp1 colocalization relevant to inhibited lipid antigen presentation.
86
87 The transcriptome of BaP-exposed human DCs strongly supported the hypothesis that
88 endocytic lipid metabolism and CD1d endocytic trafficking were impaired in BaP exposure. To

2

89 test this hypothesis, we applied imaging flow cytometry to profile the images of DC population
90 that were co-stained with multiple proteins including CD1d, endocytic markers, and DC
91 markers. Finally, co-stained cells were statistically analyzed to demonstrate the percentage of
92 intensity and areas of CD1d and Lamp1 colocalization.
93
94 **PROTOCOL:**
95
96 Human protocols in this study were approved by the Institutional Review Board of the
97 University of Cincinnati and all methods were performed in accordance with the relevant
98 guidelines and regulations. Blood samples from healthy donors were obtained from the
99 Hoxworth Blood Center at the University of Cincinnati Medical Center.
100
101 **1.   Transcriptomic Profiling of Pollutant-Exposed Human Monocyte-Derived DCs**
102
103 1.1.   Total RNA extraction of BaP-exposed DCs
104
105 1.1.1. Differentiate human DCs using cytokines GM-CSF and IL-4, and expose DCs to BaP for 4
106 days[13].
107
108 1.1.2. Sort BaP-exposed DCs using flow cytometry based on the surface expressed markers
109 (Lineage⁻HLA-DR⁺), the majority of which consists of conventional DCs (CD11c⁺CD1c⁺)[13].
110 Typically, sort 10,000 DCs into culture medium containing 10% FBS, centrifuge DCs at 600 x g
111 and 4 °C for 6 min, and immediately remove the supernatant by aspiration using a 1 mL pipette
112 tip.
113
114 1.1.3. Once the supernatant is removed by aspiration, lyse the cell pellet by adding 0.4 mL of
115 Lysis/Binding Buffer from the RNA isolation kit for storage in -80 °C before RNA extraction.
116
117 1.1.4. Use the RNA isolation kit with the total RNA extraction protocol to extract the total
118 RNA[14]. Measure the RNA integrity using a Bioanalyzer[15].
119
120 1.2.   Transcriptomic analysis of sorted DCs (**Figure 1A**)
121
122 1.2.1. Prepare the library for RNA-seq using a RNA Library preparation kit. In short, fragment
123 the isolated poly-A RNA (~200 bp), reverse transcribe the fragments to the 1st strand of the
124 cDNA, and follow by the second strand cDNA synthesis labeled with dUTP.
125
126 1.2.2. Ligate double strand cDNA to the adapter with a stem-loop structure after bead
127 purification, end-repairing and dA-tailing. Excise the uracil residues in the 2nd strand of the
128 cDNA and adapter loop with USER (Uracil-Specific Excision Reagent) enzyme to maintain strand
129 specificity and open the adapter loop for PCR.
130

131   1.2.2.1.   Perform 13 cycles of PCR using universal and index-specific primers to enrich the
132   indexed library. Clean up the library and run on a Bioanalyzer DNA High Sensitivity chip to check
133   the library quality and size distribution.

134

135   1.2.3.   Use a library quantification kit and a real-time PCR system combined with library size
136   information to calculate library concentration *via* a standard curve method.

137

138   1.2.4.   Proportionally pool individually indexed compatible libraries and adjust the final total
139   concentration to 15 pM. Perform library cluster generation and sequence the library at a setting
140   of single read 1x50 bp to generate ~25 million reads per sample.

141

142   1.3.   Sequencing

143

144   1.3.1.   Load the sequencing and indexing reagents to the SBS and PE reagent racks,
145   respectively. Place the reagents in a laboratory-grade water bath for 1 h until all the ice has
146   melted and the reagents in each bottle/tube are mixed properly.

147

148   1.3.2.   Prepare the ICB mix by adding the thawed dye and -20 °C enzyme to the bottle and mix.
149   Prepare a NaOH solution according to the sequencing instructions. Place all reagents at 4 °C
150   until ready to use.

151

152   1.3.3.   During the 1 h waiting period, power on the sequencer. Wait for the DONOTEJECT drive
153   to appear and connect the computer to a network drive.

154

155   1.3.4.   Launch the sequencer control software.

156

157   1.3.5.   Prepare 2 L of Maintenance Wash solution that contains 0.5% Tween 20 and 0.03%
158   ProClin 300 in laboratory-grade water.

159

160   1.3.6.   In the SBS reagent rack, add ~100 mL of Maintenance Wash solution to each of the 8
161   bottles, and screw funnel caps to the bottles. In the PE reagent rack, add ~12 mL of
162   Maintenance Wash solution to each of the ten 15 mL conical tubes, and discard the caps.

163

164   1.3.7.   Load the two racks with the solution filled bottles/tubes to the sequencer.

165

166   1.3.8.   From the sequencer control software, choose the Maintenance Wash; follow the
167   instructions on screen to clean the sequencer fluid system until the process is completed.

168

169   1.3.9.   Start a New Run in the **SEQUENCE** tab from the software; direct the output data to a
170   network drive. Choose parameters for single read 1x50 bp with single index multiplexed
171   libraries.

172

173   1.3.10. Optionally, log into the BaseSpace Sequence Hub so that the sequencing status can be
174   remotely monitored via a computer or smart phone.

175

1.4.   Bioinformatic analysis

1.4.1.   Perform bioinformatics RNA-seq data analysis[13].

2.   Pathway Analysis of Transcriptomic Profiles (Figure 1B)

2.1.   Use an edgeR Bioconductor to compare resultant gene expression intensity counts between BaP-exposed and non-exposed DCs from three donors. Then, identify differentially expressed genes between BaP-exposed and non-exposed DCs based on the absolute fold change (>2 folds) and the false discovery rates (FDRs)-adjusted *p*-values (<0.05).

2.2.   To predict the functional clusters of differentially expressed genes, use the ToppCluster software package to search the altered genes against several databases including KEGG and REACTOME and generate clustering data. ToppCluster uses the hypergeometric test to obtain functional enrichment achieved via the gene list enrichment analysis[16].

2.3.   Further input the results from these function clusters to Cytoscape[17,18] Version 3.3.0, a broadly used open source software platform for visualizing complex networks. Thus, the genes involved in different clusters or pathways, including endocytic clusters and lipid metabolism, are shown in a network with color annotation of the averaged fold change of gene expression (**Figure 1B**)[13].

3.   Imaging Flow Cytometry of CD1d and Lamp1 Colocalization

3.1.   Antibody labeling of BaP-exposed DCs

3.1.1.   Expose 0.5 x 10^6 human DCs to 5.94 µM BaP for 4 days at 37 °C in 2 mL of complete Dulbecco's Modified Eagle's Medium. Harvest DCs by centrifugation at 400 x g for 10 min. Block the differentiated DCs by incubating cells with human serum blocker and anti-human Fc receptor antibodies for 10 min in ice, including anti-human CD16, CD32, and CD64 antibodies.

3.1.2.   Incubate Brilliant Violet 421-anti-CD1c (L161), phycoerythrin/cyanine dye 7 (PE/Cy7)-anti-HLA-DR, and PE/Cy5-anti-CD11c with DCs for 20 min in ice. **Table 1** shows this list of antibodies.

3.1.3.   Fix DCs with 4% paraformaldehyde in PBS and permeabilize them with Permeabilization Wash Buffer. Perform the intracellular staining with a mixture of PE-labeled purified anti-human CD1d (51.1) and the Alexa Fluor 647-labeled anti-Lamp1 (CD107a) (H4A3).

3.2.   Imaging flow cytometry measurement (**Figure 2**)

3.2.1.   Analyze the stained samples using an imaging flow cytometer at the flow cytometry core of Cincinnati Children's Hospital using a 40X objective to yield 300 pixels for a cell with around 10 µm in diameter. One-pixel size is approximately 0.5 µm by 0.5 µm of the object.

3.2.3. Acquire 10,000 cellular images in an unbiased manner according to the manufacturer's instruction.

4.   Imaging Analyses of Flow Cytometry Images

4.1.   Colocalization analysis using IDEAS software (**Figure 2**)

4.1.1.   Perform compensation with single-stained versus non-stained samples by setting the fluorescence intensity of non-stained samples below the threshold, including non-stained BaP-exposed DCs with BaP autofluorescence, *e.g.*, **Analyze | Colocalization | Colocalization**.

4.1.2.   Gate stained cells to obtain the subsets of HLA-DR+CD11c+CD1d+Lamp1+ cells (**Figure 2A**) and show cellular images in the gated subsets (**Figure 2B**).

4.1.3.   Save cell images in a png format based on two technical inclusion criteria, the visual presence of strong co-stained signals and subcellular localization of CD1d and Lamp1 proteins, for the colocalization analyses using ImageJ-Fiji (**Figure 2B**).

4.2.   Use software ImageJ-Fiji to perform colocalization analysis (**Figure 3**).

262   4.2.1.   Prepare the input image files by merging the 100 saved cell images for non-exposed and
263   BaP-exposed human DCs, respectively (**Figure 3A**).

264

265   4.2.2.   Analyze CD1d (Red) and Lamp1 (Green) colocalization using a scatterplot.

266

267   4.2.2.1 Run ImageJ-Fiji program. Open the .png file with 100 cell images (**Figure 3B and** Figure
268   **4A**): **File | Open**.

269

270   4.2.2.2 Split the image with merged red and green channels into two images with either a red
271   or a green channel: **Image | Color | Split channels**.

272

273   4.2.2.3 Draw a scatterplot using the commands **Analyze | Colocalization | Colocalization**
274   **Threshold**. Save the scatterplot using the **PrintScreen** key.

275

276   4.2.3.   Calculate Mander's colocalization coefficients for each single cellular image (n=100)
277   **(Figure 4B)**

278

279   4.2.3.1 Select a single cell image on the image file with split channels using the **Oval** selection
280   tool.

281

282   4.2.3.2 Use the commands **Analyze | Colocalization | Colocalization Threshold**. Select **Channel**
283   **1** from the dialogue box of region of interest (ROI). Keep all calculation options including
284   **Mander's using thresholds** for each cell image.

285

286   4.2.3.3 Repeat this calculation for all 100 cell images.

287

288   4.2.3.4 Save and open the results using a spreadsheet.

289

290   4.2.3.5 Plot the average and standard errors for "thresholded Mander's coefficients" (n=100, 0
291   means no colocalization and 1 means perfect colocalization). Use Student's t-test to calculate
292   the p value for the comparison between BaP-exposed and non-exposed groups **(Figure 4B)**.

293

294   4.2.4.   Calculate the percent of thresholded pixel intensity co-localized between CD1d and
295   Lamp1 for multiple cell images (n=100) (**Figure 5C**).

296

297   4.2.4.1.   Use the same analysis protocol in 4.2.3 and additionally select the result
298   option **% intensity above threshold colocalized**.

299

300   4.2.4.2.   Perform this analysis together with Mander's colocalization coefficients.

301

302   4.2.4.3.   Similarly plot the average and standard errors for% intensity colocalized
303   between CD1d and Lamp1. Use Student's t-test to calculate the p value for comparison
304   between BaP-exposed and non-exposed groups **(Figure 5C).**

305

REPRESENTATIVE RESULTS:

The lipophilic pollutant BaP alters endocytic gene clusters in human DCs. Human monocyte-derived DCs from each donor (n=3) were incubated with BaP and sorted for labeled conventional DCs, which were further used for RNA extraction and transcriptomic analysis as described. Upon the normalization of gene expression, altered genes between BaP-exposed and non-exposed groups were clustered according to the functional correlation of differentially expressed genes. We input the altered gene list to the Toppcluster program[16] for the analyses of relevant cellular pathways and perform the statistical test using a false discovery rate (FDR) correction of *p* values (**Figure 1B**). Several major altered gene clusters, including lipid metabolism and endocytic functions, were identified in BaP-exposed DCs. In this study, we specifically focused on the functional test of endocytic trafficking, because CD1-mediated antigen presentation has been highly dependent on the lipid antigen loading in the endocytic pathway[5,19-21].

CD1d endocytic trafficking is demonstrated using imaging flow cytometry. Upon obtaining a large number of cell images using imaging flow cytometry, we started using the IDEAS program to visualize individual cellular images through conveniently gating cell populations co-stained with multiple antibodies (**Figure 2A**). Specifically, we were able to gate the major DC population co-stained with HLA-DR and CD11c to further show the DCs with CD1d and Lamp1 co-expression (**Figure 2A**). Co-stained signals can be enriched through gating the cell population with double stain of CD1d and Lamp1 (**Figure 2A**). We first showed the non-exposed normal DCs with minimal colocalization of CD1d and Lamp1 proteins, indicating the basal level of CD1d endocytic trafficking and high level of surface expression at physiological conditions (**Figure 2B**)[5,19-21]. To measure the colocalization of CD1d and Lamp1 proteins, we initially used IDEAS to show a slight increase of bright detail similarity between CD1d and Lamp1 proteins with BaP exposure[13]. The bright detail similarity was calculated based on the Pearson's correlation coefficient for testing the correlation of two factors, while CD1d and Lamp1 proteins were unlikely highly overlapped in physiological condition, indicating that CD1d is able to transiently traffic through late endocytic compartments to obtain functional lipid antigens. Thus, it is more biologically meaningful to test whether the CD1d protein in BaP exposure retains more in the late endocytic compartments as reflected by the increased percentage of overlapped CD1d and Lamp1 proteins using the imaging analysis software Fiji in the ImageJ package. Finally, retention of the CD1d protein in late endocytic compartments can be used to functionally confirm altered endocytic gene profiles in BaP exposure.

Co-localized intensity and area between CD1d and Lamp1 proteins was measured. To obtain a reproducible threshold setting and percentages of co-localized areas and intensity, we applied ImageJ-Fiji[22], which has also been used in several other studies[23-25]. We randomly select CD1d+Lamp1+ cells with strong co-stain and clear subcellular localization of CD1d and Lamp1 proteins as input images (**Figure 2B**). Individual cellular images can be merged to a single image file for one consistent analysis (**Figure 3A**). In our study, we merged 100 cell images and opened it in the program to analyze by setting "colocalisation thresholds" (**Figure 3B**). The colocalization of pixel intensity between two channels (PE-labeled CD1d and AF647-labeled Lamp1) was shown overall with a scatterplot by detecting the co-localized pixels from these 100

350   cell images (**Figure 4A**). In this plot, horizontal and vertical lines represent Costes's thresholds
351   and the diagonal lines represent the ratio of overall pixel intensity between two channels. The
352   Costes's threshold provides a visible setting for removing weak pixels from two overlapped
353   channels. The resulted scatterplot shows an increased colocalization of CD1d and Lamp1
354   proteins in BaP exposure. Moreover, we quantified the degree of co-localized areas between
355   CD1d and Lamp1 proteins using Mander's coefficients (**Figure 4B**). Consequently, Mander's
356   coefficients demonstrate increased colocalized areas between CD1d and Lamp1 in late
357   endocytic compartments of BaP-exposed DCs comparing with non-exposed DCs, as statistically
358   supported by Student's t-tests (**Figure 4B**). However, if protein intensity is heterogeneous
359   between colocalized and non-colocalized areas, colocalized intensity will be unparalleled to
360   colocalized areas. Therefore, it is also necessary to further exam the colocalization based on
361   intensity (**Figure 4C**). As a result, each column represents an averaged calculation of percentage
362   of colocalized intensity from multiple cell images (n=100) between BaP-exposed and non-
363   exposed conditions for a pair of co-localized proteins. Student's t-tests also show statistical
364   significance (n=100, $p<0.001$) between BaP-exposed and non-exposed groups (∗) with indicated
365   standard errors. Altogether, our data demonstrate that the CD1d protein was retained in late
366   endosomes at BaP exposure, supporting that BaP-altered gene expression further contribute to
367   the impaired endocytic function and CD1d trafficking.
368
369   In summary, CD1d-Lamp1 colocalization enhances upon BaP exposure. Multiple parameters
370   based on cell images (**Figure 2B**), including scatterplots with overlapped pixel intensity (**Figure
371   4A**), enhanced Mander's coefficients (**Figure 4B**), and increased percentage of overlapped
372   intensity (**Figure 4C**), support CD1d endocytic retention at BaP exposure. This colocalization
373   analysis of cellular images is a similar and accurate approach to determine the degree of
374   protein colocalization and demonstrate biological mechanisms of cell functions, for example,
375   endocytic function consistent with disrupted endocytic gene expression, further contributing to
376   the inhibited activation of CD1d-restricted T cells[13].
377
378   **Figure 1: Perturbation of DC transcriptomes in BaP exposure** (**A**) Flowchart of RNA-Seq. First,
379   quality control (QC) analysis was performed to examine the quality of total RNA. Using an
380   automated system and high-quality total RNA as input, poly-A RNA was isolated, which
381   demonstrated depleted rRNA in poly-A RNA QC analysis. The poly-A RNA was then subjected to
382   automated library preparation and indexing via PCR. Next, the amplified library was analyzed by
383   a Bioanalyzer for QC with expected yield and size distribution. After qPCR quantification,
384   indexed libraries were pooled and clustered onto flow cell for sequencing, and the sequencing
385   data quality was real-time monitored. The axis represents relative counts of flow cytometry
386   staining signals. **(B)** Altered genes were grouped in different functional clusters and the gene
387   clusters associated with endocytic function and lipid metabolism are shown. Red: CD1d; Green:
388   Lamp1.
389
390   **Figure 2: Analysis of cellular images using the IDEAS program and calculation of bright detail
391   similarity.** (A) Monocyte-derived DCs from healthy donors were firstly gated on HLA-
392   DR+CD11c+ cells and further gated on CD1d⁺Lamp1⁺ cells using the IDEAS program. (B) Cellular

393 images were extracted from the HLA-DR⁺CD11c⁺CD1d⁺Lamp1⁺ population for cellular image
394 visualization.
395
396 **Figure 3. Analysis of cellular images using the Fiji-ImageJ program.** Cells with strong co-stain of
397 CD1d and Lamp1 proteins were randomly selected for the image analysis using Fiji-ImageJ, but
398 cells* with invisible or weak staining for either CD1d or Lamp1 protein was excluded as in
399 **Figure 2B**. (**A**) These randomly selected cellular images were assembled into a single image file
400 as an input image. (**B**) The colocalization analysis was performed through setting the
401 colocalization thresholds for both green and red channels. The scatterplots were generated
402 with threshold calculation.
403
404 **Figure 4. Colocalization of CD1d and Lamp 1 proteins.** Use similar dataset reported in Sharma
405 *et al.*[13], co-localized pixel intensity between CD1d and Lamp1 proteins from DCs at BaP-exposed
406 and non-exposed conditions was recalculated and shown with scatterplots (**A**). Mander's
407 coefficients for CD1d and Lamp1 colocalization were calculated and are shown with an average
408 from selected cell images (**B**). The percentage of co-localized pixel intensity was also calculated
409 (**C**). Statistical significance (n=100, $p<0.001$) was obtained with Student's t-tests in comparison
410 to the non-exposed group (∗∗). Standard errors were indicated. Data are from one assay with
411 cells from a healthy donor. Two independent assays were performed with similar results.
412
413 **Table 1. Antibodies used in this study.**
414 Due to shared excitation and emission wavelengths, fluorophores PE/Cy5 and AF647 partially
415 overlapped at staining signals. We use these fluorophores to label a surface protein (CD11c)
416 and an intracellular protein (Lamp1) respectively to avoid their signal overlapping at a
417 subcellular level. We also use optimal titration and compensation to minimize the overlapped
418 signals between both fluorophores.
419
420 **DISCUSSION:**
421 Functional confirmation of an altered gene pathway is challenging, because of widely impacted
422 gene expression involving multiple pathways and the difficulty to functionally integrate
423 individual and populational cellular activities. We employed imaging flow cytometry to
424 specifically test the functional impact on CD1d trafficking in endocytic compartments. Imaging
425 flow cytometry integrates the populational measurement of cells and the individual
426 demonstration of subcellular colocalization of multiple proteins. Confocal microscopy has
427 previously provided high resolution in measuring protein colocalization, and flow cytometry is
428 able to provide comprehensive phenotyping for different cell populations. Imaging flow
429 cytometry combines the advantages of confocal microscopy and flow cytometry at acceptable
430 image resolution to analyze protein colocalization in a high-profile manner for functional
431 confirmation of transcriptomic alteration by environmental pollutants.
432
433 Analyses of colocalization of multiple proteins in a populational scale are technically
434 demanding, because (i) detailed analyses of multiple individual cells are labor-intensive and
435 relatively biased; (ii) it is difficult to define an optimal threshold for interpreting overlapped
436 pixels based on the intensity or area of positive staining; and (iii) balanced observation of

437 individual and populational cells is critical. In this study, we focused on performing a biologically
438 meaningful observation, a statistical significance test, and an imaging demonstration of co-
439 localized proteins. To achieve this goal, we first obtained thousands of images of human DCs
440 using imaging flow cytometry. Quantitative analyses of imaging files combined a bright detail
441 similarity analysis using the software IDEAS and a statistically tested colocalization analysis
442 using Fiji-ImageJ. The IDEAS program allows gating the co-stained population, calculating
443 colocalization of two proteins using Pearson's correlation coefficient-based bright detail
444 similarity, and randomly selecting co-stained cells with subcellular localization of proteins[13]. To
445 further achieve the biological relevant demonstration of co-localized proteins, we used ImageJ-
446 Fiji software and applied the Mander's coefficient to calculate the percentage of co-localized
447 areas and overlapped pixel intensity to demonstrate the degree of CD1d localization in the
448 endocytic pathway.
449
450 The critical step for using the ImageJ-Fiji program is to perform single cell imaging analysis.
451 Variation of percentage protein colocalization from individual cells can be monitored in
452 comparison to visually merged colors from single cell images. By averaging the percentage
453 protein colocalization from all 100 cells, the means and standard errors can be calculated to
454 demonstrate protein colocalization and functional correlation at a populational level. This
455 bioinformatics analysis is simple and straightforward without concerning ambiguous results,
456 because this analysis offers a reproducible threshold calculation using the Costes's method to
457 produce the same resulted thresholds for the same data sets and similar thresholds for similar
458 datasets[26]. The limitation of this technique is the labor-intensive manual input of single cell
459 images and potentially subjective selection of visually co-stained cells using the IDEAs software.
460 In our study, we analyze 100 cell images from each group and we set reproducible selection
461 criteria, including strong co-stained signals and clear subcellular localization of CD1d and Lamp1
462 proteins. Overcoming these limitations requires technical improvement of analytical software
463 to allow batch analysis and objective selection of single cells based on morphological features
464 beyond the staining intensity provided by flow cytometry. In summary, combined cellular and
465 population imaging analysis will provide highly reproducible, comprehensive, statistically
466 tested, and biologically relevant results of percentage protein colocalization. We believe this
467 CD1d-Lamp1 colocalization analysis can serve as a successful example of high profile cellular
468 imaging analyses to answer broader questions on functional and morphological alteration of
469 cellular proteins.
470
471 **DISCLOSURES:**
472 The authors have nothing to disclose
473

481

REFERENCE

481 1   Roche, *P*.A., Cresswell, *P*. Antigen Processing and Presentation Mechanisms in Myeloid
484 Cells. *Microbiology Spectrum* **4** (3) (2016).

485 2   Huang, S. *et al.* MR1 uses an endocytic pathway to activate mucosal-associated invariant
486 T cells. *The Journal of experimental medicin*e **205** (5), 1201-1211 (2008).

487 3   Nakamura, N. *et al.* Endosomes are specialized platforms for bacterial sensing and NOD2
488 signalling. *Nature* **509** (7499), 240-244 (2014).

489 4   Blum, J.S., Wearsch, *P*.A., Cresswell, *P*. Pathways of antigen processing. *Annual review of
490 immunology* **31**, 443-473 (2013).

491 5   Brennan, *P*.J., Brigl, M., Brenner, M.B. Invariant natural killer T cells: an innate activation
492 scheme linked to diverse effector functions. *Nature reviews*. *Immunology* **13** (2), 101-117
493 (2013).

494 6   Zajonc, D.M., Kronenberg, M. CD1 mediated T cell recognition of glycolipids. *Current
495 opinion in structural biology* **17** (5), 521-529 (2007).

496 7   Cox, D. *et al.* Determination of cellular lipids bound to human CD1d molecules. *PloS one*
497 **4** (5), e5325 (2009).

498 8   Yuan, W., Kang, S.J., Evans, J.E., Cresswell, *P*. Natural lipid ligands associated with human
499 CD1d targeted to different subcellular compartments. *Journal of immunology* **182** (8), 4784-
500 4791 (2009).

501 9   Huang, S. *et al.* Discovery of deoxyceramides and diacylglycerols as CD1b scaffold lipids
502 among diverse groove-blocking lipids of the human CD1 system. *Proceedings of the National
503 Academy of Sciences of the United States of America* **108** (48), 19335-19340 (2011).

504 10   de Jong, A. *et al.* CD1a-autoreactive T cells recognize natural skin oils that function as
505 headless antigens. *Nature immunology* **15** (2), 177-185 (2014).

506 11   Trombetta, E.S., Mellman, I. Cell biology of antigen processing *in vitro* and *in vivo*.
507 *Annual review of immunology* **23**, 975-1028 (2005).

508 12   Maecker, H.T., McCoy, J.*P*., Nussenblatt, R. Standardizing immunophenotyping for the
509 Human Immunology Project. *Nature* reviews *immunology* **12** (3), 191-200 (2012).

510 13   Sharma, M. *et al.* Inhibition of endocytic lipid antigen presentation by common lipophilic
511 environmental pollutants. *Science Reports* **7** (1), 2085 (2017).

512 14   Boom, R. *et al.* Rapid and simple method for purification of nucleic acids. *Journal of
513 clinical microbiology* **28** (3), 495-503 (1990).

514 15   Nachamkin, I. *et al.* Agilent 2100 bioanalyzer for restriction fragment length
515 polymorphism analysis of the Campylobacter jejuni flagellin gene. *Journal of clinical
516 microbiology* **39** (2), 754-757 (2001).

517 16   Kaimal, V., Bardes, E.E., Tabar, S.C., Jegga, A.G., Aronow, B.J. ToppCluster: a multiple
518 gene list feature analyzer for comparative enrichment clustering and network-based dissection
519 of biological systems. *Nucleic acids research* **38** (Web Server issue), W96-102 (2010).

520 17   Bauer-Mehren, A. Integration of genomic information with biological networks using
521 Cytoscape. *Methods in molecular biology* **1021**, 37-61 (2013).

522 18   Cline, M.S. *et al.* Integration of biological networks and gene expression data using
523 Cytoscape. *Nature protocols* **2** (10), 2366-2382 (2007).

524   19   Huang, S., Moody, D.B. Donor-unrestricted T cells in the human CD1 system. *Immunogenetics* **68** (8), 577-596 (2016).

526   20   Huang, S. Targeting Innate-Like T Cells in Tuberculosis. *Frontiers in immunology* **7**, 594 (2016).

528   21   Moody, D.B., Porcelli, S.A. Intracellular pathways of CD1 antigen presentation. *Nature reviews*. *Immunology* **3** (1), 11-22 (2003).

530   22   Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nature methods* **9** (7), 676-682 (2012).

532   23   Diard, M. *et al.* Stabilization of cooperative virulence by the expression of an avirulent phenotype. *Nature* **494** (7437), 353-356 (2013).
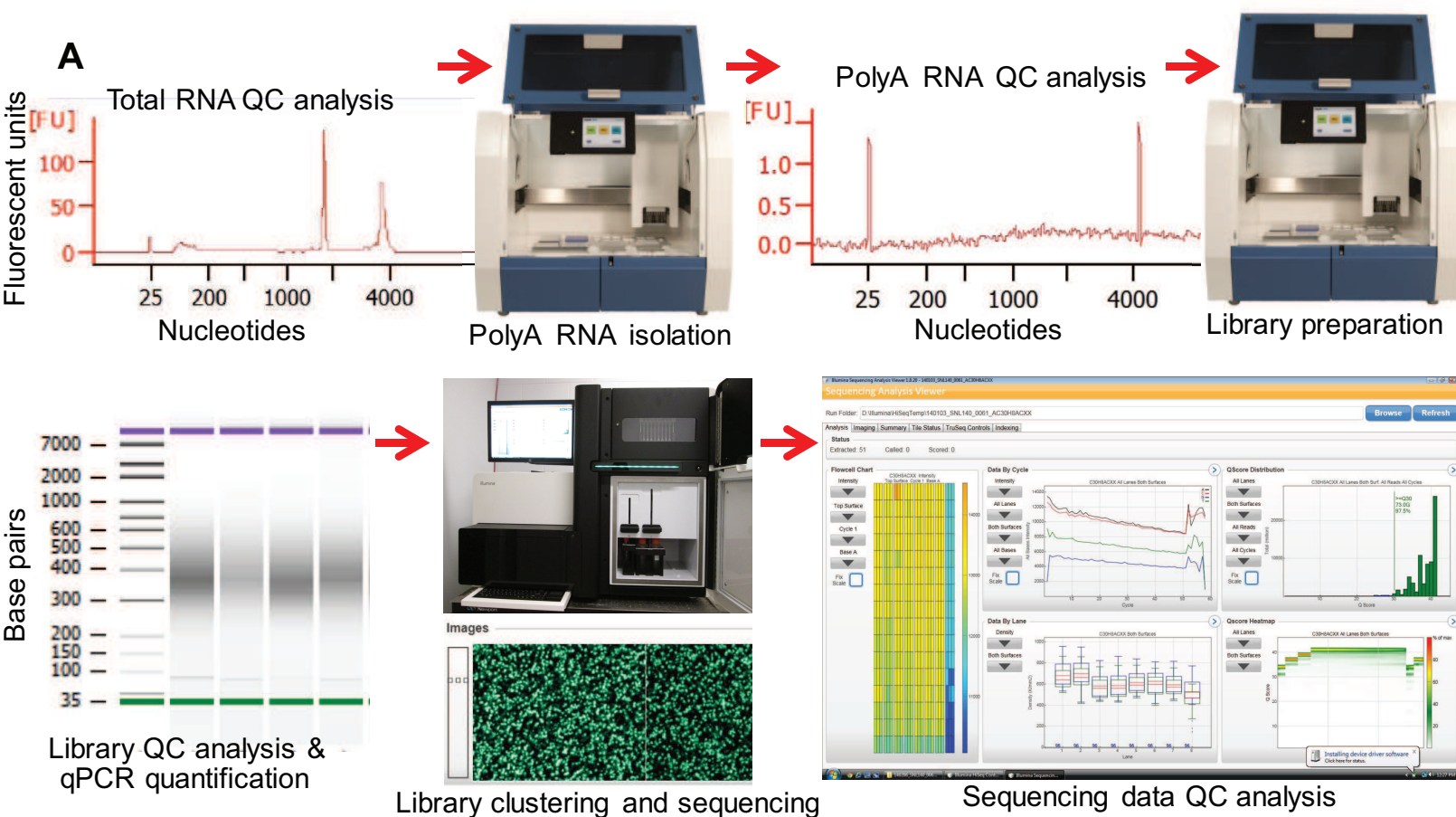
534   24   Bader, E. *et al.* Identification of proliferative and mature beta-cells in the islets of Langerhans. *Nature* **535** (7612), 430-434 (2016).

536   25   Eliceiri, K.W. *et al.* Biological imaging software tools. *Nature methods* **9** (7), 697-710 (2012).

538   26   Costes, S.V. *et al.* Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophysical journal* **86** (6), 3993-4003 (2004).

Figure1

A

Total RNA QC analysis

Fluorescent units

[FU]
100
50
0

25 200 1000 4000

Nucleotides

PolyA RNA isolation

PolyA RNA QC analysis

[FU]
1.0
0.5
0.0

25 200 1000 4000

Nucleotides

Library preparation

Base pairs

7000
2000
1000
600
500
400
300
200
150
100
35

Library QC analysis & qPCR quantification

Library clustering and sequencing

Sequencing data QC analysis

B

Apoptosis

Cell chemotaxis

Lipids

Signaling

Phosphorus

Cytokines

Hormone

Inflammation

Immunity

p53

Proliferation

Endosome

Gene

Functional Clusters

Log2(fold change) BaP (0.32 μM) vs non-exposed

-11 -4 0 4 11

Lipid metabolism

lysosome

Figure3

A

CD1d
Lamp1

B

(Fiji Is Just) ImageJ

File Edit Image Process Analyze Plugins Window Help

x=6450, y=2010, value=000,000,000 (black)

Colocalisation Thresholds

Channel 1   CD1d 1.5 BaP no LPS-03-16-17all1.png (red).png

Channel 2   CD1d 1.5 BaP no LPS-03-16-17all1.png (green).png

Use ROI   None

Channel Combination   Red : Green

☑ Show Colocalized Pixel Map
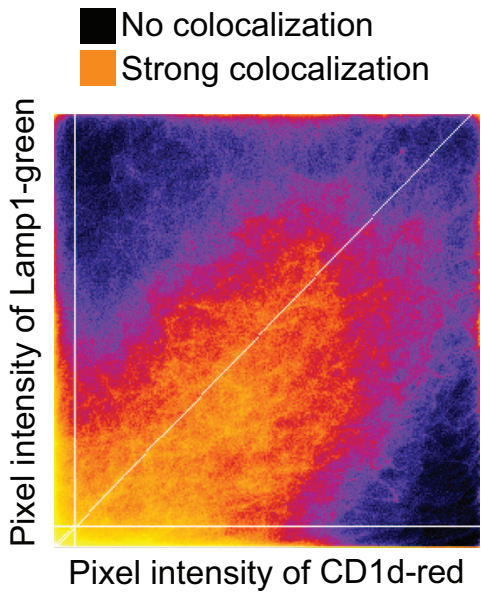☑ Use constant intensity for colocalized pixels
☑ Show Scatter plot
☑ Include zero-zero pixels in threshold calculation
☐ Set options

See: http://uhnresearch.ca/wcif/imagej

■ No colocalization
■ Strong colocalization

Pixel intensity of Lamp1-green

Pixel intensity of CD1d-red

Figure4

A Colocalization of CD1d and Lamp1

BaP (0 μM)　　　　BaP (5.94 μM)

Pixel Intensity of Lamp1

Pixel Intensity of Lamp1

Pixel Intensity of CD1d (n=100) ⟶

■ No colocalization　　■ Strong colocalization

B

n=100

Mander's Coefficient

1 –
0.8 –
0.6 –
0.4 –
0.2 –
0 –

*

0　5.94
BaP (μM)

C

n=100

% Pixel Intensity of CD1d colocalized with Lamp1

100 –
80 –
60 –
40 –
20 –
0 –

*

0　5.94
BaP (μM)

n=100

% Pixel Intensity of Lamp1 colocalized with CD1d

100 –
80 –
60 –
40 –
20 –
0 –

*

0　5.94
BaP (μM)

B　n=100　*　　**C**　n=100　*　　n=100　*

Table 1

Antibodies	Fluorophores or secondary antibody
HLA-DR	Phycoerythrin/Cyanine 7 (PE/Cy7)
CD11c	Phycoerythrin/Cyanine 5 (PE/Cy5)
CD1c	Brilliant violet 421
Purified anti-human CD1d	PE-anti-mouse IgG2b
Lamp1	Alexa Fluor 647 (AF647)

Clone
L243
3.9
L161
51.1
CD107a(H4A3)

Name of Material/ Equipment	Company
Transcriptomics	Illumina
ImagingStream X	Millipore
mirVana miRNA Isolation Kit	Thermo Fisher
Agilent RNA 6000 Pico Kit	Agilent
Veriti 96-Well Fast Thermal Cycler	Thermo Fisher
NEBNext Poly(A) mRNA Magnetic Isolati	New England Biolab
Automated SMARTer Apollo system	Takara
NEBNext Ultra RNA Library Prep Kit for I	New England Biolab
Agencourt AMPure XP magnetic beads	Beckman Coulter
2100 Bioanalyzer	Agilent
Agilent High Sensitivity DNA Kit	Agilent
QuantStudio 5 Real-Time PCR System (	Thermo Fisher
NEBNext Library Quant Kit	New England Biolab
cBot	Illumina
TruSeq SR Cluster Kit v3 - cBot – HS	Illumina
HiSeq 1000	Illumina
TruSeq SBS Kit v3 - HS (50-cycles)	Illumina
Phycoerythrin/Cyanine 7 (PE/Cy7)	Bio Legend
Phycoerythrin/Cyanine 5 (PE/Cy5)	Bio Legend
Brilliant violet 421	Bio Legend
PE-anti-mouse IgG2b	Bio Legend
Alexa Fluor 647 (AF647)	Bio Legend

Catalog Number	Comments/Description
HiSeq 2500 v4	Illumina HiSeq system
100220	Imaging flow cytometry
	Total RNA extraction
	Total RNA QC analysis
	PCR, enzyme reaction
	PolyA RNA purification
	PolyA RNA purification
	Library preparation
	DNA purification
	Library QC, size distribution analysis
	Library QC, size distribution analysis
	Library quantification
	Library quantification
	Library cluster generation
	Library cluster generation
	Sequencing
	Sequencing

L243

3.9

L161

51.1

CD107a(H4A3)

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Mar 06, 2018

Dear Editors:

We have revised the manuscript according to editorial comments. In particular, we have newly generated Fig. 1B to comprehensively show the sequenced gene network and generated Fig. 4 using different sets of 100 cell images to avoid the adaptation of previously published results. We have also revised the text to avoid repeating our published description.

Point-by-point responses to editorial comments are listed below.

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Xiang Zhang

Editorial comments:

1. There is previously published text in the manuscript that must be revised before the manuscript can be accepted. Please see the attached iThenticate report and revise the following lines: 58-60, 130-134, 137-144, 152-168, 205-219, 223-228, 230-247, 300-314, 318-321

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--corrected. We prefer standard access. We will take video using computers, because contents to be recorded are computational analyses.

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--The manuscript was also revised according to detailed comments.

4. Please revise the protocol to be completely in the imperative tense. Furthermore, we need more details for protocol replication. These details cannot be referenced out and are needed if a particular step is to be filmed. The script is directly derived from the written protocol text.

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