Supplementary Information

Tracing hematopoietic progenitor cell neutrophilic differentiation via Raman spectroscopy

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Supplementary Materials and Methods

Nuclear staining area quantification

Live 32D cells were stained with Hoechst 33342 dye (1:500 dilution) and imaged with Olympus IX81 inverted fluorescence microscope to visualize their nucleus. Resulting brightfield and fluorescence images were processed with ImageJ to determine the total area and the nuclear area of each cell to calculate the nucleus-to-cytoplasm ratio. The area of the cytoplasm was calculated by subtracting the nucleus area from the total cell area.[1]

Cytospin and Wright's stain

32D cells were collected with a cell scraper, spun down, and washed twice with warm PBS (37°C). The cells were then resuspended with ice cold 70% ethanol dropwise in a 15-mL polypropylene tube for fixation and stored at 4°C until analysis. Stored samples were cytospinned and Wright stained at the Veterinary Diagnostic Laboratory at the University of Illinois at Urbana-Champaign College of Veterinary Medicine. Wright stained cells were imaged with a light microscope for visualization.

Flow cytometric analysis for quantifying Gr-1 expression

32D cells were collected with a cell scraper, spun down, washed twice with warm PBS (37°C), and resuspended in ice cold PBS supplemented with 2% FBS and 1% Pen/Strep (PBS/FBS buffer). Cells were then incubated with Fc receptor blocking antibody (CD16/CD32) to prevent nonspecific binding followed by incubating with FITC-conjugated Gr-1 (Ly-6G/C) antibody (1:200 dilution; eBiosciences, San Diego, CA).[2] Propidium iodide (Thermo Fisher Scientific Inc.,

Waltham, MA) was used to exclude dead cells. Labeled cells were analyzed with BD LSR Fortessa Flow Cytometry Analyzer (BD Biosciences, San Jose, CA) to quantify Gr-1 expression.

Cell cycle analysis

32D cells were collected with a cell scraper, spun down, and washed twice with warm PBS (37°C). The cells were then resuspended with ice cold 70% ethanol dropwise in a 15-mL polypropylene tube for fixation and stored at 4°C until analysis. For staining, stored samples were spun down (200g, 10 minutes, 4°C), washed with ice cold PBS, and resuspended in Triton X-100/propidium iodide solution.[3] This solution was prepared by adding 0.40 mL of 500 µg/mL propidium iodide to 10 mL of 0.1% Triton X-100 solution. Stained cells were analyzed with BD LSR Fortessa Flow Cytometry Analyzer (BD Biosciences, San Jose, CA). Resulting flow cytometric data was analyzed with FCS Express software (De Novo Software, Glendale, CA) to perform cell cycle analysis.

Cell viability quantification

32D cell viability was assessed by staining cells with Live/Dead Cell Viability Assay (Life Technologies, Grand Island, NY) according to manufacturer's instructions and imaging them with Olympus BX51 upright fluorescence microscope (Olympus Corp., Waltham, MA).

BCA assay

Cells were washed twice with PBS and pelleted by centrifugation at 1800 x g for 5 minutes. RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS,

50 mM Tris, pH 8.0) was added to the cell pellets, followed by vortexing for 20 seconds and centrifugation at 8000g for 10 minutes, for cell lysis. Cellular protein concentration was measured by a BCA protein assay kit according to manufacturer's instructions (ThermoFisher Scientific, Waltham, MA).

PLD assay

To quantify total cellular phospholipid, cellular lipid extracts were prepared via the Bligh and Dyer method.[4] Briefly, 2 mL methanol and 1 mL chloroform were added to each cell pellet. The cell pellet was vortexed, sonicated for 15 minutes, allowed to sit at room temperature for 1 hour, and centrifuged at 1800 x g for 5 minutes. The cell pellet was discarded. 1 mL of chloroform and 1 mL of 18 mΩ water was added to the supernatant, followed by vortexing and centrifugation at 1800 x g for 5 minutes. The chloroform layer was recovered and dried under nitrogen gas. The lipid extracts were re-dissolved in 1:1 PLD/cholesterol buffer, vortexed, and sonicated for 15 minutes. Cellular phospholipid concentration was measured by a phospholipase D (PLD) assay according to manufacturer's instructions (Sigma Aldrich, St. Louis, MO).

Supplemental Figures

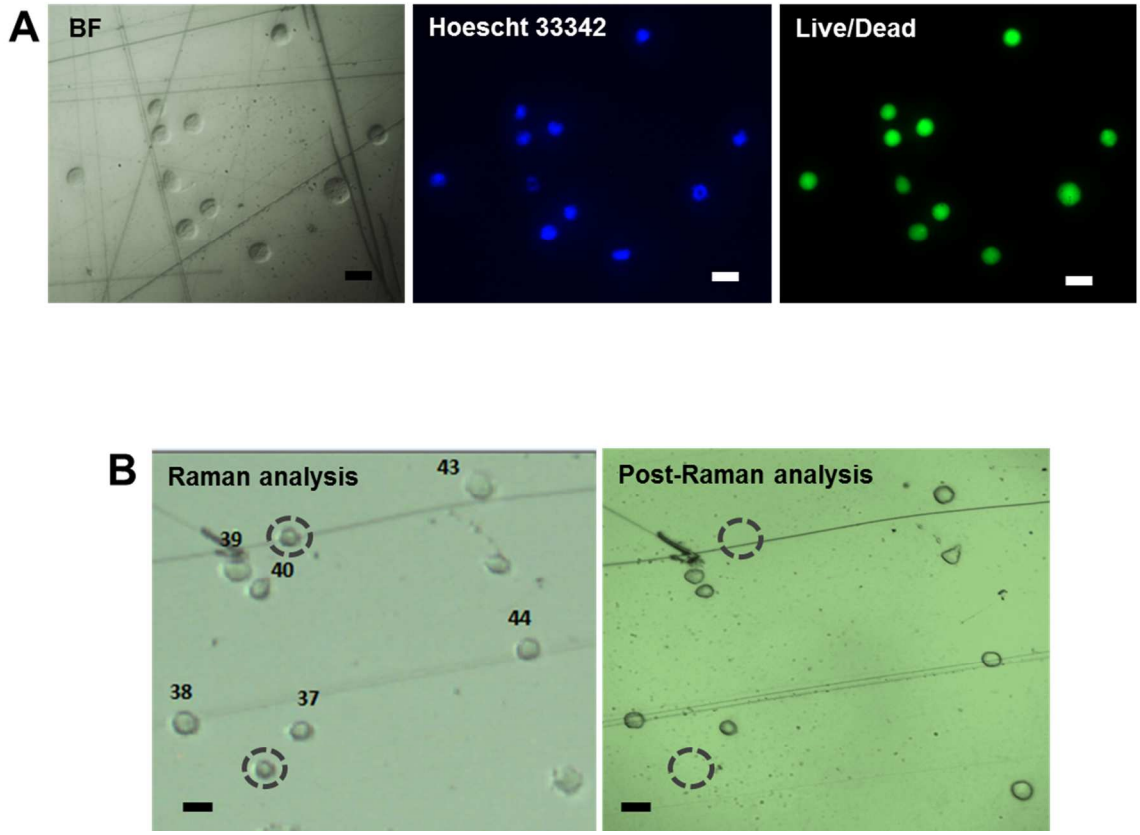

Figure S1. Effects of laser irradiation during Raman spectroscopic analysis.

Immediately following Raman spectroscopic analysis, 32D cells were stained in situ and imaged with an upright fluorescence microscope. Representative images of 32D cells are shown here. Scale bar: 10 μm. (A) No significant changes in cell viability were observed within at least 3-hours of irradiation. Nucleus stain Hoechst 33342 (Blue) was used to visualize nuclear shape. Live/Dead stain was used to visualize cell viability (Green: Live, Red: Dead, Composite image is shown here, and there were no dead cells in the field of view). (B) Some cells detached during the staining and imaging step post-Raman analysis. Detached cells are shown in dashed circles. Left: Brighfield image taken during the Raman spectroscopic analysis. Right: Brightfield image taken during the imaging step post-Raman analysis. Some cells have detached during staining and imaging.

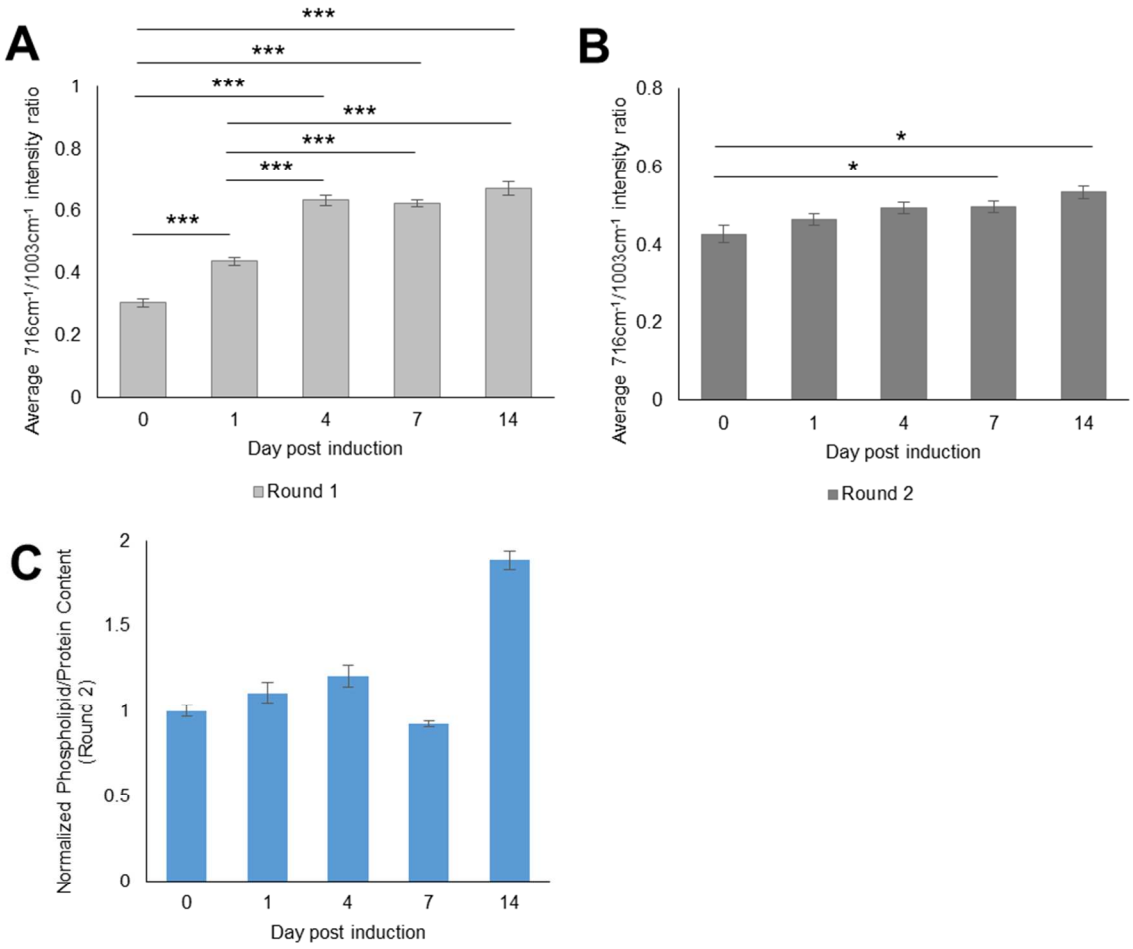

Figure S2. Changes in cellular lipid content during neutrophilic differentiation.

(A, B) Average ratios of choline (716 cm-1) to phenylalanine (1003 cm-1) Raman peak intensities on days 0, 1, 4, 7, and 14 post-induction from two independent rounds of neutrophilic differentiation (Round 1 and 2). **: $p<0.05$. ***: $p<0.001$.* (C) For Round 2, the ratio of total cellular phospholipid to protein content was determined using a PLD and BCA assay. Phospholipid-to-protein levels were normalized to Day 0 values. Errors represent standard deviations from the technical replicates.

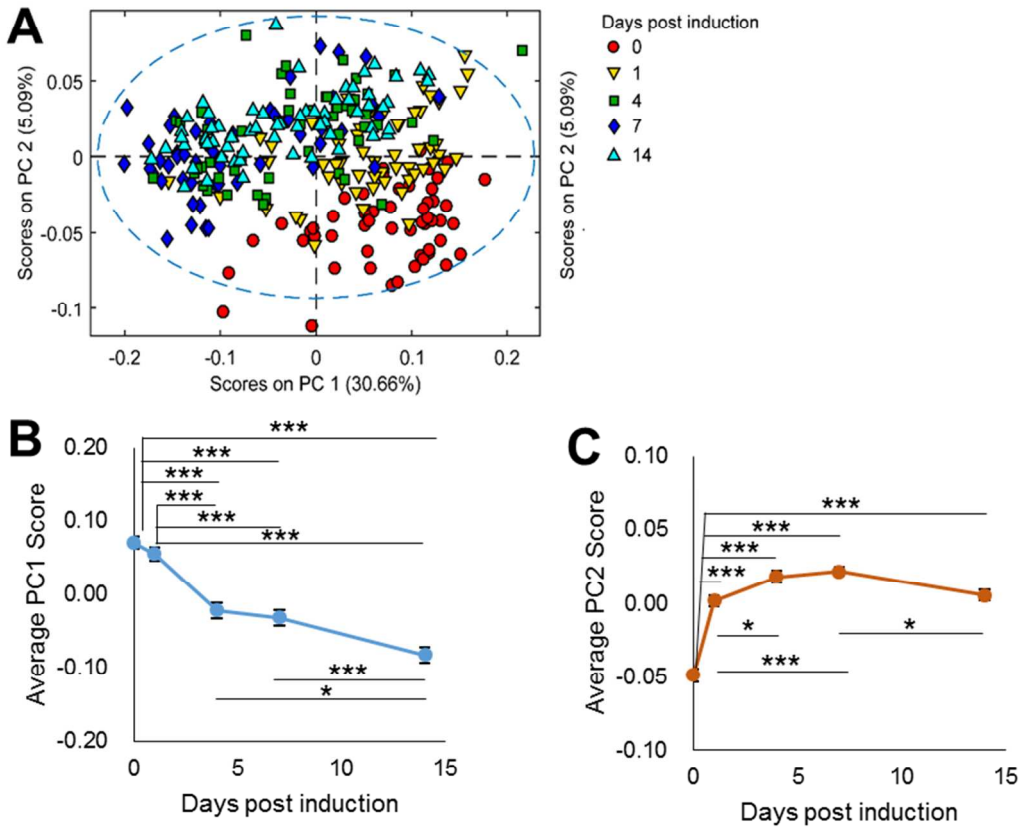

Figure S3. PCA of 32D cells grouped by day of analysis during neutrophilic differentiation.

(A) PCA of 32D cell spectra when cells were grouped by the day of analysis rather than their nucleus shape. In this analysis, cells appeared more broadly distributed. (B) Average PC 1 scores still decreased with cell maturation. (C) Average PC 2 scores increased and plateaued over time. **: p<0.05. ***: p<0.001.*

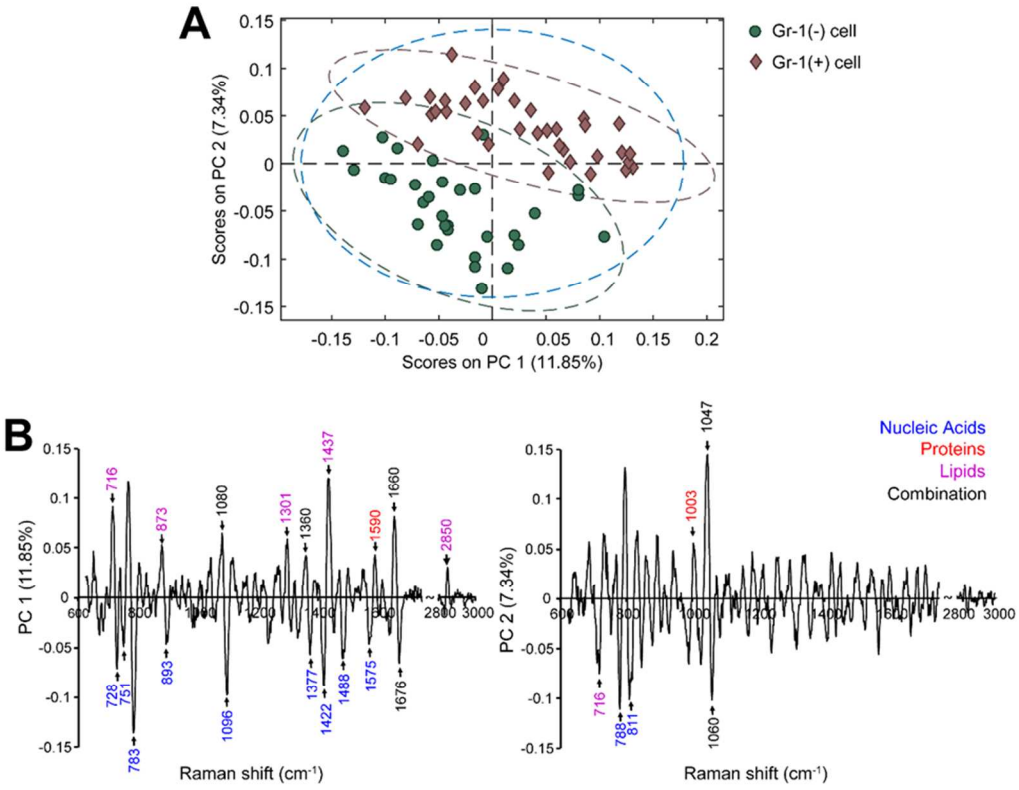

Figure S4. PCA of 32D cells sorted based on their Gr-1 expression.

(A) PCA was performed on 32D cells sorted based on their Gr-1 expression. Gr-1(-) and Gr-1(+) 32D cells from day 0 and 14 post-induction were collected using fluorescence-activated cell sorting (FACS). (B) The corresponding PC 1 and PC 2 score plots are shown. The most significant and relevant peaks are labeled based on the peak wavenumber and the corresponding biomolecule type (nucleic acid, protein, lipid, or a combination of biomolecules).

Supplemental Tables

Table S1. Raman peak assignments.

Raman Shift (cm^{-1})	Nucleic acids	Proteins	Lipids	Carbohydrates
645		C-C twist Tyr		
682	G ring br.			
701			Cholesterol ring str.	
716			Choline	
728	A ring br.	Ring br. Trp	C-N str.	
751	T ring br.	Trp.		
783	U, C, T ring br.			
788	O-P-O str. DNA			
811	O-P-O str. RNA		O-P-O	
828	O-P-O str.	Out of plane ring br. Tyr		
853		Ring br. Tyr		
873			C_2-C_1	
893	BK, deoxyrib.			
937		C-C BK str. α helix		
972			C-C	
1003		Sym. ring br. Phe		
1047		Culture media (undefined)		
1080		C-N str.	Chain C-C str.	C-O str.
1096	PO_2^- str.		Chain C-C str.	C-C str.
1128		C-N str.	Chain C-C str.	
1180		C-H in-plane bend Tyr		
1208	C, T	C-C_6H_5 str. Phe, Trp		
1285		Amide III α helix		
1296		Amide III, CH def.	CH_2 twist	
1312		CH def.		
1342	A, G	CH def.		
1367			CH_3 sym str.	
1377	A, G, T			
1422	A, G			
1437			CH def.	
1449		CH def.	CH def.	CH def.
1488	A, G			
1514	A			
1550		Amide II α helix		
1575	A, G			
1608		C=C Phe, Tyr		
1617		C=C Tyr, Trp		
1626-1640		Amide I β sheet		
1660		Amide I α helix	C=C str.	
1669-1690		Amide I turn		
1676	T			
2850			CH_2 sym str.	
2935		CH_3 sym str.	CH_3 sym str.	

Abbreviations: Phe: phenylalanine, Trp: tryptophan, Tyr: tyrosine; A, T, G, C: adenine, thymine, guanine, cytosine; sym: symmetric, asym: asymmetric, def: deformation, bk: backbone, ring br: ring breathing. Assignments are based on references.[5-11] Several measured peaks were shifted slightly from reference peaks, likely due to misalignment or limit in spectral resolution.

Table S2. A summary of PLS-DA models built for myeloid cell discrimination.

Model #	PLS-DA Model/Test	Sensitivity calibration/CV/ prediction	Specificity calibration/CV/ prediction	Prediction classification error
1	Calibration and prediction: myeloblasts and neutrophils from differentiation rounds 1,2,3 (split using venetian blinds)	0.965, 0.966 0.912, 0.879 0.893, 0.882	0.966, 0.965 0.879, 0.912 0.882, 0.893	11.3%
2	Calibration: myeloblasts and neutrophils from round 2 Prediction: myeloblasts and neutrophils from rounds 1, 3	0.887, 0.887 0.849, 0.868 0.938, 0.756	0.887, 0.887 0.868, 0.849 0.756, 0.938	15.3%
3	Calibration: Sorted Gr-1(-) and Gr-1(+) cells Prediction: myeloblasts and neutrophils from differentiation rounds 1,2,3	0.906, 0.938 0.875, 0.938 0.776, 0.904	0.938, 0.906 0.938, 0.875 0.904, 0.776	16.0%
4	Calibration and prediction: myeloblasts, promyelocytes/metamyelocytes, band cells, and neutrophils from differentiation rounds 1,2,3	0.793, 0.704, 0.771, 0.803 0.793, 0.278, 0.343, 0.724 0.797, 0.407, 0.371, 0.882	0.782, 0.781, 0.729, 0.830 0.782, 0.669, 0.681, 0.755 0.727, 0.724, 0.751, 0.743	23.8% 43.5% 43.9% 18.8%

Supplemental References

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