Introduction

Endothelial cells constantly experience dynamic mechanical stimuli, including the drag of blood flow or the stiffness of vessels. Therefore, endothelial cell mechanotransduction is essential for proper vascular function and dysregulation in integrating mechanical stimuli can result in pathology including atherosclerosis. New techniques in measuring cellular forces and advances in RNA sequencing enabled rapid progress in mechanobiology, investigating how mechanical cues drive cell function through changes in gene expression. However, recent work shows that intracellular membranous organelles can receive, modulate, and initiate mechanotransduction signaling (Phuyal et al., 2023). Endosomes are membrane-bound vesicles that internalize cell membrane proteins and sort them for recycling or degradation. The Rab family of small GTPases orchestrates endosome dynamics (e.g. identity, maturation, and motility) by localizing to diverse endosomal structures and recruiting various effectors. Rab7 and Rab11 mark late and recycling endosomes, respectively. Current methods in endosome characterization include fluorescent labeling and tracking of endocytosed cargo (Toshima and Toshima, 2024), multiplexed DNA-PAINT imaging of endosomal proteins (Bond et al., 2025), and CRISPR-Cas9-based endogenous labeling for imaging and immunoprecipitation proteomics (Hein et al., 2025). Aforementioned methods frequently use 2D imaging, where spatial morphology and localization can become mischaracterized. However, emerging methods in 3D image analysis aim to better segment and characterize endosome morphologies, making full use of high-resolution confocal microscopy and imaging techniques (Rose et al., 2024). Here, we used our 3D image analysis workflow to identify changes in Rab7 and Rab11 containing endosome morphology in endothelial cells under static and laminar fluid flow conditions.

Methods

To identify endosomal changes in response to flow stimuli, HUVECs were grown at a density of 2.5×105 cell/cm^2 on gelatin-coated Ibidi slides for 24 hours. The cells were exposed to continuous laminar flow at 15 dyn/cm^2 or maintained under static conditions for an additional 24 hours. After incubation, cells were fixed with 4 % PFA for 10 minutes and stained with specific antibodies for Rab7 and Rab11, as well as phalloidin and Hoechst stains to visualize actin and nucleus, respectively. Cells were imaged on a CrestOptics X-Light V3 Spinning Disk Confocal at 100x magnification and 0.2 µm z step size. The images were imported to Fiji for 3D segmentation and analysis (Figure 1). A Z-project was made to create whole cell ROIs before splitting the stack channels. Point spread functions for Rab7 and Rab11 endosome channels were generated using DeconWithTheoreticalPSF script. Rab7 stacks were deconvolved using Richardson-Lucy algorithm and Rab11 endosome stacks were deconvolved using Non-Linear Least-Square algorithm in Deconvolutionlab2 with their associated point spread functions. Deconvolved stacks were duplicated and processed with gaussian blur $\sigma = 1$ or $\sigma = 2$. The image calculator subtracted the intensities of the stack with blur $\sigma = 2$ from the stack with blur $\sigma = 1$. The cell ROI isolated the segmentation region before running 3D Simple Segmentation. Low threshold was set to minimum intensity endosome signal and minimum size set to 10. 3D ROI Manager measured segmentation output and means for endosome volume, compactness, elongation, and flatness were taken per cell.

Results/Discussion/Conclusions:

Morphometric analysis revealed that both Rab7 and Rab11 endosomes in laminar flow conditions tended to be significantly less compact than those in static flow conditions, instead being more elongated, flat, and voluminous. Specifically, Rab11 endosomes of cells treated with laminar flow conditions have higher average volume (P =.0005, $\underline{x_{static}}$ = .53 μm^3, $\underline{x_{Flow}}$ = .79 μm^3), elongation (P = .0496, $\underline{x_{static}}$ = 1.61, $\underline{x_{Flow}}$ = 1.63), and flatness (P = .0035, $\underline{x_{static}}$ = 1.38, $\underline{x_{Flow}}$ = 1.41), and lower compactness (P = .0002, $\underline{x_{static}}$ = .98, $\underline{x_{Flow}}$ = .91), compared to Rab11 endosomes of cells in static conditions (Figure 2a-d). Rab7 endosomes followed similar trends, with laminar flow driving Rab7 endosomes to have a higher average volume (P = .0008, $\underline{x_{static}}$ = .68, $\underline{x_{Flow}}$ = .91), elongation (P < .0001, $\underline{x_{static}}$ = 1.59, $\underline{x_{Flow}}$ = 1.63), and flatness (P = .0004, $\underline{x_{static}}$ = 1.39, $\underline{x_{Flow}}$ = 1.43), and lower compactness (P = .0002, $\underline{x_{static}}$ = .95, $\underline{x_{Flow}}$ = .89) (Figure 2e-h). The observed difference in endosome morphology between cells in static and laminar flow conditions suggests endosomes are mechanoregulated and their morphological shapes like elongation, flatness, compactness, and volume change in response to mechanical cues. Here, we used a 3D image analysis workflow to segment and analyze endosomes in 3D, then identified differences in endosome morphology between endothelial cells in static and laminar flow conditions. We found that Rab7 and Rab11 endosomes from endothelial cells treated with laminar flow were flatter, less compact, more elongated, and were more voluminous than Rab7 and Rab11 endosomes from endothelial cells in static conditions. 2D image analysis techniques lack vertical axis resolution and can only focus on one focal plane, limiting capability in accurate morphology quantification. Our 3D image analysis workflow processed stacks to interpret continuous geometry along all axes, providing accurate metrics to identify differences in endosome volume, compactness, elongation, and flatness that would not be possible with 2D image analysis.

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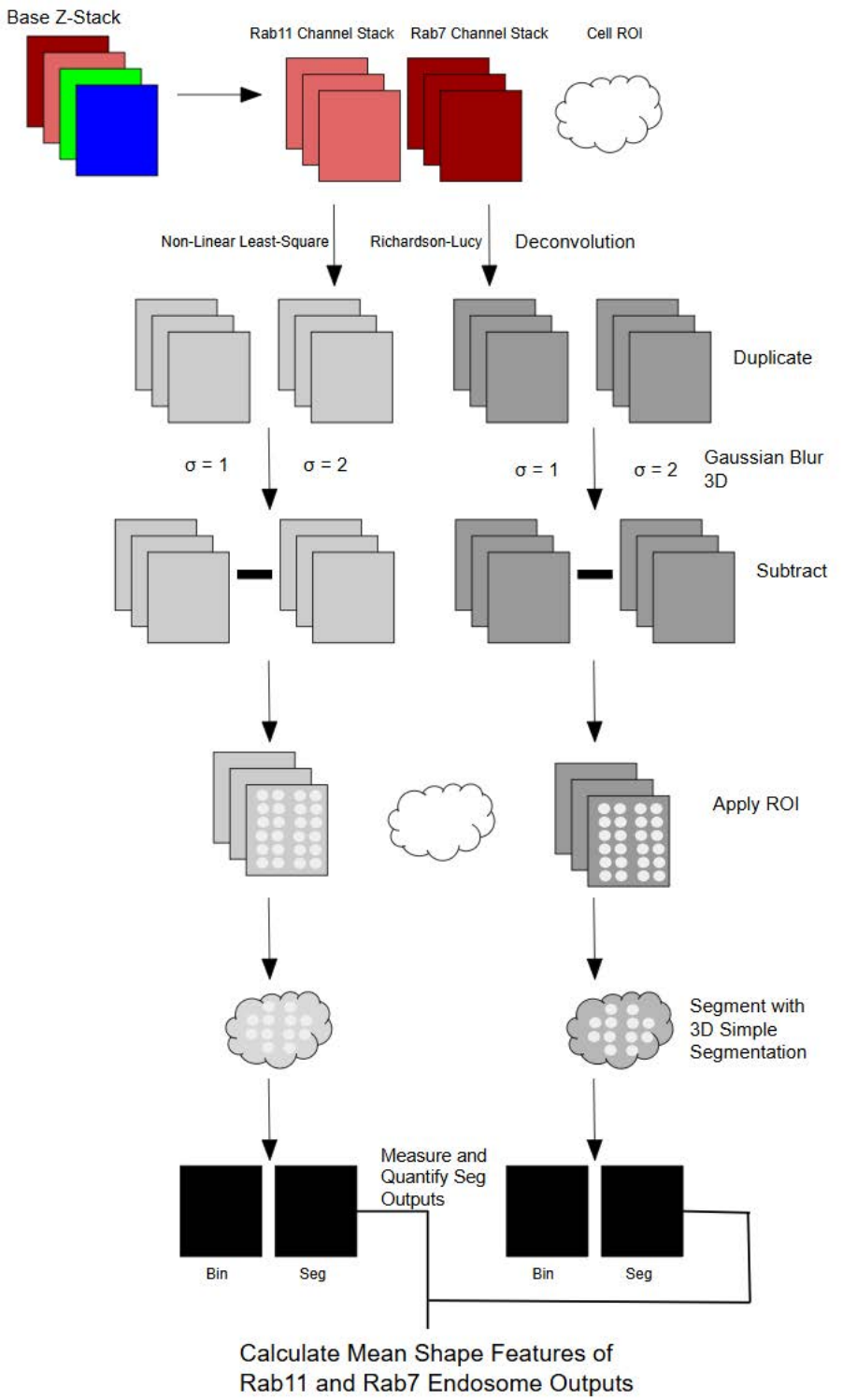
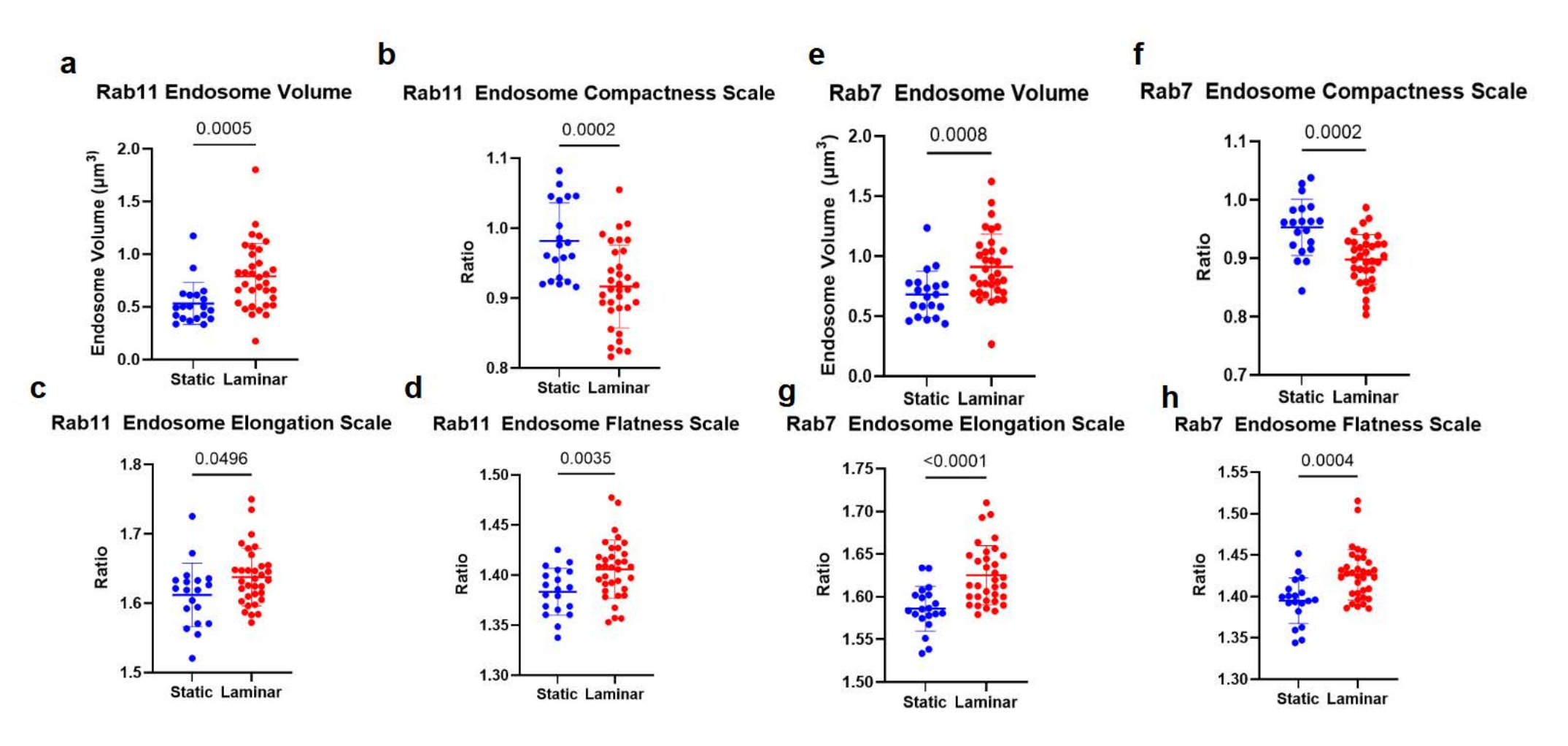

Base Z-Stack
Rab11 Channel Stack
Rab7 Channel Stack
Cell ROI
Non-Linear Least-Square
Richardson-Lucy
Deconvolution
Duplicate
σ = 1
σ = 2
σ = 1
σ = 2
Gaussian Blur 3D
Subtract
Apply ROI
Segment with 3D Simple Segmentation
Measure and Quantify Seg Outputs
Bin
Seg
Bin
Seg
Calculate Mean Shape Features of Rab11 and Rab7 Endosome Outputs


a
Rab11 Endosome Volume
0.0005
Endosome Volume (µm³)
2.0
1.5
1.0
0.5
0.0
Static Laminar
b
Rab11 Endosome Compactness Scale
0.0002
Ratio
1.1
1.0
0.9
0.8
Static Laminar
e
Rab7 Endosome Volume
0.0008
Endosome Volume (µm³)
2.0
1.5
1.0
0.5
0.0
Static Laminar
f
Rab7 Endosome Compactness Scale
0.0002
Ratio
1.1
1.0
0.9
0.8
0.7
Static Laminar
c
Rab11 Endosome Elongation Scale
0.0496
Ratio
1.8
1.7
1.6
1.5
Static Laminar
d
Rab11 Endosome Flatness Scale
0.0035
Ratio
1.50
1.45
1.40
1.35
1.30
Static Laminar
g
Rab7 Endosome Elongation Scale
<0.0001
Ratio
1.75
1.70
1.65
1.60
1.55
1.50
Static Laminar
h
Rab7 Endosome Flatness Scale
0.0004
Ratio
1.55
1.50
1.45
1.40
1.35
1.30
Static Laminar