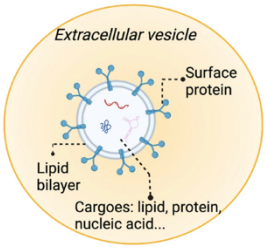
EXTRACELLULAR VESICLES, NOW IN 3D

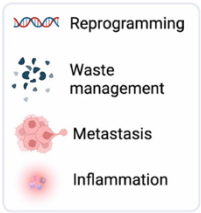
Extracellular vesicles (EVs) are nanoscale structures (~30–200 nm) released by cells that are thought to play an important role in intercellular communication, with their functional contents (proteins, lipids and nucleic acids) and shape indicative of specific pathologies. To use EV geometry as a diagnostic marker, it is therefore imperative to establish reliable quantitative measurement and classification methods. Previous work in this area has primarily focused on 2D measurements, revealing approximately circular EVs. However, in reality, EVs are complexly shaped 3D particles that are composed of a lipid bilayer membrane, genetic information, and surface receptors.

In response to these challenges, Double Helix Optics has developed new quantitative methods for characterizing EVs imaged in 3D using single-molecule localization microscopy (SMLM) powered by Double Helix Optics' engineered point spread function technology.

To demonstrate these methods, we imaged labeled tetraspanin proteins on the surfaces of EVs from human embryonic kidney (HEK) cells and used their 3D morphology to obtain descriptive parameters that can be used for EV classification.



Structure of EVs

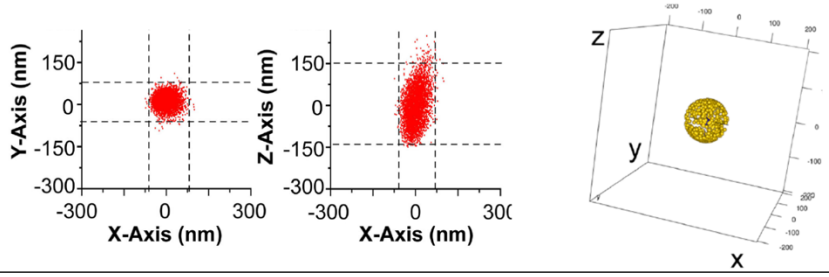


Biological functions

EVs are complex transport structures that can have varied molecular cargoes. They have been implicated in an array of biological processes, including genetic reprogramming, cellular waste management, cancer metastasis, and inflammation.

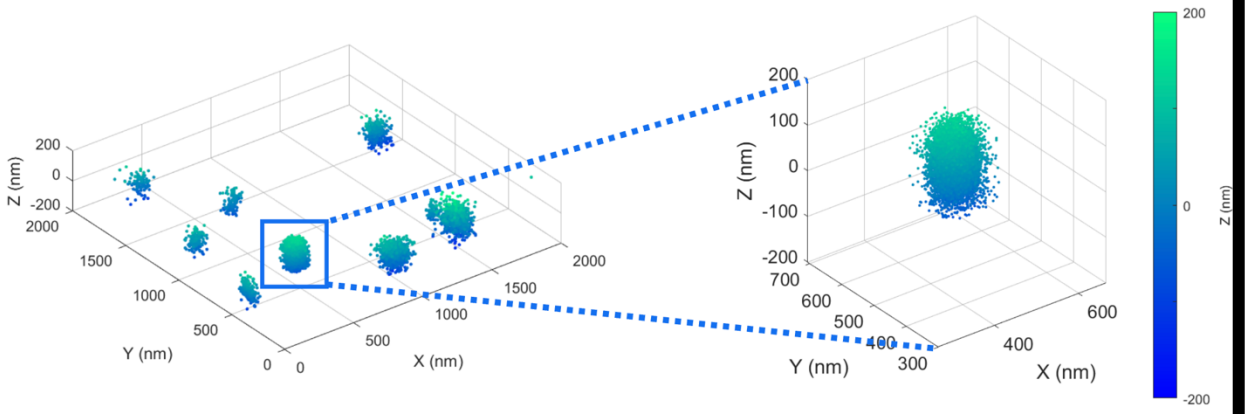
3D ISOTROPIC RESOLUTION

To date, super-resolution characterization has been limited in its ability to the reveal the complete structure of EVs. 2D imaging methods remain standard, but they provide an incomplete and often biased view of EV morphology, capturing only projections of 3D objects. The primary 3D method used to date employs cyclindrical lenses to create astigmatic point spread functions that rapidly lose precision with depth. Furthermore, EVs are typically assumed to be perfect spheres and simplistic depth calibrations are applied, resulting in apparent Z-axis elongation.



In published 3D imaging studies, EVs appear circular in the XY plane but elongated along the Z-axis (red), necessitating assumptions of EV sphericity (yellow). Plots from McNamara et al., Journal of Extracellular Vesicles (2022).

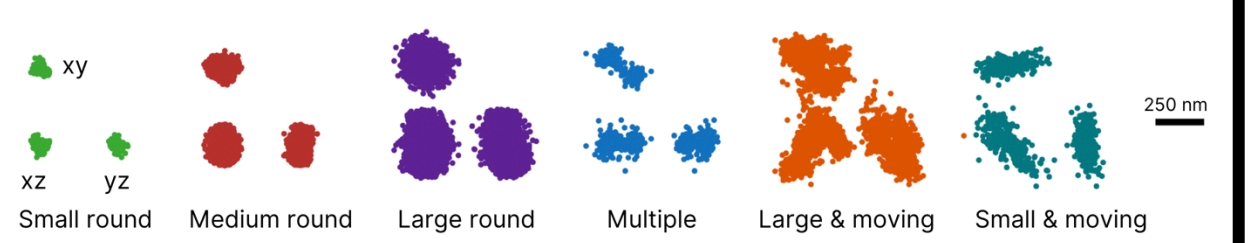
Using DHO's 3D Double Helix PSF (DH-PSF) phase mask in combination with a DHO SPINDLE® module and 3DTRAX recovery software, we were able to achieve sub-10 nm *spatially isotropic localization* precision with accurate depth calibration across varying EV structures. The result: high-resolution reconstruction of fields of EVs reliably quantified without making assumptions about EV shape or size.



Left: A selected volume of 3D-imaged EVs show well-defined EVs of varying shapes and sizes. Right: a closeup of one of the EVs shows excellent localization coverage that facilitates clear definition of EV boundaries.

3D MORPHOLOGY QUANTIFICATION

For quantitative analysis, localized PSFs were first segmented into individually identifiable EVs using density-based clustering. Varied EV morphologies were immediately semi-quantitatively identifiable from this first step.

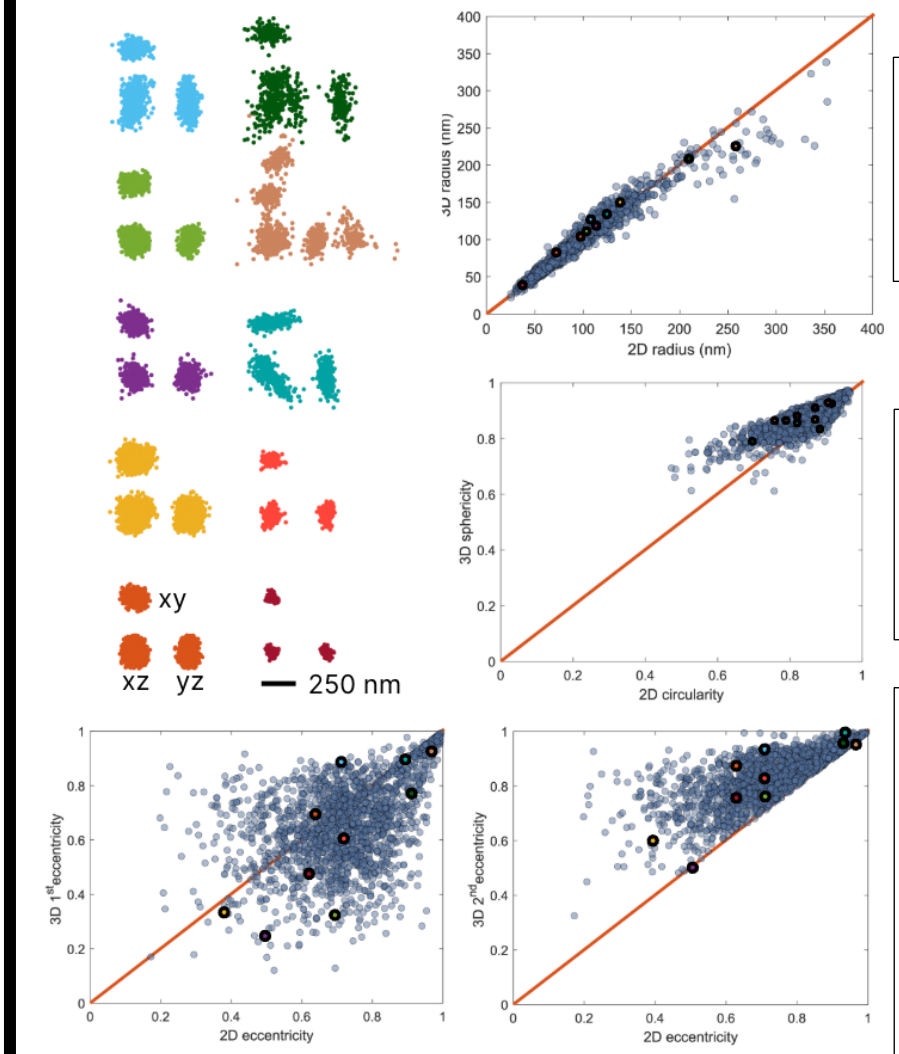


Selected characteristic EV morphologies likely correspond to different biological states (size- & motion-based) or indicate poorly segmented EVs due to close spatial proximity (multiple).

Individual EV morphologies were quantitatively parametrized using the 3D extensions of standard 2D measures of shape, including volume, sphericity, and eccentricity. Analogous 2D measures were also determined by only considering the XY projection for each EV.

Plotting the 3D parameters for each EV as a function of the corresponding 2D parameters clearly shows that the 2D parameters are not good predictors of 3D morphology. For example:

- 2D area is not a good predictor of 3D volume when EVs are large
- As 2D circularity decreases, it becomes a worse predictor of 3D sphericity
- 2D eccentricity (one parameter) is a poor predictor of 3D eccentricity (two parameters)



After M.L.P. Bailey et al. ISEVtech 2024 poster presentation.

Deviation from 1:1 correlation and increasing variance of 3D radius with 2D radius indicates that 2D area is not a good predictor of 3D volume when EVs are large.

Increasing variance of 3D sphericity with decreasing 2D circularity indicates that apparent circularity in 2D is not a good predictor of true 3D sphericity when EVs are less circular .

Increasing variance of 1st and 2nd 3D eccentricity with decreasing 2D eccentricity indicates that that 2D eccentricity is not a good predictor of true 3D eccentricity. For example, three different EVs have similar 2D eccentricity of ~0.7, but different 3D eccentricities, ranging from the bottoms to the tops of the distributions.

REALIZE A MORE COMPLETE UNDERSTANDING OF EVs

By utilizing DHO's full technology platform of precision-engineered optics and cutting-edge computation, we have shown that 2D imaging of 3D EVs provides an incomplete and potentially misleading picture of biological reality. DHO's more complete super-resolved 3D imaging provides a baseline for enhanced study and quantitative analysis of EVs for applications ranging from basic science to diagnostics and drug delivery.

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