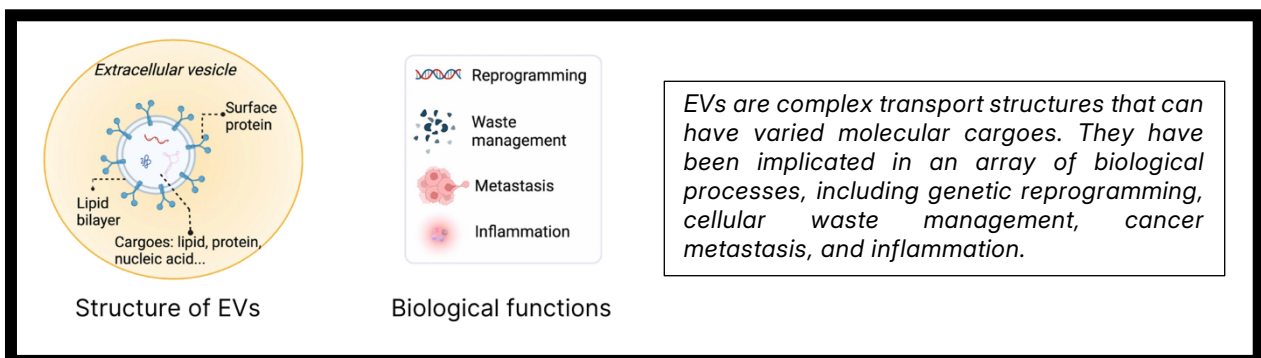


EXTRACELLULAR VESICLES, NOW IN 3D

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.

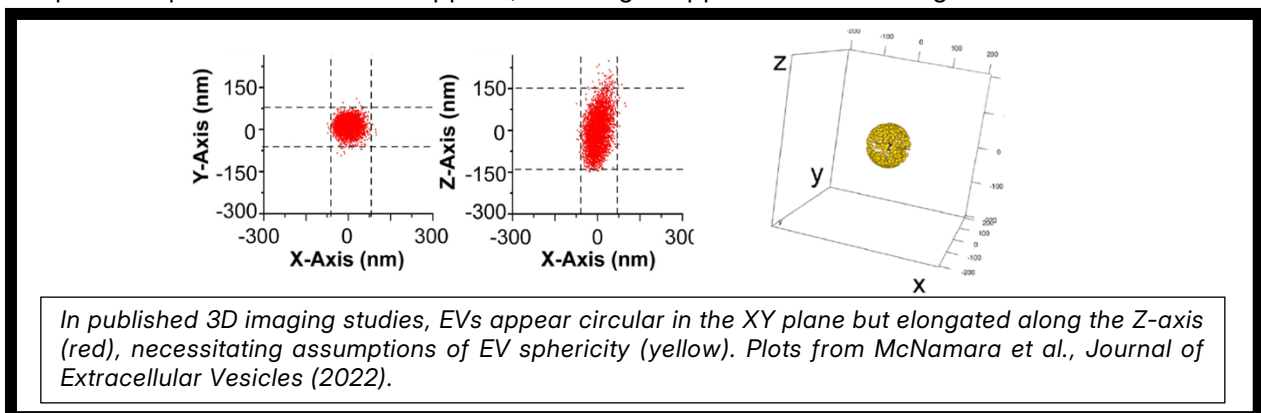
In response to these challenges, Double Helix Optics has developed new quantitative methods for characterizing EVs imaged in 3D using single-molecule localization microscopy (SMLM) powered by Double Helix Optics' engineered point spread function technology.

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.

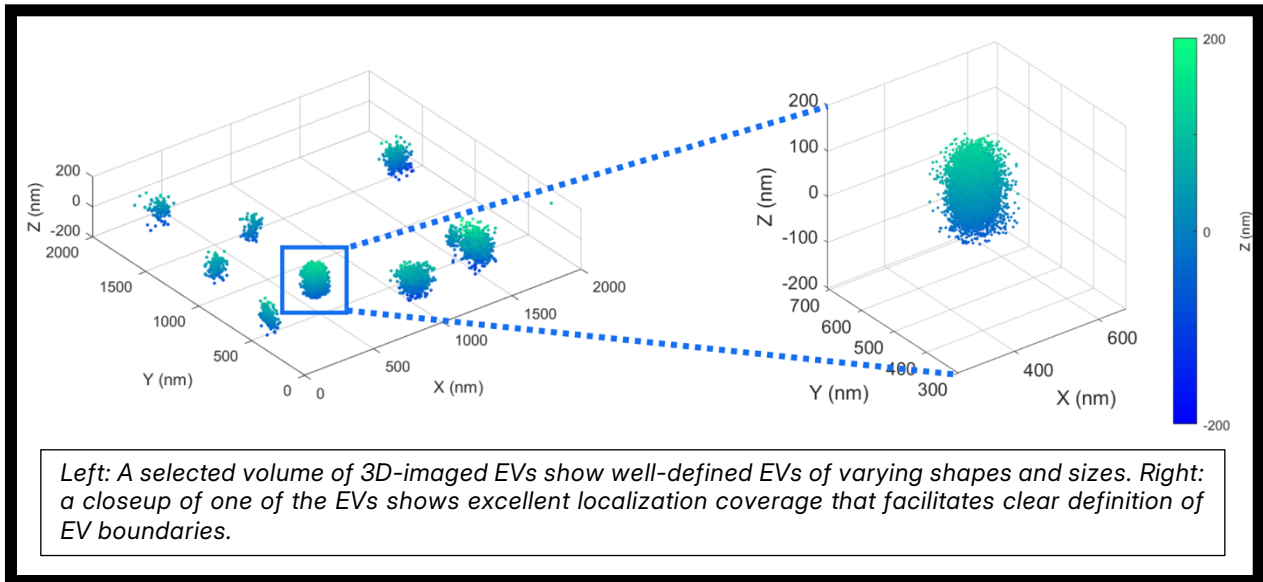


3D ISOTROPIC RESOLUTION

To date, super-resolution characterization has been limited in its ability to 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 cylindrical 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.

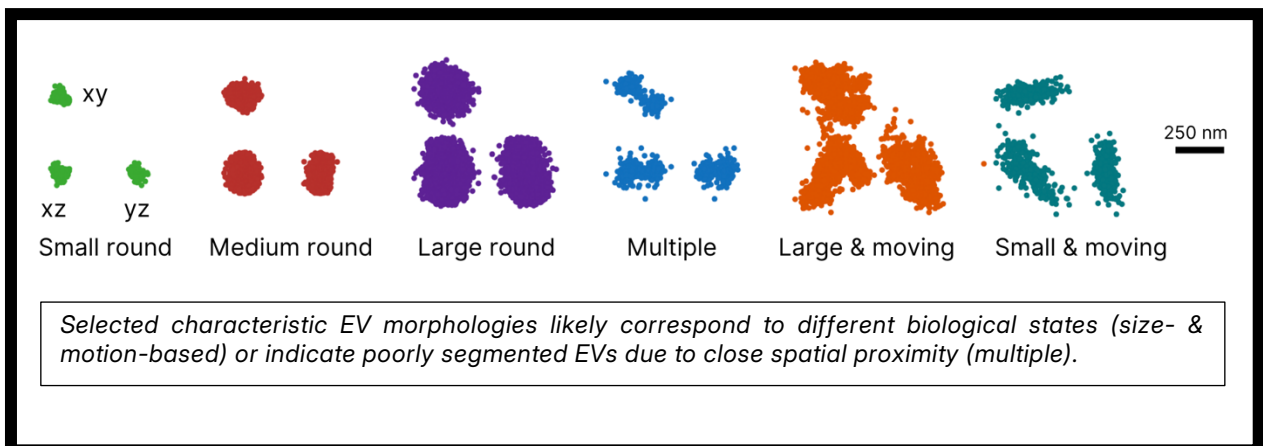


Using DHO's 3D Double Helix PSF (DH-PSF) phase mask in combination with a DHO SPINDLE[®] module and 3DTRAX recovery software, we were able to achieve sub-10 nm spatially isotropic localization precision with accurate depth calibration across varying EV structures. The result: high-resolution reconstruction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



3D MORPHOLOGY QUANTIFICATION

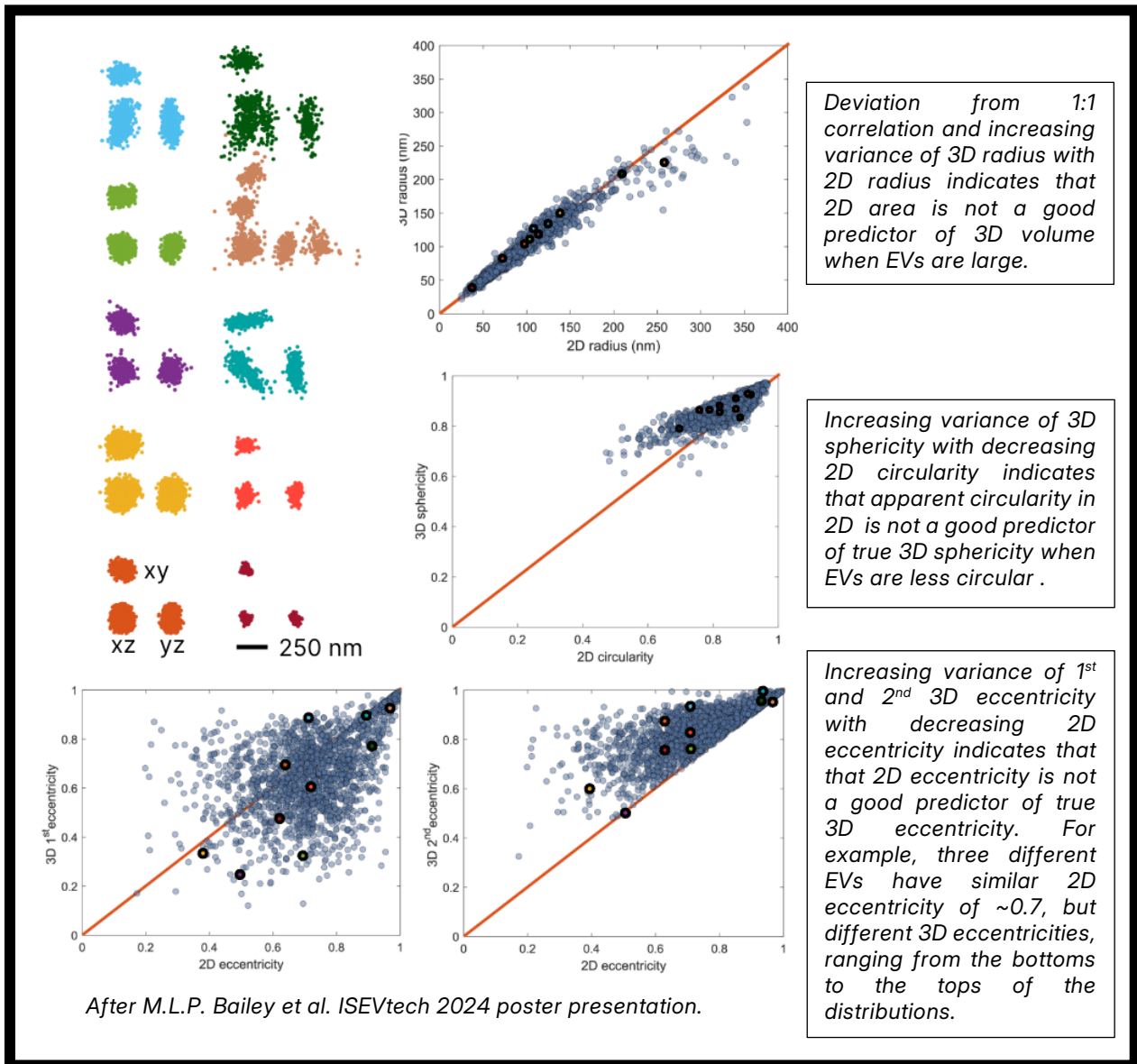
For quantitative analysis, localized PSFs were first segmented into individually identifiable EVs using density-based clustering. Varied EV morphologies were immediately semi-quantitatively identifiable from this first step.



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)



REALIZE A MORE COMPLETE UNDERSTANDING OF EVs

By utilizing DHO's fully 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.