Supplementary Materials

High-content imaging platform to discover chemical modulators of plasma membrane rafts

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No unexpected or unusually high safety hazards were encountered.

GPMV preparation for high throughput screening
HeLa cells were obtained from ATCC (cat # CCL-2) and cultured at 37 °C in 5% CO₂ in a humidified tissue culture incubator. HeLa cells were maintained in DMEM (Gibco cat # 11885-084) supplemented with 10% fetal bovine serum (FBS, Gibco 26140-079), 1% penicillin/streptomycin (P/S), and 1% L-glutamine. GPMVs were prepared from HeLa cells using the DTT/PFA method as described before.

In brief, cells were plated in 150 mm tissue culture dishes the day before the experiment, to yield a confluence of 70-80% the day of the experiment. To generate GPMVs, cells were rinsed twice with 10 ml of GPMV buffer (2 mM DTT, 25 mM formaldehyde, 150 mM NaCl, 10 mM HEPES, 2 mM CaCl₂, pH 7.4) and subsequently incubated in 12 ml of GPMV buffer at 37°C with gentle shaking at 70 rpm for 1.5 h. The raft marker NBD-DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoazadiazol-4-yl) (Avanti Polar Lipids) and the non-raft marker DiD (1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate; cat # D307 Thermo Scientific) were added from stock solutions in ethanol to the GPMV suspension at final concentrations of 5-6 µg/ml and 1 µg/ml, respectively, for the initial screenings. For some experiments, cells were prelabelled with 5 µg/ml DiD for 8 min at 4 °C and washed several times prior to GPMVs generation. Screening was performed the same day that GPMVs were generated.

High-throughput screening of GPMVs
High throughput screening was performed at the Vanderbilt High Throughput Screening Facility using their Cayman Bioactive Lipid Library (Cayman Chemical, cat # 10506). Compounds dissolved in DMSO (stock concentration of 1 mM) were preloaded into 96-well plates using an acoustic droplet ejection liquid dispenser (ECHO 555, Labcyte) at volumes of 50 - 80 nl per well. 50 - 80 µl GPMV suspensions in GPMV buffer was then added to each well of a 96-well plate to obtain a final drug concentration of 1 µM. After a brief mixing, the GPMVs were allowed to settle to the bottom of the plates for 1.5 h prior to imaging. Samples were prepared and imaged at RT. The screen was repeated on three different days using three separately prepared batches of GPMVs (3 biological replicates).

Image acquisition was performed with an ImageXpress Micro XL automated microscope (Molecular Devices) using a Nikon 40X 0.6 NA Super Plan Fluor ELWD objective and a Lumencor Sola solid state light source. Laser autofocus was employed at each site to obtain the best focus z-plane for each channel. NBD-DSPE fluorescence was excited and detected using a Semrock GFP filter set (GFP-3035B) consisting of a 472/30 excitation filter, 442-488 / 502-730 dichroic, and 520/35 emission filter, and DiD fluorescence was excited and detected using a Semrock Cy5 filter set (Cy5404A) consisting of a 628/40 excitation filter, a 594-691/669-726 dichroic, and 692/40 emission filter. Sixteen sites were captured per well using a 4.2MP widefield scientific cMOS camera with a camera binning of 1. Each plate took approximately 1.3 h to image. Multiple plates were loaded to a plate carousel (ThermoScientific), shuttled to/from the ImageXpress via a ThermoScientific CRS F3 robot arm, and automatically run through the Momentum scheduling software (Thermo). To analyze the data, images and metadata were exported as TIFF files and subjected to image analysis using a custom MATLAB-based program as described below. Wells containing ≤ 50 useable GPMVs were excluded from further analyses.

Compounds identified in the initial screens as potential hits were re-ordered and their identity verified by mass spectrometry by the Vanderbilt Chemical Synthesis Core. Ten-point dose response curves using 1:2 serial dilutions were generated producing a range of final concentration of 30 nM to 30 µM and backfilled to yield equivalent concentrations of DMSO. Data were collected in duplicate for each concentration. Two independent dose response experiments were performed.
High-content image analysis of GPMVs
To perform high content image analysis of GPMVs, we developed a custom algorithm called VesA using MATLAB (MathWorks). The program, installation instructions, a quickstart userguide, and test datasets will be made available for download upon acceptance. VesA automatically loads images, detects GPMVs, records their position and diameters, measures the fluorescence intensity of the membrane, local background, and lumen, determines whether they are phase separated or not, calculates partition coefficients, and outputs the relevant data in tabular format. A flowchart describing the overall steps in data processing is provided in Figure S1, and the individual steps used to process the images described in more detail below.

Disc identification, contour detection, and vesicle exclusion criteria. In the initial step of the analysis, a 2D Gaussian filter was used to smooth the images. Disc-shaped objects were identified using the built in MATLAB two-stage circular Hough transform (Figure S2b) and the location of each disc, its diameter, and its circularity were recorded.

For each detected disc, the position of the membrane contour was refined by generating a radial intensity profile as a function of each angle 2πR, where R is the vesicle radius in pixels (Figure S3a-c). A circle was then fit into the position defined by the maxima of the radial intensity profile (Figure S3d). This step provides a smoothing of the position of the membrane and also facilitates further analysis of phase separated vesicles, which by definition contain regions of both high and low membrane fluorescence. Bilinear interpolation was used to estimate the intensity on the circle at a given angle for each 2πR angle (Figure S3e). The luminal (intravesicular) fluorescence, and the local background fluorescence were then measured and recorded for each circle together with its position and radius.

GPMVs were excluded from further analysis if they were closer than 5 pixels to one another, the fluorescence signal in the membrane was > 98% of the maximum attainable intensity, the background fluorescence was > 75% of the maximum membrane fluorescence, or if the interior (luminal) fluorescence vesicle was > 85% of the maximum membrane fluorescence for a given GPMV. Additional constraints were also included to exclude vesicles where the magenta and green channels did not overlap due to drifting between imaging in the two channels, the center of the disks in the red and green channels were separated from each other by more than 10 pixels, or if there was more than a 15% deviation in the radii of the green and red channels for that same disk. This step also allows for correction of registration of GPMVs in the red and green channel that are offset due to drifting as well as re-scaling the disk radii to overlap to correct for small shifts in z that may occur between collection of the red and green images. Altogether, application of these criteria led to the exclusion of a significant fraction of GPMVs (for an example, compare Figures S2b and S2c).

Identification of phase separated vesicles and measurement of partition coefficients. Two methods were used to quantify the percentage of phase separated vesicles and partition coefficients.

In the first method, termed the “distribution” method in the software, the algorithm generates a series of line profiles bisecting the GPMV at each 2πR angle $\phi$. The peaks in fluorescence intensity $I$ corresponding to the position of the membrane along the line profile are then used to calculate angular partition coefficients, $p_{ang}$ (Figure 1f, i) according to

$$p_{ang}(\phi) = \frac{I(\phi)}{I(\phi) + I(\phi + 180^\circ)}$$

Using this approach, non-phase separated vesicles yield a single population of angular partition coefficients centered around 0.5 (Figure 1f), whereas phase separated vesicles typically yield three populations of partition coefficients (Figure 1i). To computationally distinguish between these possibilities for each vesicle, histograms of angular partition coefficients were generated, and the histograms fit to determine if they were best described by one or three Gaussians. Each vesicle was
then scored either as single phase (one Gaussian peak) (Figure 1g) or phase separated (two or more symmetric Gaussians peaks) (Figure 1j), and the partition coefficients were determined from the position of the maxima of the peaks. GPMVs in which a single Gaussian peak was obtained that was not symmetric around 0.5 were excluded from further analysis. This method would be expected to underestimate the fraction of phase separated vesicles since the measurements are made in a single focal plane bisecting the GPMVs. However, this effect should be consistent across wells and treatments.

A second approach termed the “colocalization” method was used to score the vesicles as phase separated or not for experiments where the average radius of the GPMVs was less than 10 pixels (Figure S1). In this method, the fluorescence intensity in the red and green channels were measured at each point along the membrane contour (Figure S1b and e). The intensity of red versus green fluorescence was then plotted for each datapoint (Figure S1c and f). For phase separated vesicles, the datapoints fall into two separate quadrants corresponding to pixels with high red signal but low green signal, and vice versa (Figure S1c), whereas for membranes containing a single uniform phase, all the datapoints fall on the diagonal (i.e. they are colocalized) (Figure S1f). This method was only used to score the percentage of phase separated vesicles and was not used to calculate partition coefficients.

**Determination of % phase separated vesicles and partition coefficients per well, and correction for positional effects.** For each plate, plots of the percent of phase separated vesicles per well were generated in the order they were imaged (Figure S1a). Such plots typically yielded higher values in the initial wells that flattened to a plateau in the later part of the plate. This effect was observed across multiple plates, indicating it is not linked to the compounds within a specific plate but rather is related to changes that occur when plates are initially moved into the microscope. We speculate this may result from changes in temperature as each plate is moved into the sample chamber, which is at least 1° C higher than RT, that level off once the plate temperature has equilibrated. To ensure our approach works irrespective of the cause of the position dependent effect, we implemented a correction that would help any researcher using our work. To correct for these positional effects, the datasets for each well were fit with a simple exponential decay function

\[ y_{trend} = y_0 \pm Ae^{-\left(\frac{x-x_0}{t}\right)} \]

where \( x \) is the well number and \( y \) is the percentage of phase separated vesicles for a single well. The data were then normalized to yield relative change in \( y \) per well, \( \Delta y \) (Figure S1b):

\[ \Delta y = \frac{y - y_{trend}}{y_{trend}} \]

Following these corrections, wells that exhibited either very high or very low relative changes in phase separation were apparent (Figure S1c, d). A similar approach was used to correct for positional effects in measurements of partition coefficients.

**Z-score determination and final ranking of hits.** Z-scores of the percentage of phase separated vesicles (Figure S1d) and partition coefficients were determined on a per-plate basis as

\[ Z = \frac{\Delta y}{SD_{plate}(\Delta y)} \]

Compounds with z-scores > 2 or < -2 were deemed to be raft modulators. Some variability was observed between screens, in part because not all wells contained ≤ 50 usable GPMVs in every
Compounds that we identified as hits in at least 2 of the 3 independent screens were included in the final list of raft modulators.

Comparison of widefield versus confocal images
GPMVs were prepared from HeLa cells and labeled with NBD-DSPE and DiD as described above. GPMVs were added into a 96-well plate and imaged using an ImageXpress Micro Confocal High Content Screening System (Molecular Devices, San Jose CA) with a Nikon 40X 0.95 NA Plan Apo Lambda objective and an Andor Zyla 4.2MP 83% QE sCMOS camera, and an 89-North LDI 5 channel laser light source. Nine fields were imaged per well. The whole plate was first image using a spinning disc confocal module with a 60 µm pinhole size. Immediately after the plate was imaged again in widefield. NDB-DSPE was excited and detected using a FITC filter set consisting of Semrock a 460-488 nm excitation filter and 502-548 nm emission filter (widefield) or a 536/40 nm emission filter (confocal). DiD was excited and detected using a Cy5 filter set consisting of a 633-650 nm excitation filter and 653-730 nm emission filter (widefield) or a 692/40 nm emission filter (confocal). Imaging in each modality took approximately 45 minutes. Both image sets were analyzed in VesA as described above.

Tmisc measurements
GPMVs were generated from HeLa cells and either labeled with NBD-DSPE and DiI as described above (TLCK experiments) or labelled with DiI-C12 (C6-ceramide experiments). They were then treated with 1 µM C6 ceramide (Sigma Aldrich) or 10 µM TLCK (Cayman) for 1-2 h, mounted in a chamber formed from two coverslips separated by a silicone spacer in the continued presence of drug or an equivalent volume of DMSO (final concentration = 0.1 mol%), and allowed to settle for 1 h prior to being imaged.

Tmisc measurements for GPMVs treated with C6-ceramide were performed using a Zeiss LSM 510 confocal microscope. Images were collected using a 40X 1.2 NA Zeiss Plan-Neofluor objective with a confocal pinhole setting of 5 airy units. DiI-C12 fluorescence was excited using the 543 nm line of a HeNe laser and images were collected at 1X digital zoom. The final image resolution was 0.290 µm/pixel. A Linkam Peltier Cooling system (Tadworth, UK) was used to vary the temperature between 25 and 40 °C. More than 5 fields of view were collected at each temperature. For these datasets, the number of phase separated GPMVs was scored manually. Data are reported as ∆T from 2 independent experiments.

Tmisc measurements for GPMVs treated with TLCK were performed using a Zeiss A120 confocal microscope. At each temperature, a 3x3 tile of images was collected using 20X Air objective at a final pixel resolution of 0.138 µm/pixel. NBD-DSPE fluorescence was excited using a 458 Argon laser line and detected using a 458/548 dichroic and 482-614 nm band pass filter. DiD fluorescence was excited using a 633 nm HeNe laser and emission was collected using a 633/698.5 dichroic and 639-759 nm band pass filter. A Peltier temperature system was used to vary the temperature between 5 and 38 °C. For these datasets, the percentages of phase separated GPMVs were scored using the “colocalization” method using the MATLAB algorithm described above. Data are reported from 2 independent experiments.

Effects of hits on GPMVs from other cell types
GPMVs were generated from HeLa (Human Cervical cancer cells, ATCC), hTERT-RPE-1 (Human Retinal Pigmented Epithelial cells, kindly provided by Dr. Ethan Lee, Vanderbilt University) and BSC1 (Monkey Kidney cells) (ATCC). Prior to GPMV preparation, cells were seeded in 150 x 25 mm plates and cultured in DMEM containing 10% FBS and 1% Penicillin/ streptomycin in a tissue culture incubator with humidified air supplemented with 5% CO2 at 37°C.

To prepare GPMVs, cells were washed twice with active GPMV buffer (2 mM DTT, 25 mM formaldehyde, 150 mM NaCl, 10 mM HEPES, 2 mM CaCl2, pH=7.4) and plates with cells containing 10 ml of active GPMV buffer were incubated at 37°C with gentle shaking at 90 rpm for 1.5 hours. GPMVs

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were collected and labeled with 9.7 μg/ml NBD-DSPE (Avanti Polar lipids # 810141) and 1 μg/ml DiD (Thermo scientific # D307).

To test the effects of the putative hits and negative control compound, 1 µl of a 1 mM stock solution of TLCK or C6 ceramide, or an equivalent volume of vehicle (DMSO) was loaded per well in duplicate in a 96 well plate. 99 µl of GPMV suspension was added per well to obtain a final concentration of 10 µM of the compounds to be tested. After allowing the vesicles to settle down for 1-1.5 hours, they were imaged at RT using an Operetta CLS High Content Imaging System (Perkin Elmer HH16000000) fitted with 14-bit CMOS camera (1.3 Mega Pixel, 6.5 µm/pixel). Epi-fluorescent images of 16 different sites of each well were acquired using a 40X water objective (NA 1.1). Fluorescence was excited with an LED light source and fluorescence emission was collected using Alexa 488 and Alexa 647 filter sets provided by the manufacturer. Data were exported as TIFF files and processed as described above. Data are reported from a single experiment performed in duplicate.

Assessment of assay robustness using SSMD*

GPMVs were prepared as described above and incubated for 2 hours at RT. They were then seeded into a flat bottom, black-walled 96-well plates (Greiner Bio-One, Kremsmünster Austria) coated with 0.1% bovine serum albumin and pre-loaded in a checkerboard pattern with DMSO, 10 µM TLCK, or 10 µM C6 ceramide (Figure S9a). GPMVs were imaged using the ImageXpress Micro XL automated imager using a Nikon 40x super plan fluor ELWD objective and analyzed using the software described above to determine the percentage of phase separated vesicles in each well (Figure S9b). An estimate for robust strictly standardized mean difference (SSMD*) was then calculated based on the percentage of phase separated vesicles per well as reported in the NBD-DSPE channel according to the following equation:

\[
SSMD^* = \frac{\bar{X}_p - \bar{X}_N}{\sqrt{s_p + s_N}}
\]

Here, \(\bar{X}_p\) and \(\bar{X}_N\) represent the medians of the percentage of phase separated vesicles for the positive controls (C6 ceramide or TLCK) and negative control (DMSO), respectively, whereas \(s_p\) and \(s_N\) correspond to the absolute deviations from the median for the positive and negative controls. Three independent GPMV preparations were analyzed on separate days to evaluate reproducibility (Figure S9c).

Supplementary References

1. Sezgin, E.; Kaiser, H. J.; Baumgart, T.; Schwille, P.; Simons, K.; Levental, I., Elucidating membrane structure and protein behavior using giant plasma membrane vesicles. Nat Protoc 2012, 7 (6), 1042-51.

2. Zhang, X. D., A pair of new statistical parameters for quality control in RNA interference high-throughput screening assays. Genomics 2007, 89 (4), 552-61.

3. Zhang, X. D., Illustration of SSMD, z score, SSMD*, z* score, and t statistic for hit selection in RNAi high-throughput screens. J Biomol Screen 2011, 16 (7), 775-85.
Table S1. Efficiency of detection of phase separated GPMVs using DiD (red) fluorescence versus NBD-DSPE (green) fluorescence across screens.

|            | Mean number of GPMVs/well<sup>1</sup> | Phase-separated red GPMVs<sup>2</sup> | Phase-separated green GPMVs |
|------------|--------------------------------------|--------------------------------------|-----------------------------|
| Screen 1   | 232 ± 193 (697)                      | 89.4 ± 6.2%                          | 52.0 ± 8.6 %                |
| Screen 2   | 214 ± 98 (849)                       | 88.6 ± 3.9 %                         | 69.1 ± 5.2 %                |
| Screen 3   | 172 ± 95 (806)                       | 94.5 ± 3.6 %                         | 73.0 ± 6.7 %                |
| Average    | 206 ± 139 (2355)                     | 90.8 ± 5.3 %                         | 65.4 ± 11.2 %               |

<sup>1</sup> Mean ± SD (n = number of wells) of the number of GPMVs that are both red and green.

<sup>2</sup> The large percentage of phase separated GPMVs in the red channel reflects systematic exclusion of red GPMVs after applying selection criteria using the image processing algorithm (see Figure S2 for examples of GPMVs that are detected in the green channel but not the red channel).
### Table S2. Metrics for hits.

| Chemical Name¹ | CasRn | VU Number | Found disks | Number of used GPMVs | % phase separated GPMVs | P ordered z-score | Repeats |
|----------------|-------|-----------|-------------|----------------------|-------------------------|-------------------|---------|
| **Compounds that decrease % phase separated GPMVs** |
| TOFA           | 54857-86-2 | VU0454815 | 25663       | 3081                 | -3.41                   | 1.20              | 3       |
| 2-thio-PAF     | 96801-55-7 | VU0454596 | 15924       | 1266                 | -3.15                   | 0.72              | 3       |
| C6 Ceramide (d18:1/6:0) | 124753-97-5 | VU0454551 | 16444       | 986                  | -3.01                   | 0.76              | 3       |
| C8 Ceramide (d18:1/8:0) | 74713-59-0 | VU0454580 | 11224       | 1551                 | -2.87                   | 0.72              | 2       |
| Cigitazone     | 74772-77-3 | VU0454703 | 24290       | 3578                 | -2.73                   | 1.71              | 3       |
| Oleyl Trifluromethyl Ketone | 177987-23-4 | VU0454635 | 28111       | 2418                 | -2.65                   | 1.25              | 3       |
| OMDM-1         | 61684-62-9 | VU0454354 | 15245       | 838                  | -2.62                   | -0.54             | 3       |
| cis-trimethoxy Resveratrol | 94608-23-8 | VU0454575 | 31300       | 1657                 | -2.45                   | -1.61             | 2       |
| 11(Z),14(Z),16-Eicosadienonic Acid | 2091-39-6 | VU0454750 | 28101       | 2363                 | -2.15                   | -0.46             | 2       |
| (2S)-OMPT      | 121747-69-6 | VU0454882 | 28604       | 2510                 | -2.12                   | 0.27              | 3       |
| O-1602         | 317321-41-8 | VU0454839 | 24490       | 2925                 | -2.10                   | 0.27              | 3       |
| N,N-Dimethylthiophosphinate (d18:1) | 119567-63-4 | VU0454609 | 25783       | 2020                 | -2.09                   | -0.70             | 3       |
| SU6656         | 330161-87-0 | VU0455031 | 21976       | 1628                 | -2.06                   | -0.42             | 3       |
| PAF C-16       | 74389-68-7 | VU0454565 | 17334       | 997                  | -2.00                   | 1.87              | 2       |
| **Compounds that increase % phase separated GPMVs** |
| TLCK hydrochloride | 4238-41-9 | VU0454477 | 9682        | 1781                 | 6.73                    | 5.69              | 2       |
| (−)-Epigallocatechin Gallate | 989-51-5 | VU0244129 | 14772       | 2379                 | 4.33                    | 1.42              | 2       |
| N-Stearyl Taurine | 63155-80-6 | VU0454824 | 26313       | 2125                 | 3.21                    | 2.89              | 3       |
| XAV939         | 264028-89-3 | VU0454979 | 24084       | 1487                 | 2.33                    | 0.58              | 3       |
| N-Palmityl Taurine | 83982-06-3 | VU0454833 | 25582       | 3185                 | 2.19                    | 1.66              | 3       |
| 1-piperazin-1-yl-isouquinoline (hydrochloride) | 936643-79-7 | VU0454937 | 22084       | 1464                 | 2.14                    | 1.61              | 3       |
| thyo-Mi1fesine | 943022-11-5 | VU0455039 | 29277       | 2406                 | 2.12                    | 2.78              | 3       |
| **Compounds that decrease NBD-DSPE partition coefficient** |
| CAY1044        | 298186-80-8 | VU0454788 | 24223       | 2407                 | -1.61                   | -2.04             | 3       |
| FR122047 (hydrochloride) | 130717-51-0 | VU0454334 | 14553       | 707                  | 0.12                    | -2.03             | 3       |
| **Compounds that increase NBD-DSPE partition coefficient** |
| TLCK hydrochloride | 4238-41-9 | VU0454477 | 9682        | 1781                 | 6.73                    | 5.69              | 2       |
| N-Stearyl Taurine | 63155-80-6 | VU0454824 | 26313       | 2125                 | 3.21                    | 2.89              | 3       |
| thyo-Mi1fesine | 943022-11-5 | VU0455039 | 29277       | 2406                 | 2.12                    | 2.78              | 3       |
| d-O-methyl PAF C-16 | 78058-44-3 | VU0454585 | 28677       | 1217                 | -1.06                   | 2.66              | 3       |
| S-ethyl thioisourea (hydrobromide) | 1071-37-0 | VU0243742 | 12660       | 799                  | 0.85                    | 2.57              | 2       |
| Arachidonyl-N-3-Fluorothiols | 166100-37-4 | VU0454718 | 28628       | 2478                 | 1.90                    | 2.38              | 3       |
| Methylcarbamy PAF C-16 | 91575-58-5 | VU0454566 | 18050       | 1523                 | -0.58                   | 2.20              | 3       |
| MK-886         | 118414-82-7 | VU0254002 | 14472       | 1337                 | 0.67                    | 2.10              | 2       |

¹ Abbreviations: TOFA: 5-(Tetradecyloxy)-2-flurancarboxylic acid; 2-thio-PAF: 1-Octadecyl-2-deoxy-2-thio-S-acetyl-sn-glycerol-3-phosphorylcholine; OMDM-1: (S)-N-(1-(4-hydroxyphenyl)-2-hydroxyethyl) oleamide; (2S)-OMPT: 2Z-Octadecenonic acid; (25)-3-(4-hydroxycapric acid)-5-2-methoxypropyl ester; triethyl ammonium salt (1:2); O-1602: 5-methyl-4-[(1',6,6'R)-3-methyl-6-(1'-methylpentyl)2-cyclohexen-1-yl]-1,3-benzenediol; SU6656: 2,3-dihydro-N,N-dimethyl-2-oxo-3-[4,5,6,7-tetrahydro-1H-indol-2-yl]methylene]-1H-indole-5-sulfonamide; PAF C-16: platelet activating factor C-16 or 1-O-hexadecyl-2-O-acetyl-sn-glycerol-3-phosphorylcholine; TLCK: N-Tosyl-Lys Chloromethyl Ketone; XAV939: 3,5,7,8-tetrahydro-2-[4-(trifluoromethyl)phenyl]-4H-tetrahydropyran[4,3-d]pyrimidin-4-one; CAY1044: 2-undecyl-thiazolidine-4-carboxylic acid; FR122047: 1-(4,5-bis[(4-methoxyphenyl)-2-thiazolyl]carbonyl)-4-methyl-piperazine, mono-hydrochloride; MK-886: 1-(4-chlorophenyl)methyl)-2-(1,1-dimethylthio)thio-Q,α-dimethyl-5-(1-methylthio)-1H-indole-2-propanoic acid
Figure S1. Workflow for image analysis and data processing from high throughput screen. (a) Vesicle detection and selection. (b) Analysis of individual vesicles. (c) Statistical analysis performed across populations of vesicles.
Figure S2. Automated vesicle selection process. (a) Zooms of a portion of the image of GPMVs shown in Figure 1b. (b) Disc-like structures identified during the first round of automated image analysis are highlighted by white circles. (c) GPMVs that pass all exclusion criteria and are included in the final analysis are highlighted in yellow circles. Note that due to the high interior fluorescence signal, GPMVs labeled with DiD often do not pass all selection criteria, especially when the membrane signal is weak and uniform. This effect leads to an undercounting of non-phase separated vesicles, and consequently an overestimation of the percentage of phase separated vesicles as reported by the DiD fluorescence signals (Table S1). Examples of double-labeled GPMVs where the GPMV is detected in the NBD-DSPE channel but not in the DiD channel are highlighted with arrows. Scale bar, 20 μm.
Figure S3. Contour detection and refinement of membrane position of individual GPMVs. (a) A radial scan is performed for each found disc to find the maxima and estimate the membrane position for both dyes. The yellow arrow shows an example of the line used to perform the radial scan. (b) A radial profile plot is used to identify the fluorescence peak corresponding to the position of the membrane in the DiD channel (magenta circles) and NBD-DSPE channel (green squares). (c) Found position of the membrane contour (white dots) and background (yellow line) for the GPMV shown in panel a. (d) To refine the position of the membrane in each vesicle, a circle (dotted white line) is fit into the contours identified in the initial scan shown in panel c. Arrow indicates angle = 0° (e) Intensity profiles as a function of angle along the membrane are then generated. For consistency, GPMVs are identical to the ones shown in Figure 1. Scale bar, 5 μm.
Figure S4. The percentage of phase separated vesicles in the DiD channel is systematically overestimated in wide field images due to the under-detection of DiD-labeled vesicles in the data analysis pipeline. GPMVs derived from HeLa cells were labeled with NBD-DSPE and DiD and imaged via (a) widefield microscopy or (b) spinning disc confocal microscopy. Images are from the same plate of GPMVs. High internal fluorescence intensity of red channel seen in the wide field images (white arrows) is not present in the confocal images. Scale bar, 50 µm. (c) Confocal and widefield images were analyzed in VesA. VesA detects more usable vesicles in the red channel in confocal images. (d) VesA overestimates the percentage of phase separated vesicles in DiD-labeled widefield images. This overestimation is eliminated when confocal images are analyzed. Data are shown for 9 fields of GPMVs.
Figure S5. TLCK and C6-ceramide alter $P_{\text{ordered}}$ for NBD-DsPE in a dose-dependent manner. Data from the same experiments shown in Figure 2 were plotted to show the dependence of $P_{\text{ordered}}$ for NBD-DsPE on the concentration of TLCK (blue circles) or C6-ceramide (red squares). Data were normalized to reflect relative changes and represent the mean ± SD from 2 independent experiments run in duplicate.
Figure S6. DMSO has no observable effect on membrane phase behavior over the range of concentrations used in this study. GPMVs derived from HeLa cells were incubated with the indicated concentration of DMSO prior to imaging at RT. Plots shows the percentage of phase separated vesicles (black circles) and P_{ordered} for NBD-DSPE (magenta squares) as a function of DMSO concentration. Each datapoint represents the mean ± SD. Data are from a single experiment performed in triplicate.
Figure S7. Representative temperature scan illustrating the effect of TLCK on $T_{misc}$. HeLa cell-derived GPMVs were incubated with 10 μM TLCK (magenta) or an identical volume of DMSO (black) and imaged using a confocal microscope as a function of temperature. The percentage of phase separated GPMVs at each temperature was quantified using high content imaging software. Data are shown for one of two independent experiments for TLCK. Solid lines show Boltzmann fits to the data. $\Delta T_{misc}$ was defined as the difference in $T_{misc}$ (i.e. the temperature at which 50% of the vesicles are phase separated) for the control and treated samples.
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Figure S13. Overview of approaches used to correct for positional effects and calculate Z-scores. (a) Representative example of positional effects. The percentage of phase separated vesicles per well is plotted as a function of the order in which they were imaged in a representative high throughput screen. Each datapoint (black circle) reports on the total number phase separated vesicles in a single well. Vertical dotted lines demarcate the beginning and end of each 96-well plate. The blue lines are the trendlines fit to the data for each plate. (b) Plot of the relative change in the percentage of phase separated vesicles per well, obtained by normalizing the data in panel a by the fitted trendline to correct for positional effects. (c) Data from plate 1 of panel b, replotted as a histogram. Outliers corresponding to wells with very high (magenta) or very low (green) percentage of phase separated vesicles are highlighted. (d) Representative plot of z-score as a function of well number for a single plate (plate 1 in panel b). Z-scores were calculated on a per-plate basis as described in the Materials and Methods. The dotted horizontal lines mark the position of Z = |2|. Compounds with a Z score > |2| are highlighted in magenta and green.
VesA
Quick user guide and demos

For software described in

High-content imaging platform to discover chemical modulators of plasma membrane rafts

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VesA installation guide and requirements

Operating system and software requirements

- VesA has been tested on multiple operating systems including Windows 10 Enterprise, Linux, CentOS 7, and macOS Catalina.
- Required software: MATLAB
  - Required MATLAB plugins:
    - 'Image Processing Toolbox'
    - 'Curve Fitting Toolbox'
    - 'Parallel Computing Toolbox'
- Typical installation time (assuming MATLAB has already been installed) is less than 1 min.

Installation instructions:

1. Copy the zipped “VesA” folder and “DemoSingleImage” (demo dataset) folder onto your desktop.
2. Unzip both folders.
3. Locate the VesA.m file in the “Software sourcecode” folder.
4. Double click on VesA.m. This will automatically launch MATLAB and load the VesA code.
5. To start the software, click on the "Run" button in MATLAB (highlighted below). This will launch the VesA GUI.

Troubleshooting:

You may receive the following message when you first launch VesA in MATLAB. If you do, select “Change Folder”.
MATLAB cannot run this file because /Users/.../sourcecode/ToPublic12012020/VesA.m shadows it in the current working folder.

To run this file, you must change the MATLAB current folder.
Preface

- VesA is designed to analyze single vesicles, populations of vesicles within a single image, stacks consisting of multiple images (“well”), or sets of image stacks (“plate”).
- A wide range of image types can be analyzed, including microscope-specific formats.
- Information from image headers is automatically loaded.
- Images can include up to three channels; for demonstration purposes, two-channel images are used.
- VesA automatically displays channel 1 as red, channel 2 as green, and channel 3 as blue.
- The steps required to analyze images are organized under series of tabs. Starting from left to right, they include:

  “Database” => “Detect Vesicles” => “Analyze Vesicles” => “Results Vesicles” => “Plates Summary”

- The final tab, “Configuration”, allows for saving or loading previously saved parameters for data analysis.

- Three demo datasets are available. They consist of real experimental data containing GPMVs labeled with red and green dyes, collected under conditions identical to that analyzed in the manuscript:
  - A single image (“DemoSingleImage” folder, provided with software.)
  - A stack of images (“DemoStackImages” folder, available upon request.)
  - A demo dataset consisting of a series of image stacks collected from different wells in a multiwell plate from a dose-response experiment (“demo/DemoSetOfStacks”, available upon request).
- Expected results for each demo dataset are included in the “Expected results” folder and consist of sets of tables and a screenshot from the “Results vesicles” tab.
- Instructions on how to analyze the demo datasets are provided in italics at the bottom of the description of the functionality of each tab in the VesA software.
- The software uses three methods to calculate “partition coefficients.” Two of these methods (distribution method and colocalization method) are described in more depth in accompanying paper. A third method, termed the threshold method, is also available for use in the software at the user’s discretion.
- For simplicity, the term “partition coefficient” is used in this manual and software to describe the preference of markers for the ordered versus disordered phases. For the case of the distribution analysis, this corresponds to the “phase preference coefficient” or “Pordered” described in the accompanying manuscript.
1. **Launching VesA**
   - Please see accompanying “Read Me” file for VesA installation guide and requirements
   - When starting VesA, a window will pop up enabling several different preset parameters to be chosen.
   - The “Demos” setting will load preset parameters to analyze the demo images.
   - “HTS” will load the settings used to analyze HTS data collected from 96-well plates using the Operetta microscope for the associated manuscript.
   - “Temperature” loads settings used to collect data from the temperature scans collected using a Zeiss 880 reported in the associated manuscript.
   - The “Free” setting enables the user to freely define parameters used for data analysis.

**Demo:**
*For demo1, demo2, or demo3 choose “demo”.*
2. Database tab

- Using the “database” tab, images are loaded for analysis by selecting the “Load folder” (2a) or “Load files” (2b) buttons.
- “Load plate” (2c) can be used to load stacked images, but requires a setting in the configuration tab, in particular the definition of free parameters in the image filenames, by regular expressions.
- Once loaded, the image will be shown on the upper right hand corner of the window (2d).
- The user can select which image is shown, and what information is shown on the image using the check boxes directly underneath the image (2e) in the stack (plate) modus. In the standard modus the user can click on the smaller thumbnail images on the left (in demo 1 and 2 there is only one image). The numbers in the information box also depend on the method chosen in the “Results vesicles” tab. For the demos they are fixed.
- Loading large images for the very first time may require some time. Since thumbnails are created during the first loading, subsequent loading of those images will be much faster.
- If the image was analyzed in a former session, some statistical information will be shown in the lower right corner of the window (2f). This was mainly used for software development purposes.

Demo:
For demo1, select “Load Folder” (2a) and then select the “DemoSingleImage” folder. This dataset consists of a single image.
For demo2, select “Load plate” (2c) and then select the “DemoStackImages” folder. This dataset consists of a single stack with 16 images. The first image is automatically previewed. Other images in the stack can be selected using the box in 2e.
For demo3, select "Load plate" (2c) and choose the "DemoSetOfStacks" folder (not the timepoint_1). Stacks can be switched between by left clicking on the preview thumbnails in "database". Other images in a stack can be selecting using the box in 2e.
3. Detect Vesicle tab

On the “Detect Vesicle” tab, parameters used to detect the vesicles can be optimized and the contour/membrane intensity profiles can be visualized.

- First, vesicles need to be detected using the “Pre-Detect” button (3a). This performs a Hough transform of the superpositioned green and red channels. (https://www.mathworks.com/help/images/ref/imfindcircles.html)
  1. \( R_{\text{min}} \) and \( R_{\text{max}} \) (units of pixels) must be set manually according to the size of the vesicles in the image.
  2. The “auto” setting for sensitivity works for well in many instances and can be used in initial testing. This parameter can also be manually adjusted if desired, for example if not enough vesicles are detected.

- The “Pre-Selection” function (3b) performs a Hough transform of the green channel and red channels individually.
  1. Using the dropdown menu and checkboxes, this step enables the user to define whether the vesicles to be analyzed are required to contain signal in both the green and red channel (“all”) or any channel, i.e. green or red (“any”).
  2. Pre-selection is not necessary for initial, simple tests, and can be skipped by selecting “none” in the dropdown menu under “needed channels”.

- Next, the membrane/contours need to be identified (3c).
  1. As a first step, the “maximum” method should be selected, since it is much faster than the “fit” method. “/1 /2 ...” indicate how many points/angles are skipped doing the detection, and are only important for the “fit” method.
  2. Left clicking on a pre-detected vesicle in the large image will start the contour detection process. In addition to thumbnail image of the chosen vesicle (3d), the results of the contour detection will be shown graphically for each channel (3e).

- “Background distance” (3f) defines the distance from each vesicle at which background should be measured. The distance is defined either as terms of absolute number of pixels or as a percentage of the vesicle radius. Background can be measured locally for each vesicle, or globally as a mean value.

- “Filter” (3g) can be used to perform two-dimensional image Gaussian filtering. The “line filter” option is applied to the contour (angular) and the radial intensities to find the contour position (radial).

- “Constraints” (3h) can be applied to narrow the population of vesicles to be analyzed. This process is important for analyzing whole images or sets of images but not for single vesicles.

- Following the predetection and preselection steps, the identified objects can be visualized in the image (3i) by choosing the corresponding checkbox (3j). The total number of vesicles detected and those remaining after preselection are shown in the bottom right panel (3k).
Demo (applies to demo 1, 2, and 3):

- If the image has not yet been fully analyzed, the first thing to do is click on the **“Pre-detect”** button (3a).
- Selecting the “Pre-detected” checkbox (3j) below the image provides a preview of all found circular objects as white circles in the image (3i) – (re)drawing the image will take longer.
- Left clicking on any detected (white) circle in the image runs the full contour/membrane detection.
- Clicking on “Pre-Select” (3b) runs the full contour detection for each detected circle. Results can be visualized by the “Pre-selected” checkbox (3j) below the image as red/green circles in the image (3i).
- In demo mode, all settings have been pre-set and cannot be changed.
4. Analyze Vesicle tab

- On the “Analyze vesicle tab,” individual vesicles can be fully analyzed by left clicking on the vesicle in the large image on the right. The selected vesicle will be shown in the thumbnail image.
- Results obtained from three different analysis methods (threshold, distribution, and colocalization) are shown for the chosen vesicle. They include:
  - A graphical depiction of the fluorescence intensities in Ch1 and Ch2 for the chosen vesicle for the “threshold” method (4a, 4b, 4c), “distribution” method (4d, 4e) and “colocalization” method (4f, 4g).
    - If desired, these graphs can be saved for individual vesicles.
    - Note that only results obtained using the “distribution” and “colocalization” methods are used for data analysis in the accompanying manuscript.
    - Also note that even though the thumbnail image will update for all GPMVs when they are clicked on, the graphs will only be updated for GPMVs that pass all exclusion criteria are selected.
  - The calculated partition coefficients (± SD) are also reported (4h).
    - Ch1/Ch1 reports the partition coefficient for the fluorophore in Ch1 in the phase where it is brightest, i.e. Ch1/Ch1 reports the partition coefficient of fluorophore in Ch1 relative to the position of the enriched phase defined by Ch1.
    - Ch2/Ch1 reports the partition coefficient for Ch1 in the phase where Ch2 is brightest (i.e. the opposite phase).
  - The percentage of the vesicle membrane (± SD) that is enriched in red fluorescence (Ch1) or green fluorescence (Ch2) is also provided (4i).
- An opportunity to save a summary of the numerical results in 4h and 4i for all of the vesicles in the image is provided on the next tab (“Results vesicles”).
- The table in the lower right will automatically be updated with results for the vesicle population (4j).
- It is not necessary to click the (Re)run button.
Demo (applies to demo 1, 2, and 3)

- Left mouse click on a pre-detected vesicle will run the full analysis and will show all measurements and accompanying graphs obtained for all three analysis methods.
5. Results Vesicles tab

- In the “Results vesicles” tab, a single image, “well” (stack of images) or “plate” (set of image stacks) can be automatically analyzed by clicking on “(Re) analyze” button (5a) after selecting the associated list entry (5b).
  Since all data is saved during the analysis of an image, it is not necessary to repeat the detection, preselection, and analysis steps from the previous tabs.
- Statistical analysis for the entire population of selected vesicles can be obtained by clicking on “(Re)Sum” button (5c) after choosing the associated list entry (5d), methods (5e) and channels of interest (5f). Graphs showing the results will then be shown. In this example, graphs showing the partition coefficients (5g) and percent phase separated vesicles (5h) are shown together with a plot of the size distribution of selected vesicles (5i). Results of the analysis can be saved as images or output the data in table format (5j).
- It is also possible to output a table that provides the results for individual vesicles on a vesicle-by-vesicle basis by selecting “Sav sel vesicles” (5k).

Demo 1:

- Choose “Image” in the drop down menu (5b) and then press the “(Re)Analyze” button (5a). This will start the full analysis using the settings defined on the previous tabs for the image. (Detection=>selection=>Analysis)
- To obtain statistics on all of the vesicles, press the “(Re)Sum” button (5c) after selecting “Image” in the drop down menu next to that button (5d). This will sum up statistical information of all vesicles with the chosen analysis methods (threshold, distribution, or colocalization (5e). Previews of the data will be shown as graphs (5g-5i) which can be saved as images or in tabular format.
Demo 2:

- Choose “Well” in the drop down menu (5b) and then press the “(Re)Anlyse” button (5a). This will start the full analysis with the settings defined on the previous tabs for the whole stack of images. Note: parallel computing will be used, and will require some time to be initiated for the first time the dataset is analyzed. The progress can be seen in the Matlab program.
- To obtain statistics on all of the vesicles, press the “(Re)Sum” together with “well” in the drop down menu next to that button (5d). This will sum up statistical information for all vesicles with the chosen analysis method. Previews of the data will be shown as graphs (5g-5j) which can be saved as images or in tabular format.

Both demos:

- “Save sel Vesicles” (5k) will save all information of the individual selected vesicles of the image or stack (well) of images.
- “Collate Database” (5k) will save the collated and statistically analyzed date of the image(s) in the database as a single table file. This yields a single number for each parameter, per image.

Demo 3:

- To fully analyze the whole dataset, choose “Plate” in the drop down menu (5b) next to the “(Re)Analyse” button (5a). The software will ask you to select the "plate" (set of stacks). Again choose "DemoSetOfStacks", not "timepoint_1. This will start the full analysis with the settings defined on the previous tabs for the whole stack of images. (Note: This step has been already done and data is saved.)
- To preview statistics for single images ("image") or a stack of images ("well"), press the "(Re)Sum" button (5c) and select the appropriate entry from the drop down menu (5d).
6. **Plate summary tab**

- This tab collates and statistically analyzes sets of images stacks (for example as obtained for multiwell plates).
- This information can combined with external information (such as concentration, temperature, drug treatment, etc.) in table format.
- Because this step is designed to collate data across multiple stacks of images, a single image or single image stack will be represented as a single data point when displayed on this tab.

**Demos (only applies to demo 3):**

- This tab shows statistics for all of the stacks. The demo dataset consists of 12 stacks, thus 12 points will be included in the graphs.
- The plate to be examined is already selected and displayed in the "edit" window; there's no need to "select plates" again
- "Check Plates" button is redundant for the demo too.
- "(Re)Collate" button is necessary to show or update graphs.
- "Re-calculate all data" checkbox is only needed for the first time, or when methods from which to examine statistics are changed on the "Results Vesicles" tab.
- "Save to file" checkbox is only needed if data should be saved as a table.
- Further checkboxes are explained elsewhere and not required for the demo.
7. Configuration tab

The configuration tab can be used to set internal parameters used by VesA, such as the patterns to identify external parameters in the image filenames and the cross-talk matrix for the channels.

Save/load settings:

The “Save Settings” [72] and “Load Settings” [73] buttons can be used to save or load all values in the graphical interface. It might be useful to save the settings for a full analysis for each run. VesA also retains all settings when the program is closed and restores them when it is started again.

“Collate Files/Wells” frame – filename patterns:

In the “Parameter(s)” box [63] a filename pattern can be set that identifies up to two free parameters used to identify a “well” belongings. Here, the free parameters are defined in curly brackets {}. The patterns are in the style of regular expressions:

Depending on whether the parameter are capital letters, small letters or numbers the notations are {A}, {a} or {0} with any repetitions, for example a three-digit number would be defined as {000}, or a combination of capital letters would be {AA}. A mix of numbers and letters as a parameter is not allowed.
(yet)! Also, the use of underscores or decimal points are not supported (yet)! The number of digits or letters defining the parameter (not the filename itself) must be the same for all images. (a variable length of numbers will be implemented soon).

An asterisk “*” represents any expression with a variable length. In case there is no asterisk at the beginning or end of the pattern, the filename must begin, or end respectively, with the given expression.

Examples:

- “*_{A}{00}_s*”:
  Would identify “crc-1234_B12_s_w1.png” as “B” for the first and “12” for the second parameter.
  It would not identify “crc-1234_B1_s.png”, “crc-1234_d12_s.png”, “crc-1234_CC12_s.png”

- “T{000}C*.lsm”:
  Would identify “T037CtestImage.lsm” or “T037C_testImage.lsm” as “037” for the first and only free parameter.
  It would not identify “ImageT037Ctest_Image.lsm”, or “T37CtestImage.lsm”, or “T037CtestImage.png”, or “T37_0CtestImage.lsm”

In the “Combine Channels” box [64] a pattern for filenames to combine channel separated image files can be defined. It follows similar rules as for the “Parameter(s)” patterns.

Examples:

- “*_w{1}.tif”:
  Would identify file “crc-1234_B12_s_w1.png” as channel 1 and “crc-1234_B12_s_w2.png” as channel 2.
  It would not identify “crc-1234_B12_s_w01.png”, or “crc-1234_B12_s_w1_a.png”

In the “Plate name pattern” box [65] an extraction of a certain plate name pattern of the folder containing the plate/images can be defined. Next to that box preview will be shown after the “test” button [68] is used.

Here, no asterisks “*” are allowed (yet)!

The content in the curly brackets {} define the plate name. It can be any combination of capital letters “A”, small letters “a”, numbers with of defined digits “0” or numbers with a variable length “d”.

Examples:

- “_{00000AAAd}_plate”:
  Would set “12345ABC15” as the plate name of folder “test-12345ABC15_plate12ab” or “12345CBA1” of folder “test-12345CBA1_plate”.
• It would not identify “test-1234ABC15_plate12ab”, “test_12345ABC15_plate12ab”, or “test-12345ABC15_images”

If the box is left blank, or the pattern could not be found in the folder name the whole folder name will considered as a plate name.

The “Image folder” [66] and “Result folder” [67] subfolders can be defined where VesA is looking for images and saves all internal tables, respectively. In case the result folder does not exist, it will be generated automatically. Both boxes can be kept empty. In that case VesA won’t look for image subfolders and will save the tables aside the image files.
8. Troubleshooting

If the demo datasets do not yield the expected results:

0. Manually input the settings.

Or

1. Manually load the settings.
   - Go to the "configuration" tab.
   - Click on "load settings" (Mid bottom)
   - Load "DemoSettings.mat" in the "presets" folder.
9. **Output csv File Headers**

*Note: when running in Free mode selecting “Any” in the Results Vesicles tab will result in data that are calculated from vesicles that have signal in any of the selected channels (i.e. they could contain red signal OR green signal). These files headers will contain “Ch1 w/Ch1” or “Ch2 w/Ch2”. Selecting “All” will refine the results and provide data from vesicles which have all of the selected channels (i.e., only vesicles that are both green and red will be included in the analysis). These file headers will contain “w/all channels”.*

| First Row Header | Second Row Header | Explanation |
|------------------|-------------------|-------------|
| Plate            | Plate             | Plate name from input files selected in Database tab |
| Well             |                   | Alpha-numeric well designations |
| Vesicle detection | Nr of found vesicles | Total number of vesicles detected |
| Vesicle detection | Nr of used vesicles w/ all channels | Total number of vesicles that are used for measuring membrane partition function (see note above for Any vs. All Channels) |
| Radial max; Circular refine | Mean radius of all channels (px) | Mean radius of the vesicles in pixels |
| Radial max; Circular refine | Mean radius of all channels STD (px) | Standard deviation of above |
| Vesicle detection | Nr of used vesicles w/ all channels | Total number of vesicles that are used for measuring membrane partition function |
| Radial max; Circular refine | Mean radius of all channels (px) | Mean radius of the vesicles (repeat) |
| Radial max; Circular refine | Mean radius of all channels STD (px) | Standard deviation of above (repeat) |
| Vesicle detection | Nr of used vesicles w/ all channels | Total number of vesicles that are used for measuring membrane partition function |
|                  | Nr of phase separated vesicles Ch1 w/ all channels | Number of vesicles that are phase separated |
| Distribution method w/ max val. of double Gauss fit | Fraction of phase separated vesicles Ch1 (%) | Number of vesicles that are phase separated/Total number of used vesicles |
| Distribution method w/ max val. of double Gauss fit | Mean Phase Sep. of separated vesicles Ch1 w/ all channels (%) | Average percentage of membrane in phase separated vesicle that is covered in a lipid preference |
| Distribution method w/ max val. of double Gauss fit | Mean Phase Sep. of separated vesicles Ch1 w/ all channels STD (%) | Std Dev of above |
| Distribution method w/ max val. of double Gauss fit | Mean Phase Sep. of all vesicles Ch1 w/ all channels (%) | Average percentage of membrane in all vesicles that is covered in a lipid preference |
| Distribution method w/ max val. of double Gauss fit | Mean Phase Sep. of all vesicles Ch1 w/ all channels STD (%) | Std Dev of above |
| Distribution method w/ max val. of double Gauss fit | Nr of phase separated vesicles Ch2 w/ all channels | Number of vesicles that are phase separated using marker 2 |
| Distribution method w/ max val. of double Gauss fit | Fraction of phase separated vesicles Ch2 (%) | Fraction phase separated vesicle |
| Distribution method w/ max val. of double Gauss fit | Mean Phase Sep. of separated vesicles Ch2 w/ all channels (%) | Average percentage of membrane in phase separated vesicle that is covered by marker 2 |
| Distribution method w/ max val. of double Gauss fit | Mean Phase Sep. of separated vesicles Ch2 w/ all channels STD (%) | Std Dev of above |
| Distribution method w/ max val. of double Gauss fit | Mean Phase Sep. of all vesicles Ch2 w/ all channels (%) | Average percentage of membrane in all vesicles that is covered by marker 2 |
| Distribution method w/ max val. of double Gauss fit | Mean Phase Sep. of all vesicles Ch2 w/ all channels STD (%) | Std Dev of above |
| Distribution method w/ max val. of double Gauss fit | Partition coefficient of Ch1 w/ all channels | Partition coefficient of channel 1 marker in channel 2 (Ch1/Ch2) from distribution method = 1 – Part. Coeff. of Ch1/Ch1 (as reported in Analyze Vesicles tab) |
| Distribution method w/ max val. of single Gauss fit | Partition coefficient of Ch1 w/ all channels STD | Std Dev of above |
| Distribution method w/ max val. of single Gauss fit | Partition coefficient of Ch2 w/ all channels | Partition coefficient of channel 2 marker in channel 2 (Ch2/Ch2) from distribution method = Part. Coeff. of Ch2/Ch2 (as reported in Analyze Vesicles tab) |
| Distribution method w/ max val. of single Gauss fit | Partition coefficient of Ch2 w/ all channels STD | Std Dev of above |
| Contour detection | Noise/Signal Ch1 w/ all channels (%) | Signal to noise ratios average for marker 1 |
| Contour detection | Noise/Signal Ch1 w/ all channels STD (%) | Std Dev of above |
| Contour detection | Intravesicular Intensity Ch1 w/ all channels (abs) | What is the fluorescence intensity inside vesicle using marker 1 |
| Contour detection | Intravesicular Intensity Ch1 w/ all channels STD (abs) | Std Dev of above |
| Radial fits | Detected fraction of membrane Ch1 /w all channels (%) | empty |
| Radial fits | Detected fraction of membrane Ch1 /w all channels STD (%) | empty |
| Contour detection | Membrane intensity Ch1 /w all channels (%) | Average Membrane intensity defining cutoff for profile using marker 1 |
| Contour detection | Membrane intensity Ch1 /w all channels STD (%) | Std Dev of above |
| Radial fits | Membrane thickness Ch1 /w all channels (px) | empty |
| Radial fits | Membrane thickness Ch1 /w all channels STD (px) | empty |
| Contour detection | Intravesicular/Membrane intensity Ch1 /w all channels (%) | empty |
|--------------------|--------------------------------------------------------|-------|
| Contour detection | Intravesicular/Membrane intensity Ch1 /w all channels STD (%) | empty |
| Contour detection | Noise/Signal Ch2 w/ all channels (%) | Signal to noise ratios average for marker 2 |
| Contour detection | Noise/Signal Ch2 w/ all channels STD (%) | Std Dev of above |
| Contour detection | Intravesicular Intensity Ch2 w/ all channels (abs) | Internal fluorescence intensity inside vesicle using marker 2 |
| Contour detection | Intravesicular Intensity Ch2 w/ all channels STD (abs) | Std Dev of above |
| Radial fits        | Detected fraction of membrane Ch2 /w all channels (%) | empty |
| Radial fits        | Detected fraction of membrane Ch2 /w all channels STD (%) | empty |
| Contour detection | Membrane intensity Ch2 /w all channels (%) | Average Membrane intensity defining cutoff for profile using marker 2 |
| Contour detection | Membrane intensity Ch2 /w all channels STD (%) | Std Dev of above |
| Radial fits        | Membrane thickness Ch2 /w all channels (px) | empty |
| Radial fits        | Membrane thickness Ch2 /w all channels STD (px) | empty |
| Contour detection | Intravesicular/Membrane intensity Ch2 /w all channels (%) | empty |
| Contour detection | Intravesicular/Membrane intensity Ch2 /w all channels STD (%) | empty |
| PIRow              | Well letter – useful for sorting data |
| PICol              | Column number – useful for sorting data |