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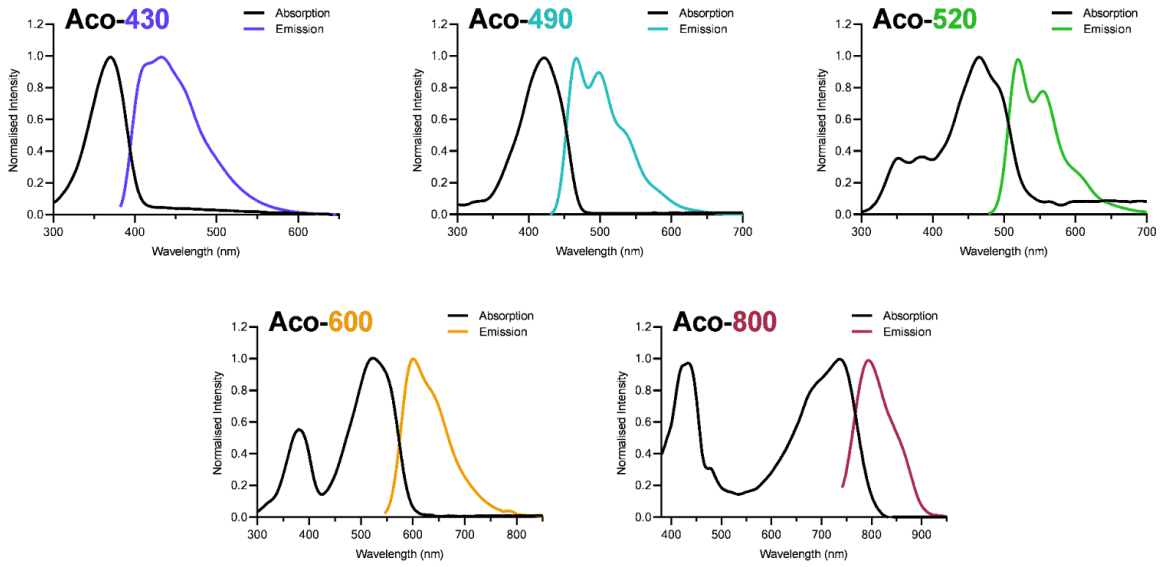
Technical Datasheet

Aco-Dyes™ for Membrane Labelling

Introduction

Acoerela's **Aco-Dyes** are a family of conjugated oligoelectrolytes (COEs) that intercalate into lipid bilayers. These **lipophilic** dyes fully embed themselves across the lipid bilayer of their target membranes, minimising dye dissociation or "leakage". Aco-Dyes are **water-soluble** and do not form micelles, thereby reducing the risk of false positive signals. They are also **fluorogenic**, exhibiting maximum fluorescence only upon intercalation into a lipid bilayer.

Acoerela also offers a range of **Reference Liposomes, AcoRL™,** designed as positive and negative controls for fluorescence-based analyses of nanoparticles such as extracellular vesicles (EVs). These include pre-stained fluorescent liposomes and unstained liposomes, which may be stained alongside biological samples to provide a reliable reference.



Absorption (UV-Vis Spectrometer) and Emission (Fluorometer) spectra were obtained for the Aco-Dyes in SUVs.

Product	Aco-430™	Aco-490™	Aco-520™	Aco-600™	Aco-800™
Appearance	Colourless	Yellow	Orange	Purple	Green
$\lambda_{abs\ (SUV)}$ (nm)	369	422	465	525	735
$\lambda_{em\ (SUV)}$ (nm)	403 - 460	458 - 508	511-530	586 - 635	775 - 818
Recommended Lasers	UV (355 nm)	Violet (405 nm)	Blue (488 nm)	Blue / Yellow (488 / 561 nm)	Red (638 nm)
Quantum Yield (%)	94	60	76	27	13.8
Fluorescence Lifetime (ns)	1.1	0.9	1.3	1.9	1.6
IC_{50} (μM)	>256	>256	>128	170	>272

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Storage Conditions

Dye State	Storage	Shelf Life
Unopened dry dyes	2 to 8°C, protected from light	Refer to expiry date on vial
Reconstituted dyes	2 to 8°C, protected from light	Up to 1 month
Frozen reconstituted dyes	-80°C, protected from light	Up to 3 months, avoid freeze-thaw cycles

Dye Reconstitution

Ensure that your fluorescence-based instruments are equipped with the appropriate lasers and detectors for the chosen Aco-Dye(s) and/or AcoRL(s) before proceeding with your experiment.

1. Allow all buffers and reagents to come to room temperature before use.
2. To get a 25 µM stock solution, reconstitute the dried dye in 100 µL of filtered 1X PBS or water.
3. Vortex, then perform a quick spin to collect the dye stock solution at the bottom of the tube.
4. Sonicate the dye stock solution at 40°C for 15 minutes.

   Sonication is highly recommended *to ensure complete dissolution. If a sonicator is not available, incubate the dye stock solution at room temperature for 15 minutes to allow the dye to solubilise.*
5. Repeat Step 3.
6. Aco-Dyes perform best when freshly reconstituted. While reconstituted dyes can be stored for up to 1 month at 2 to 8°C, perform Steps 3 to 5 prior to using previously reconstituted dyes.

EV Sample Preparation

7. Dilute EV to 1e10 particles/mL using a filtered buffer of your choice.
8. Dilute a suitable volume of 25 µM stock solution to 10 µM in a clean Eppendorf tube, using the same filtered buffer. *(e.g. 6 µL of 25 µM dye stock + 9 µL of filtered buffer)*
9. Incubate 1e10 particles/mL diluted EV with 1 µM* final dye concentration for 1 hour at 37°C, protected from light. *(e.g. 5 µL of 10 µM diluted dye solution + 45 µL of 1e10 particles/mL EV)*

   If an incubator is not available, incubate the stained samples at room temperature.

   It is essential to* ***optimise the staining concentration for your EV type, dye and instrument* *by performing a titration. Suggested final dye concentrations are 0.1 µM, 0.5 µM, 1 µM, 1.5 uM, 2 uM.*
10. Include the corresponding controls to identify background signals:
    - Dye Only *(e.g. 5 µL of 10 µM diluted dye solution + 45 µL of filtered buffer)*
    - EV Only *(e.g. 5 µL of filtered buffer + 45 µL of 1e10 particles/mL EV)*
    - Buffer Only

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11. **For Flow Cytometry:**

    a. As a starting point, dilute stained EV sample 100X using a filtered buffer of your choice.

    b. Acquire the 100X diluted sample and collect data as per instrument's user guide.

    c. Optimise the dilution of the stained EV sample to achieve instrument manufacturer's recommended nanoparticle acquisition parameters (e.g. abort rate, event count).

    Each flow cytometer requires an optimal event rate to avoid:

    a) Swarming from overly concentrated samples,
    b) Very weak signals from excessively diluted ones.

    *E.g. The recommended event rate for stained EV samples on the **CytoFLEX nano** is 1,000-1,500 events/second, the dilution factor to achieve this should be optimised for specific sample types.*

    d. Apply this optimised dilution factor uniformly to all related samples, including controls.

    e. Use the same instrument settings (gain, threshold and width) and acquisition time across all samples to ensure reliable comparisons.

12. **For Plate Reader:** Prepare samples with reaction volume 100 µL per well in a 96-well polystyrene non-tissue culture treated plate (clear flat bottom), avoiding the outer wells of the plate. After incubation, measure the full fluorescence spectrum or fluorescence at peak maxima.

Guidelines for EV Labelling with Aco-Dyes

- Filter all buffers through a 0.2 µm filter before use.
- Sonicate, vortex and spin down the Aco-Dyes (Steps 3 to 5) before every use to ensure low background in Dye Only controls.
- EVs perform best when used fresh after isolation or preparation. Make a fresh 1e10 particles/mL dilution on each test day, as particle counts tend to drop over time.
- Each Aco-Dye has its own unique brightness and staining efficiency. Titrate and optimise the labelling concentration for each dye, EV type, study condition and instrument.
- To improve staining efficiency, it may be helpful to incubate the EV sample with the dye for a longer duration (e.g. 2 hours - overnight).
- Consider purifying the EV sample using Size Exclusion Chromatography (SEC) to remove any residual particles, such as cryoprotectants, saccharides or remnants from the cell culture medium that might interfere with dye binding.

Technical Support

FAQs and relevant publications can be found on the Support page of our website www.acoerela.com. Feel free to reach out to us at hello@acoerela.com for technical support and troubleshooting.

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Data and Figures

Figure 1. PC-3 Exosomes (Abcam Cat#: ab239689) were stained with **Aco-490** and analysed on the **Amnis® ImageStream®X Mk II Imaging Flow Cytometer**. All lasers were set to maximum power and data was acquired using 60X magnification, a 7 µm core size and low flow rate. **Aco-490**-stained PC-3 Exosomes were excited using a 405 nm violet laser and emission was collected on Channel 2 (480-560 nm filter).

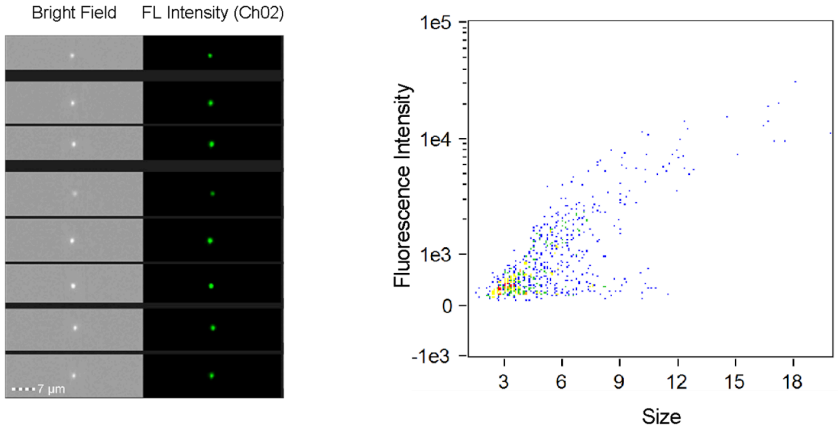

Figure 2. Liposomes of different sizes (50 nm, 100 nm, 200 nm, 400 nm) were stained with **Aco-490** and analysed on the **Amnis® ImageStream®X Mk II Imaging Flow Cytometer**. All lasers were set to maximum power and data was acquired using 60X magnification, a 7 µm core size and low flow rate. **Aco-490**-stained liposomes were excited using a 405 nm violet laser and emission was collected on Channel 2 (480-560 nm filter).

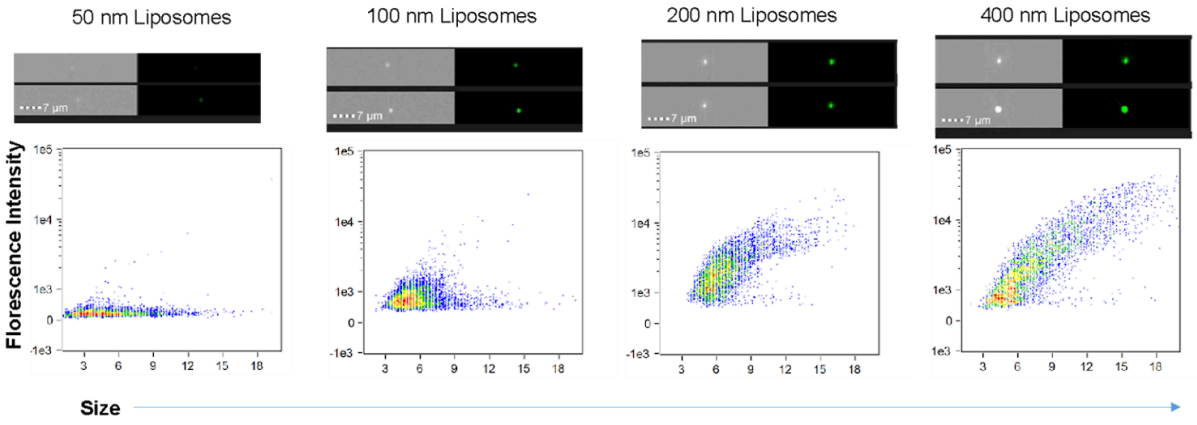

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Figure 3. Red Blood Cell-derived Extracellular Vesicles (RBCEV) and AcoRL-Neg (unstained) liposomes were both diluted to 1e10 particles/mL and stained with **Aco-490** (0.5 µM), **Aco-520** (0.5 µM) or **Aco-600** (1 µM) for 1 hour at 37°C. Stained samples and controls were acquired on the **Beckman Coulter CytoFLEX LX Flow Cytometer** configured with VSSC, at slow flow rate. The samples were collected at an event rate of 2,000-10,000 events/µL.

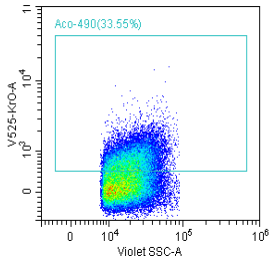
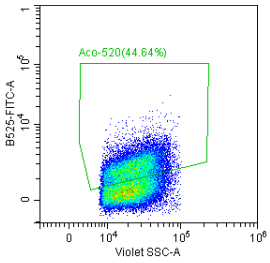
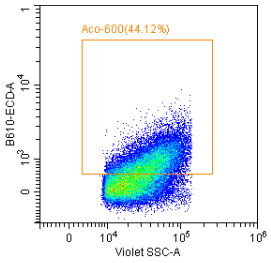
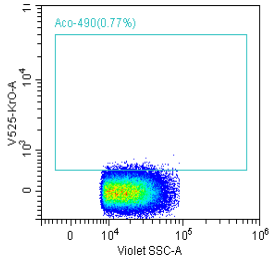
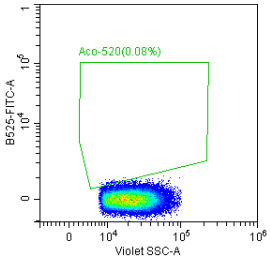
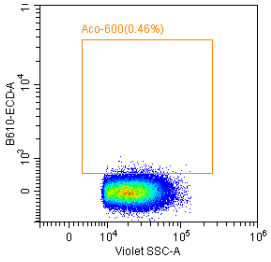
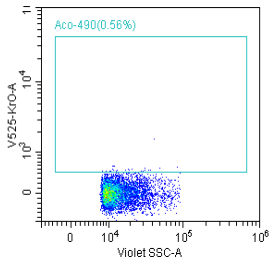
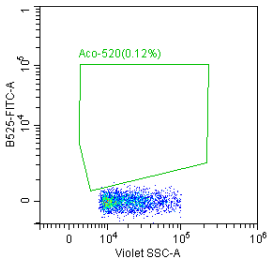
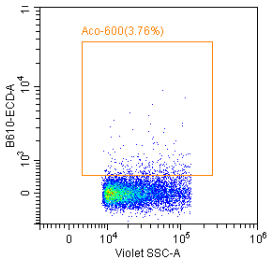
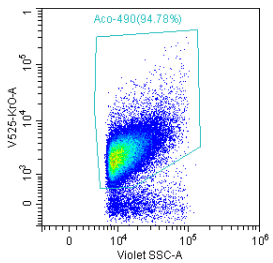
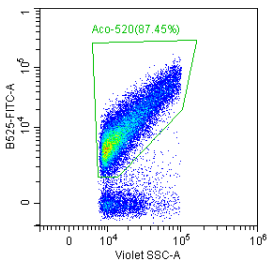
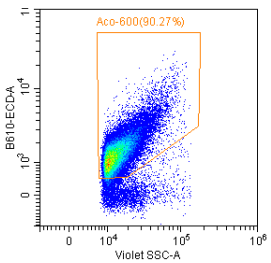
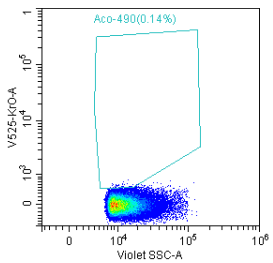
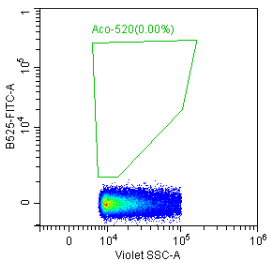
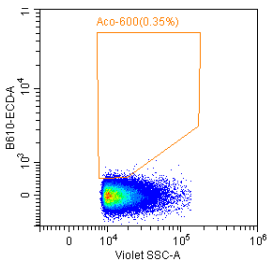
Aco-Dye	**Aco-490 (0.5 µM)**	**Aco-520 (0.5 µM)**	**Aco-600 (1 µM)**
Detector	**V525 Channel** *Laser: Violet (405 nm)* *Filter: 525/40 nm*	**B525 Channel** *Laser: Blue (488 nm)* *Filter: 525/40 nm*	**B610 Channel** *Laser: Blue (488 nm)* *Filter: 610/20 nm*
RBCEV + Aco-Dye			
RBCEV Only			
Aco-Dye Only			
AcoRL-Neg + Aco-Dye			
AcoRL-Neg Only			

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Figure 4. Red Blood Cell-derived Extracellular Vesicles (RBCEV) and AcoRL-Neg (unstained) liposomes, both diluted to 1e10 particles/mL, were stained with **Aco-520** (0.1 µM), **Aco-600** (0.2 µM) or **Aco-800** (0.2 µM) for 1 hour at 37°C. Stained samples and controls were acquired on the **NanoFCM Flow NanoAnalyzer** at an event rate of 2,000-10,000 events/µL. ***NOTE:*** *To combat stickiness of samples,* ***1X TAE buffer*** *can be used in place of 1X PBS for diluting samples before acquisition.*

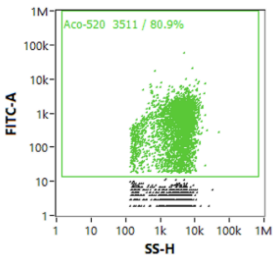
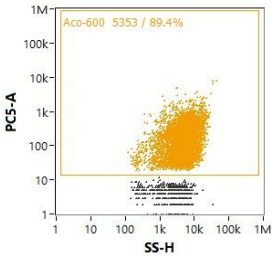
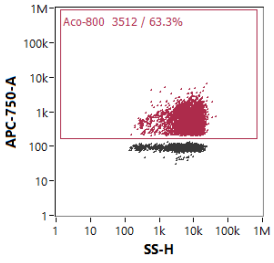
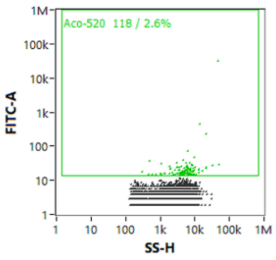
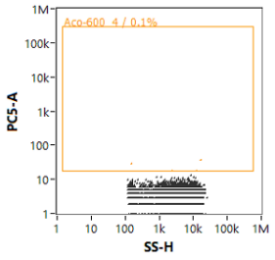
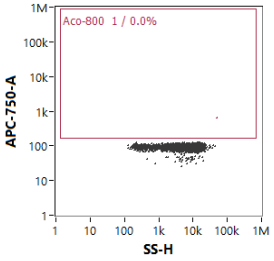
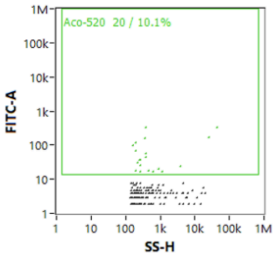
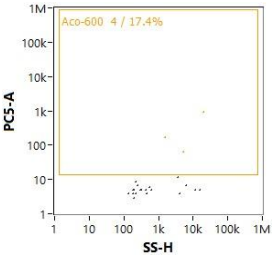
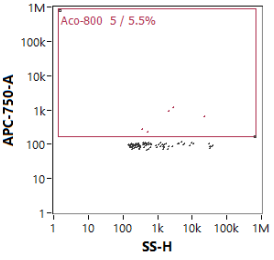
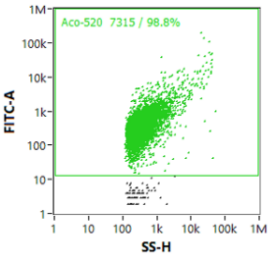
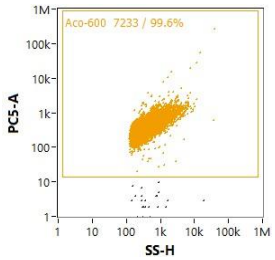
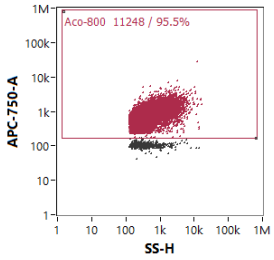
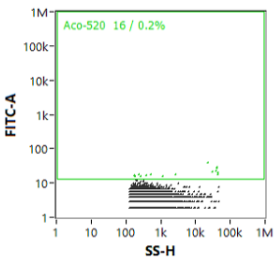
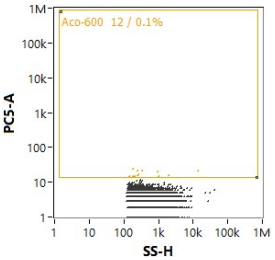
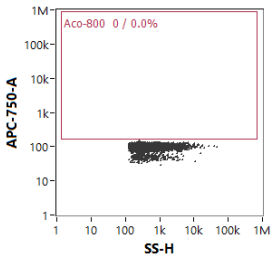
Aco-Dye	**Aco-520 (0.1 µM)**	**Aco-600 (0.2 µM)**	**Aco-800 (0.2 µM)**
Detector	**FITC Channel** *Laser: Blue (488 nm)* *Filter: 525/40 nm*	**PC5 Channel** *Laser: Blue (488 nm)* *Filter: 613/18 nm*	**APC750 Channel** *Laser: Red (640 nm)* *Filter: 783/56 nm*
RBCEV + Aco-Dye			
RBCEV Only			
Aco-Dye Only			
AcoRL-Neg + Aco-Dye			
AcoRL-Neg Only			

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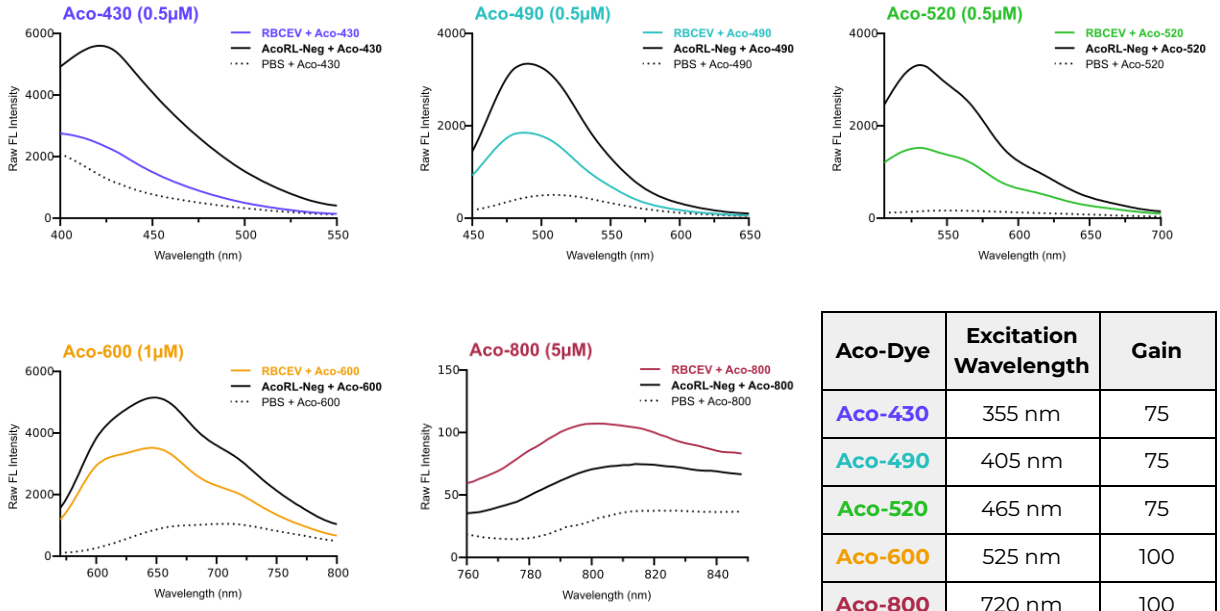
Figure 5. Red Blood Cell-derived Extracellular Vesicles (RBCEV) and AcoRL-Neg (unstained) liposomes were both diluted to 1e10 particles/mL and stained using **Aco-430** (0.5 µM), **Aco-490** (0.5 µM), **Aco-520** (0.5 µM), **Aco-600** (1 µM) or **Aco-800** (5 µM) for 1 hour at 37°C. The fluorescence emission was measured on the **Tecan Spark® Multimode Microplate Reader** with instrument settings as in the table below.



Aco-Dye	Excitation Wavelength	Gain
Aco-430	355 nm	75
Aco-490	405 nm	75
Aco-520	465 nm	75
Aco-600	525 nm	100
Aco-800	720 nm	100