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Protocol for EV Uptake Experiments using AcoDyes™

List of Products	Storage Conditions
Aco-430™ Membrane Labelling Dye (100 Tests Size) **Aco-490™ Membrane Labelling Dye (100 Tests Size)** **Aco-520™ Membrane Labelling Dye (100 Tests Size)** **Aco-600™ Membrane Labelling Dye (100 Tests Size)** **Aco-800™ Membrane Labelling Dye (100 Tests Size)**	Solubilised dyes can be stored for up to 1 month at **2–8°C**. Dry dyes are stable for at least 1 year at **2–8°C**.

1. Prepare cell culture samples 24 hours prior to treatment in the imaging chamber of choice.
2. Allow all buffers and reagents to come to room temperature before use.
3. To get a **25 µM stock solution**, reconstitute the dried dye in **100 µL of an aqueous buffer** of your choice (e.g. sterile, filtered PBS).
4. Vortex the dye for 1 minute to fully dissolve the solid, then do a quick spin to collect the dye at the bottom of the tube.
5. Incubate the dye for 15 minutes at RTP# to allow the dye to solubilise.
6. Repeat Step 4.
7. Dyes perform best when freshly reconstituted. Solubilised dyes can be stored for up to 1 month at 4°C. However, dyes should be allowed to equilibrate to RTP# before each use.
8. Aliquot a suitable volume of 25 µM stock solution into a clean Eppendorf tube and **dilute to 10 µM** using the same aqueous buffer.
9. Dilute EV sample to approximately **10^{10} particles/mL** using the same aqueous buffer.
10. Prepare the samples according to Table 1 on the next page, mixing thoroughly.
    a. Ensure that the **ratio of dye particles to EV particles** remains constant if you plan to deviate from the suggested volumes in Table 1.
    b. *AcoDyes™ are provided in vials of 25 µM stock concentration (post-reconstitution). We recommend **optimising staining for your sample prior to conducting EV uptake** by diluting the dye stock in aqueous buffer and adding it directly to your sample (suggested final dye dilutions are 0.1 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM). Incubate the sample for 1 hour at RTP immediately after the addition of the dye.*

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Table 1. Suggested EV sample preparation protocol.

Samples to Run	Final Dye Conc (µM)	Dye Solution		Volume of PBS (µL)	Volume of 10^{10}/mL EV (µL)	Incubation
		Diluted Stock Conc (µM)	Volume to Add (µL)			
EV + Aco-430™ Aco-490™ Aco-520™ Aco-600™ Aco-800™	**1.0**	10	5	0	45	1 hour at RTP, protected from light
EV Only	**-**	-	-	5		
Dye Only	**1.0**	10	5	45	-	

11. Remove excess dye from the stained samples using any one of the following methods:
    a. Perform ultrafiltration using the Vesi-SEC micro: Size Exclusion Chromatography spin-columns.
        i. Add 300 µL filtered PBS into the gel column and centrifuge at 1,000 × g for 1.5 minutes.
        ii. Add up to 100 µL of the stained samples into the gel column.
        iii. Centrifuge at 1,000 × g for 1.5 minutes and collect the flowthrough in a separate microcentrifuge tube.
    b. Perform ultrafiltration using the Amicon® Ultra-0.5 Centrifugal Filter Unit (100 KDa cutoff).
        i. Add stained samples into the filter unit and centrifuge at 3,000 × g for 10 minutes.
        ii. Wash the filter twice by adding 400 µL filtered PBS and spinning again.
        iii. Invert the filter and perform a recovery spin at 1,000 × g (collect ~15 µL per sample).
    c. As an alternative to ultrafiltration, perform overnight dialysis at 4°C using a 10 KDa cutoff.
12. Verify particle/mL concentration of the stained EV samples e.g. by flow cytometry or dynamic light scattering (DLS).
13. Resuspend stained EV samples in exosome-free cell culture media.
14. Aspirate cell culture media from cells to be treated and replace with the prepared media containing the stained EV samples.
15. Image as desired.

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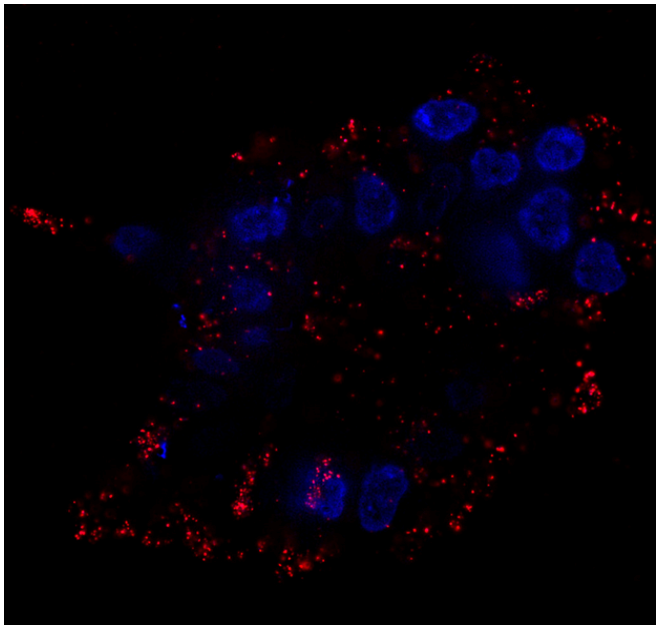

Figure 1. *Fluorescence micrograph of HEK-293T cells treated with 20 µg/mL (~10^9 particles/mL) of PC-3 EV stained with* ***Aco-600™*** *(red) after 24 hours of incubation. Nuclei stained with Invitrogen™ SYTO™ Deep Red Nucleic Acid Stain (blue). Image taken on Leica THUNDER Imager Fluorescence Microscope and processed using the built-in THUNDER computational clearing method and ImageJ.*

Guidelines for Using AcoDyes™

- #If a sonicator is available, sonicate the dye stock solution for 5 minutes at 40°C in pulse setting to ensure complete dissolution before each use.
- AcoDyes™ vary in intensity and staining efficiency. It is recommended to optimise the method for each dye, EV sample and study condition.
- To improve staining efficiency, it may be helpful to incubate the EV sample with the dye at 37°C and/or incubate 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.
- Ensure consistency in cell passages and confluency prior to EV uptake experiments.
- For very low volumes, use the reverse pipetting technique.
- AcoDyes™ are not compatible with detergent or detergent-based permeabilisation buffers.

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Table 2. Photophysical properties of Aco-430™, Aco-490™, Aco-520™, Aco-600™ and Aco-800™.

Dye Name	**Aco-430™**	**Aco-490™**	**Aco-520™**	**Aco-600™**	**Aco-800™**
Appearance	Colourless	Yellow	Orange	Purple	Green
$\lambda_{abs,\ with\ SUVs}$ (nm)	369	422	465	525	735
$\lambda_{em,\ with\ SUVs}$ (nm)	403 - 460	458 - 508	511 - 530	586 - 635	775 - 818
Suitable Laser(s)	UV (355 nm)	Violet (405 nm)	Blue (488 nm)	Blue or Yellow (488 or 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

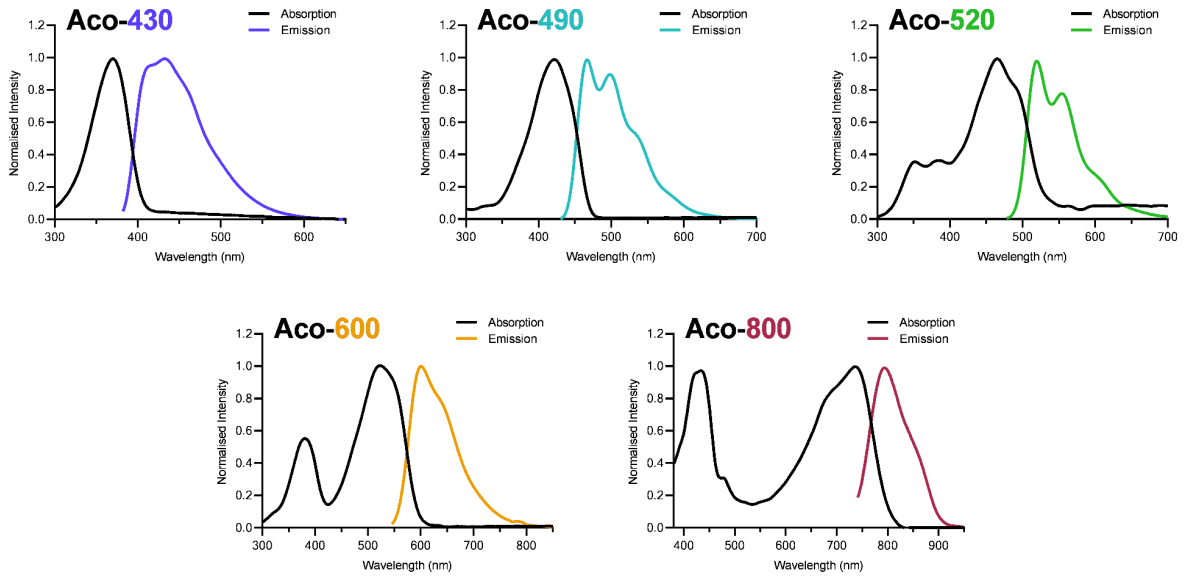

Figure 2. *Absorption and emission spectra of Aco-430™, Aco-490™, Aco-520™, Aco-600™, Aco-800™.*

Protocols and research publications can be found on our website www.acoerela.com. Please feel free to email hello@acoerela.com for assistance with troubleshooting of the staining protocol.