TECHNICAL NOTE

Expanding imaging multiplexing capabilities with PhenoVue Fluor 400LS - Phalloidin.

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Introduction

The cellular cytoskeleton is a complex network of protein filaments that provide structural support and perform essential cellular functions. The cytoskeleton is composed of three main types of filaments: microtubules, intermediate filaments, and microfilaments, also known as actin filaments (F-actin). Each filament type has distinct functions and contributes to the overall organization and dynamics of the cell.

Actin filaments are composed of globular actin monomers that polymerize to form long, flexible, and dynamic filaments. Actin network is essential for a wide range of cellular processes, including cell shape maintenance, cell movement, cell division, intracellular transport, cell adhesion, and cell signaling.

Dysfunction of actin dynamics can contribute to the development of various diseases such as cancers, neurological disorders (e.g., Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis), immune disorders, cardiovascular diseases as well as kidney diseases.

Understanding the role of actin dynamics in disease processes can provide insights for developing targeted therapeutic strategies.

Several methods can be utilized to study actin filaments and their dynamics within cells. Various biochemical assays can be used to study actin filaments and their properties. Fluorescence microscopy is widely used to visualize actin filaments in living or fixed cells. Fluorescent probes, such as fluorescently labeled phalloidin or fluorescently tagged actin-binding proteins, can be used to label actin filaments, and visualize their distribution, organization, and dynamics.

Here we describe a new PhenoVue® Fluor 400LS - Phalloidin stain which specifically binds F-actin and allows multiplexing applications with five channels and minimal crosstalk. This technical note offers tips and best practices during staining and acquisition steps to optimize fluorescent signal quality and obtain meaningful biological insights in your research.

PhenoVue Fluor 400LS - Phalloidin - general properties

Specificity

PhenoVue Fluor 400LS - Phalloidin specifically stains F-actin filaments in fixed and permeabilized cells, allowing visualization with high intracellular resolution when combined with high resolution imaging systems like the Opera Phenix® Plus and Operetta® CLS™ high-content screening instruments.

The selectivity of F-actin staining was achieved using a phalloidin peptide which specifically binds F-actin (Cano et al. Cell Motility and Cytoskeleton 1992; Melak et al. J of Cell Science 2017) conjugated to the new PhenoVue Fluor 400LS fluorophore. This fluorophore was designed to provide a bright fluorescent signal and extremely low unspecific binding, resulting in a drastically low background and very high signal to background ratio. The negative control in Figure 1A, where cells were pre-incubated with a large excess of unconjugated phalloidin prior to PhenoVue Fluor 400LS - Phalloidin, shows specificity of F-actin binding with high resolution as well as no background signal.

A titration or dose-response curve experiment was performed to determine the optimal staining concentration (Figure 1B). In these experimental conditions, the results indicate that 165 nM of PhenoVue Fluor 400LS - Phalloidin is an optimal concentration to reach maximum staining. Depending on experimental settings, the recommended optimization range is 40-400 nM.

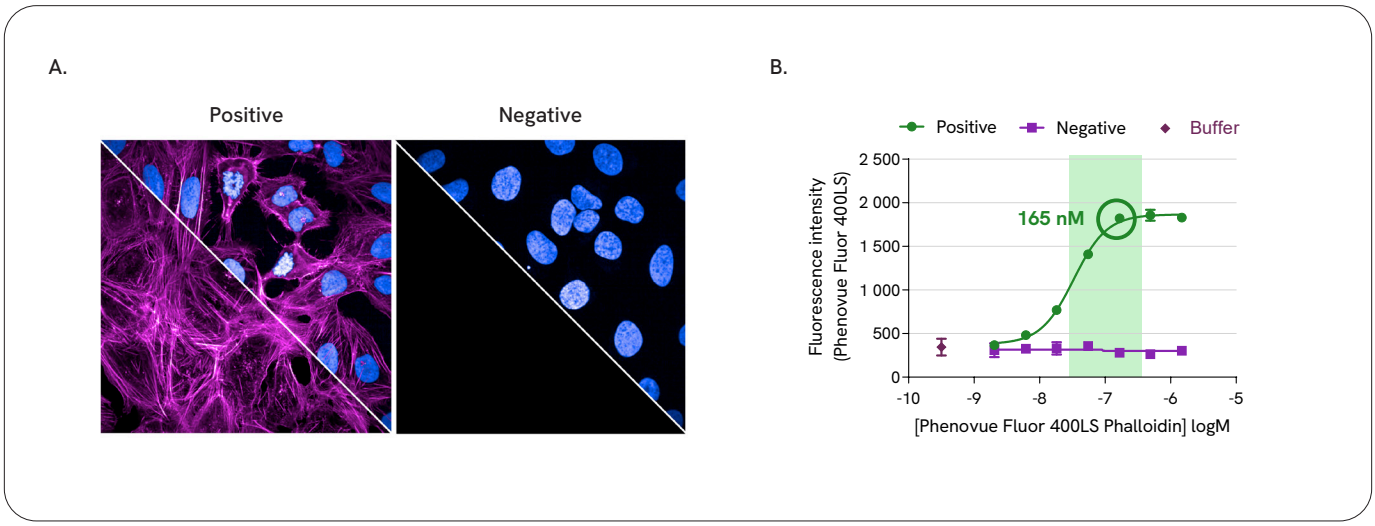


Figure 1: Specificity of F-actin filaments staining with PhenoVue Fluor 400LS - Phalloidin. U2OS cells were seeded into a PhenoPlate™ 96-well microplate (15,000 cells/well), then fixed with PhenoVue paraformaldehyde 4% solution (15 min, RT). Cells were permeabilized (PhenoVue permeabilization 0.5% Triton X-100 solution -15 min, RT), followed by a blocking step (PBS-1%BSA, 1h, RT), then stained with 165 nM PhenoVue Fluor 400LS - Phalloidin and 20 ng/mL PhenoVue Hoechst 33342 in PBS-1%BSA (45 min at RT + PBS washing). Negative control (right panel) was pre-incubated with 100-fold excess of unconjugated phalloidin (1h at RT) to compete with PhenoVue Fluor 400LS - Phalloidin binding. (B) The quantification of the fluorescent signal shows a dose dependent increase in fluorescence signal with increasing PhenoVue 400LS - Phalloidin which is blocked by preincubation with unlabeled phalloidin clearly showing the F-actin specific binding of the fluorescent probe. From these results the recommended concentration Is 165 nM. Images were acquired on an Opera Phenix Plus system using a 63X water immersion objective in confocal mode.

Signal stability

PhenoVue Fluor 400LS - Phalloidin is a bright and stable fluorophore allowing the stained cells to be imaged immediately after staining or within few days. As shown in Figure 2, PhenoVue Fluor 400LS - Phalloidin fluorescence intensity, as well as staining pattern (images and SER Ridge texture), did not change significantly over 48h (4 °C). Furthermore, PhenoVue Fluor 400LS fluorophore was specifically designed to exhibit high photostability, as illustrated in Figure 3 where fluorescent signal is stable even after 100 successive excitation cycles.

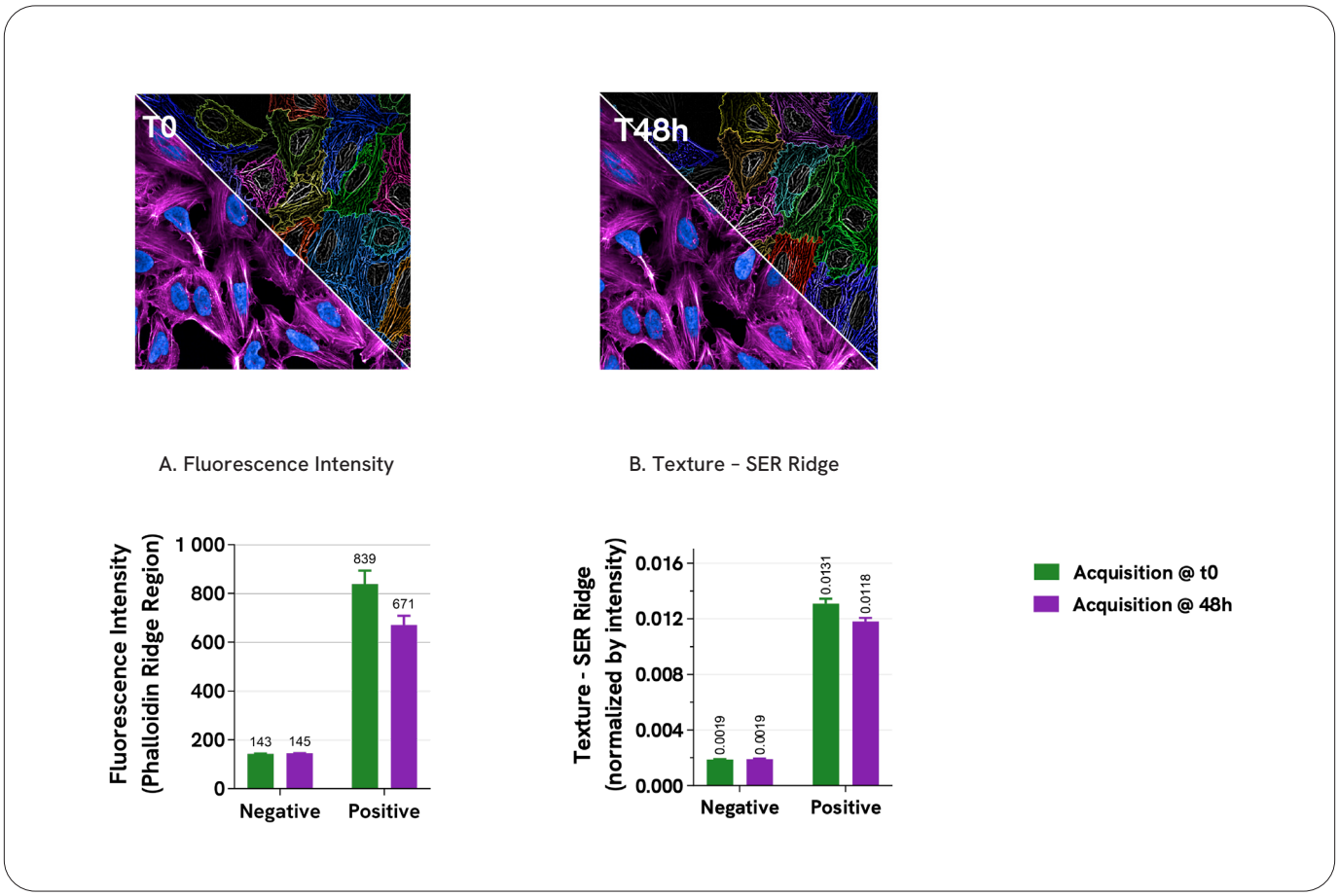


Figure 2: PhenoVue Fluor 400LS - Phalloidin staining is stable over time. U2OS cells were seeded into a PhenoPlate 96-well microplate (15,000 cells/well) for 24h at 37 °C, 5% CO_2 then fixed, permeabilized and stained as described in Figure 1. Negative control was pre-incubated with 100-fold excess of unconjugated phalloidin (1h at RT) to compete with PhenoVue Fluor 400LS - Phalloidin binding. Images were acquired on an Opera Phenix Plus using a 63X water immersion objective in confocal mode either directly after staining (left panel - T0) or after 48h (4 °C) (right panel). Graph A shows the quantification of the fluorescent signal confirming the stability over 48h. The positive condition indicates equivalent number of Ridge objects quantified at T0 and 48h. Graph B shows the quantification of SER-Ridge texture (F-actin filament) confirming the stability of staining texture over 48h (upper right part of images).

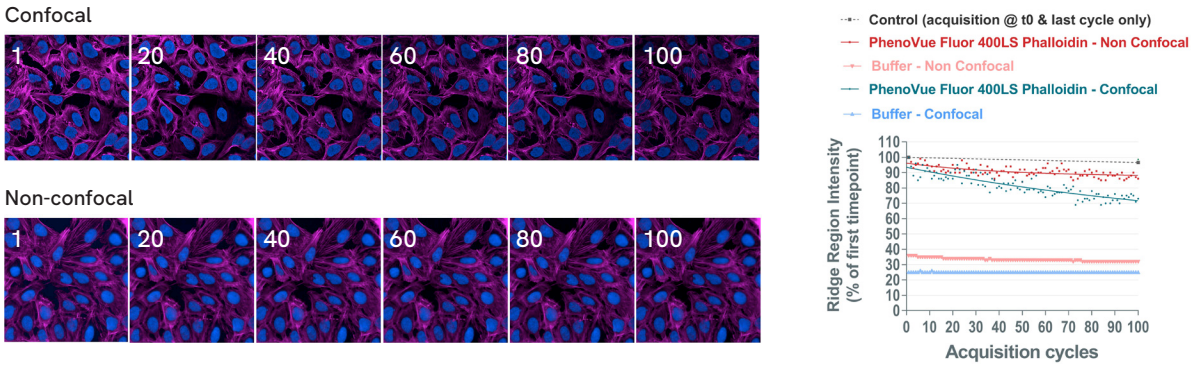
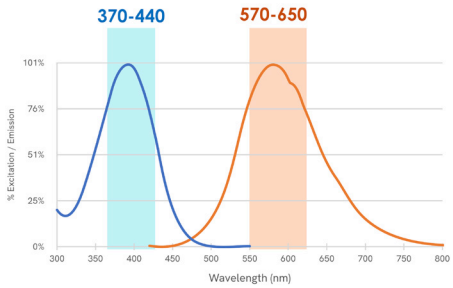


Figure 3: PhenoVue Fluor 400LS - Phalloidin is photostable. U2OS cells were seeded into a PhenoPlate 96-well microplate (15,000 cells/well) for 24h at 37 °C, 5% CO_2 then fixed, permeabilized and stained as described in Figure 1. Images were acquired on an Opera Phenix Plus using a 63X water immersion objective in either confocal or non-confocal mode for 100 successive excitation cycles. The graph shows the quantification of the fluorescence signal over the acquisition cycles, confirming the photostability of PhenoVue Fluor 400LS.

Multiplexing capability

As shown by the excitation and emission spectra (Figure 4), PhenoVue Fluor 400LS is a long Stokes shift dye with maximum excitation and emission peaks at 395 nm and 585 nm, respectively. Consequently, it increases multiplexing possibility in combination with other existing PhenoVue Fluor dyes like PhenoVue Hoechst 33342, PhenoVue Fluor 488, PhenoVue Fluor 555 (or PhenoVue Fluor 568) and PhenoVue Fluor 647 from 4 to 5 or more channels, while ensuring minimal spectral overlap and crosstalk. The following sections provide valuable guidelines (e.g., dye concentrations and acquisition settings) to further minimize spectral overlap and obtain the highest quality results.

Table 1 displays the recommended excitation and emission settings on the different configurations of Opera Phenix Plus and Operetta CLS instruments for multiplex applications with 5 channels.



Excitation max / Emission max	Common filter set for acquisition	Fluorescence lifetime	Solubility	Molar extinction coefficient (ε in $M^{-1}.cm^{-1}$ at λ_{max})	Molecular weight (g/mol)
395 nm 585 nm	Ex: 370-440 Em: 550-650	Nanosecond range	DMSO, Water, PBS, HBSS	26,000	≈ 1200

Figure 4: PhenoVue Fluor 400LS - Phalloidin spectrum (with recommended excitation and emission range) and photophysical features.

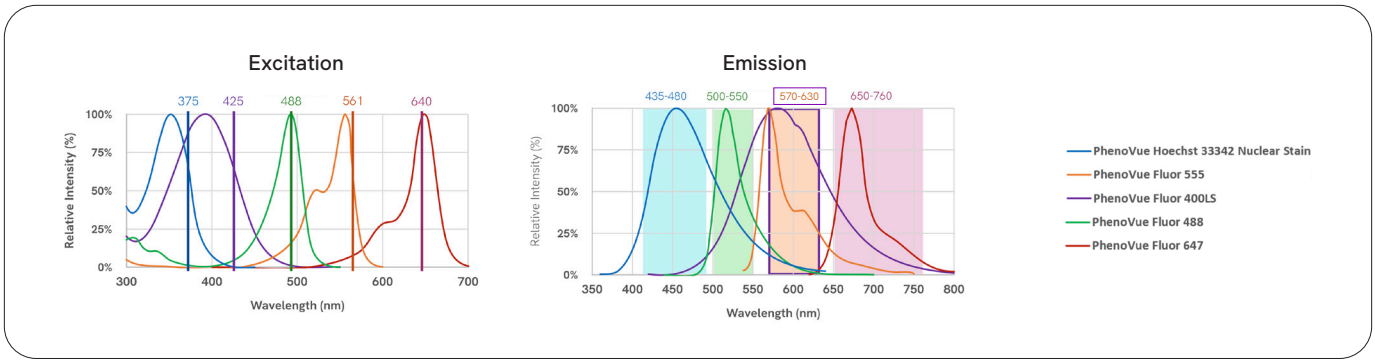


Figure 5: PhenoVue Fluor 400LS - Phalloidin enables easy multiplexing with up to 5 channels. Excitation and emission spectra of PhenoVue Fluor 400LS (purple) with additional four different PhenoVue Fluor dyes: PhenoVue Hoechst 33342 (blue), PhenoVue Fluor 488 (green), PhenoVue Fluor 555 (orange), PhenoVue Fluor 647 (red). Shown are the excitation and emission settings for Opera Phenix Plus system with 5 lasers.

Table 1: Optimal excitation and emission settings of PhenoVue Fluor 400LS on Revvity's high content screening systems.

HCS instruments		PhenoVue Hoechst 33342	PhenoVue Fluor 400LS Phalloidin	PhenoVue Fluor 488	PhenoVue Fluor 555 PhenoVue Fluor 568	PhenoVue Fluor 647
Opera Phenix Plus 5 lasers	Excitation laser	375	425	488	561	640
	Emission filter	435-480	570-630	500-550	570-630	650-760
Opera Phenix Plus 4 lasers	Excitation laser	405	405	488	561	640
	Emission filter	435-480	570-630	500-550	570-630	650-760
Operetta CLS 8 LED - 1600	Excitation LED (filter)	370 (355-385)	405 (390-420)	475 (460-490)	550 (530-560)	630 (615-645)
	Emission filter	430-500	570-650	500-550	570-650	655-760
Operetta CLS 8 LED - 1601	Excitation LED (filter)	370 (355-385)	440 (435-460)	475 (460-490)	550 (530-560)	630 (615-645)
	Emission filter	430-500	600-640 or 570-650	500-550	570-650	655-760
Operetta CLS 4 LED	Excitation LED (filter)	370 (355-385)	370 (355-385)	475 (460-490)	550 (530-560)	630 (615-645)
	Emission filter	430-500	570-650	500-550	570-650	655-760

Critical aspects for a successful 5-plex experiment

Protocol

1. For best image quality, seed cells in imaging microplates such as PhenoPlate. Incubate under appropriate cell culture conditions, usually 37 °C, 5% CO_2 until 50-70% confluency. Phalloidin conjugates are not cell permeable. Staining requires a prior fixation and permeabilization step.

2. **Pre-fixation/permeabilization staining:** Aspirate cell culture medium and add fluorescent probes which requires cell integrity for proper staining (if any). Particularly in the multiplex example presented herein, PhenoVue 641 mitochondrial stain (500 nM - CP3D1) in PhenoVue dye diluent A (1X - PVDDA1) was incubated for 30 min at 37 °C, 5% CO_2 then washed three times.

3. **Fixation:** Aspirate medium and add ready to use PhenoVue paraformaldehyde 4% methanol-free solution (PVPFA41) for 10-15 min at room temperature. Avoid methanol-based fixation methods since methanol can disrupt actin.

4. **Washing:** Three times with PBS or HBSS.

5. **Permeabilization:** Add ready to use PhenoVue permeabilization 0.5% Triton X-100 solution (PVPERM051) for 10-15 min at room temperature.

6. **Washing:** Three times with PBS or HBSS.

7. **Staining:** Incubate with a solution containing PhenoVue Fluor 400LS - Phalloidin (165 nM - CP24001) previously diluted in PhenoVue dye diluent A. In the featured example, PhenoVue Hoechst 33342 nuclear stain (20-70 ng/mL - CP71), PhenoVue Fluor 488 - Concanavalin A (5 µg/mL - CP94881), PhenoVue Fluor 555 - WGA (1.5-5 µg/mL - CP15551) were added additionally. Incubate for 30-60 min @ RT (protected from light).

8. **Washing:** Three times with PBS or HBSS.

9. Acquire images on a compatible imaging device such as Operetta CLS or Opera Phenix Plus high content screening instruments. See Table 1 for recommended acquisition settings.

Hoechst / DAPI concentration

When using laser or LED excitation between 350-415 nm (e.g., Opera Phenix/Plus with 405 laser or Operetta CLS with 405 or 370 LED), it is important to reduce the concentration of Hoechst 33342 (or DAPI) to limit bleed through in the 570-630 nm detection band. Hoechst 33342 is a bright dye with a broad emission spectrum which otherwise may cause spectral crosstalk into the PhenoVue Fluor 400LS channel (Figure 6, with the symbol ⚠). The 375 laser or 370 LED requires even lower concentration of Hoechst 33342 (or DAPI) than the 405 laser and LED since Hoechst 33342 excitation is more efficient at these wavelengths. A PhenoVue Hoechst 33342 nuclear stain concentration of 20-80 g/mL (incubated for 30-60 min) typically gives good nuclear staining while reducing spectral crosstalk to a minimum (Figure 6). Certain cell lines might require further Hoechst concentration optimization.

If the 425 laser (Opera Phenix 5 laser) or 440LED (Operetta CLS 8LED - 1601) are used for excitation, no PhenoVue Hoechst 33342 optimization is required since it is not excited at such wavelengths.

To objectivate our observation, quantification was performed as shown in Figure 6 A, B and C. Phalloidin fluorescence signal over background (S/B) in different acquisition settings indicate that 405 and 440LED excitations are the most efficient (Figure 6A). PhenoVue Hoechst 33342 used at 20 ng/mL enables a comfortable S/B when acquired with excitation 370LED (355-385) / Emission 430-500) (Figure 6B).

PhenoVue Hoechst 33342 (20 and 70 ng/mL) spills over into different PhenoVue Fluor 400LS channels. The signal is normalized by the PhenoVue Fluor 400LS - Phalloidin at the channel indicated on the graph (=100%). Here only the 370LED excitation displays significant crosstalk of PhenoVue Hoechst 33342 which can be significantly reduced by decreasing Hoechst 33342 concentration.

In conclusion, 70 ng/mL of PhenoVue Hoechst 33342 is feasible when using the 405 or 440LED, whilst 20 ng/mL PhenoVue Hoechst 33342 is preferred if using the 370LED.

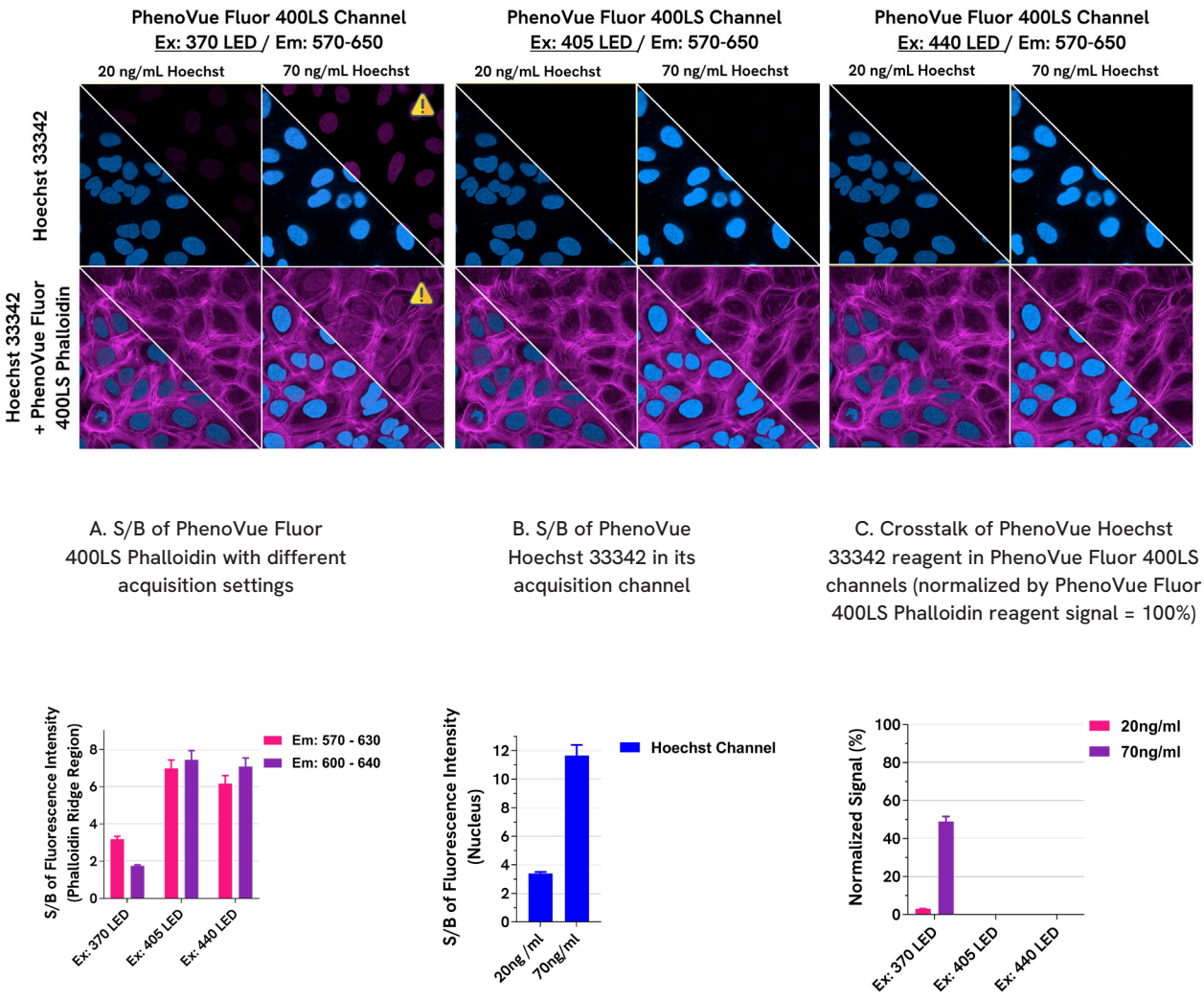


Figure 6: Hoechst 33342 concentration optimization to limit spectral crosstalk into the PhenoVue Fluor 400LS - Phalloidin channel. U2OS cells were fixed, permeabilized and stained with either PhenoVue Hoechst 33342 (20 or 70 ng/mL) alone (upper panels) or in combination with PhenoVue Fluor 400LS (165 nM) (lower panels) for 30 min at RT. Images were acquired on an Operetta CLS instrument using the indicated excitation (Ex) and emission (Em) options for PhenoVue Fluor 400LS channel (Fuchsia); Hoechst channel (Ex: 370LED / Em: 430-500 nm, blue) and a 63X water immersion objective in confocal mode.

Graph A represents the PhenoVue Fluor 400LS - Phalloidin signal over background (S/B) in the indicated acquisition settings. Graph B shows the PhenoVue Hoechst 33342 signal over background (S/B) acquired with Ex: 370LED (355-385) / Em: 430-500. Graph C shows the crosstalk of PhenoVue Hoechst 33342 reagent in the different PhenoVue Fluor 400LS channels normalized by the signal of the PhenoVue Fluor 400LS - Phalloidin reagent in each channel (=100%).

Limiting PhenoVue Fluor 488 crosstalk with Operetta CLS 8LED - 1601 440LED

If using the 440LED excitation (Operetta CLS 8LED-1601 only) for the PhenoVue Fluor 400LS fluorophore, the use of PhenoVue Fluor 488 conjugates which stain abundant cellular molecules can potentially lead to spectral crosstalk into the PhenoVue Fluor 400LS channel (see Figure 7, annotated with the symbol ⚠). In this case, PhenoVue Fluor 488 conjugate concentration should be reduced.

Alternatively, a red shifted emission filter like 600-640 nm can be used to image the PhenoVue Fluor 400LS (instead of a standard emission filter range of 570-650 nm) to reduce spectral crosstalk (see Figure 7).

However, if using the 370 or 405 excitation lasers or LED there is no risk of spectral crosstalk from PhenoVue Fluor 488.

The PhenoVue Fluor 400LS - Phalloidin fluorescence signal over background (S/B) in different acquisition settings indicate that 405 and 440LED excitations are the most efficient (Figure 7A).

PhenoVue Fluor 488 Concanavalin A spills over into different PhenoVue Fluor 400LS channels. The signal is normalized by the PhenoVue Fluor 400LS - Phalloidin at indicated channel (=100%). Here only the 440LED excitation displays significant crosstalk of PhenoVue Fluor 488 Concanavalin A which can be significantly reduced by using the 600-640 emission filter.

In conclusion, if detecting an abundant biomolecule using the 440LED excitation, then the 600-640 emission filter should be used.

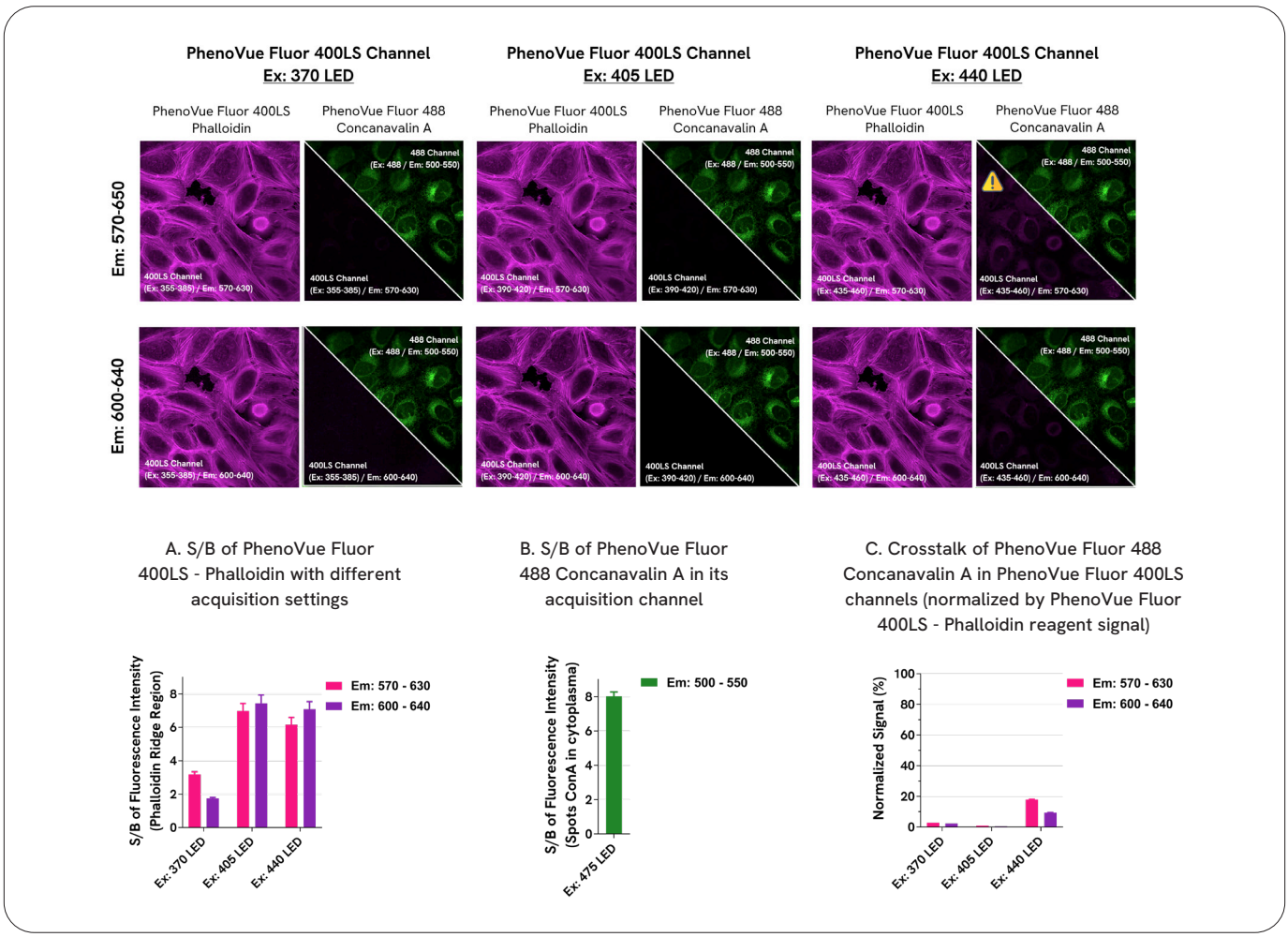


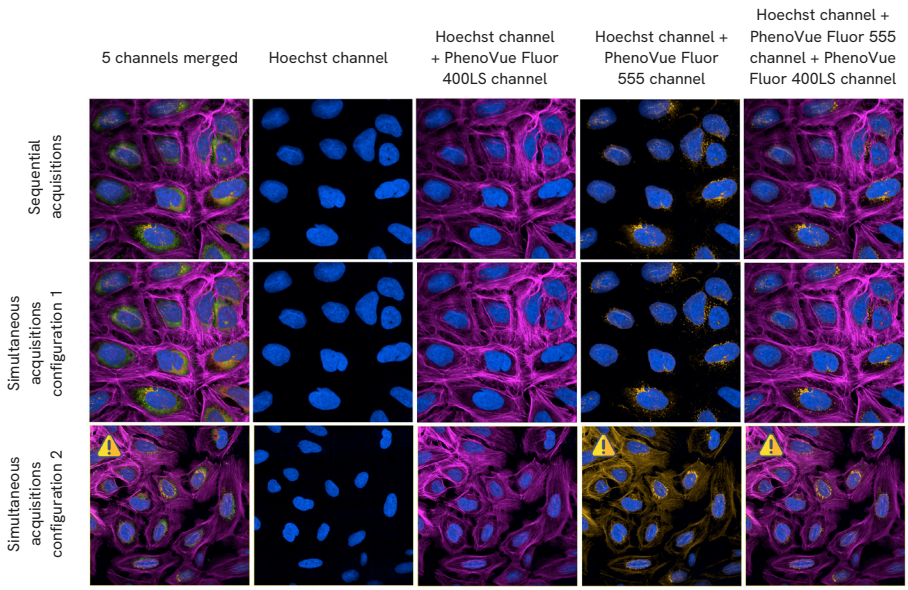
Figure 7: PhenoVue Fluor 488 crosstalk when PhenoVue Fluor 400LS is excited with 440LED (Operetta CLS 8LED - 1601): U2OS cells were fixed, permeabilized and stained with a mix of PhenoVue Hoechst 33342 (20 ng/mL, nuclei, channel not displayed on the images) and PhenoVue Fluor 400LS (165 nM, F-actin, fuchsia) or PhenoVue Fluor 488 Concanavalin A (5 µg/mL, endoplasmic reticulum, green) for 30 min at RT. Images were acquired on an Operetta CLS instrument using the indicated excitation (Ex) and emission (Em) options and a 63X water immersion objective in confocal mode. Graph A shows the PhenoVue Fluor 400LS - Phalloidin signal over background (S/B) in the indicated acquisition settings. Graph B shows the PhenoVue Fluor 488 Concanavalin A S/B acquired with Ex: 475LED / Em: 500-550. Graph C shows the crosstalk of PhenoVue Fluor 488 Concanavalin A in the different PhenoVue Fluor 400LS channels normalized by the signal of the PhenoVue Fluor 400LS - Phalloidin reagent in each channel (=100%).

Limiting PhenoVue Fluor 400LS crosstalk with 475LED (Operetta CLS) high power and longtime exposure

If using the 475LED (460-490 excitation filter-Operetta CLS instruments) combined with high excitation energy (high power and time exposure) to excite the PhenoVue Fluor 488 conjugates, the PhenoVue Fluor 400LS - Phalloidin may generate fluorescence crosstalk. Thus, we recommend decreasing PhenoVue Fluor 400LS - Phalloidin concentration while increasing acquisition power to maintain comfortable fluorescent signal. This phenomenon is not observed with the Opera Phenix Plus instrument since the 488 laser does not excite the PhenoVue Fluor 400LS fluorophore (see spectrum in Figure 5).

Sequence acquisition for Hoechst and PhenoVue Fluor 555 when combined with PhenoVue Fluor 400LS

When using simultaneous acquisition mode on the Opera Phenix Plus (equipped with 2, 3 or 4 cameras), it is important to separate Hoechst 33342 (Ex 405/425 nm ; Em: 435-480 nm) and PhenoVue Fluor 555 / 568 (Ex: 561 nm ; Em: 570-630 nm) channels since the 405/425 laser may excite PhenoVue Fluor 400LS - Phalloidin which will then emit in the 570-630 emission filter range used for PhenoVue Fluor 555 / 568 detection. This may result in the detection of actin filaments from PhenoVue Fluor 400LS - Phalloidin signal in the PhenoVue Fluor 555 / 568 images (Figure 8, annotated with the symbol ⚠). We also recommend to separate Hoechst and PhenoVue Fluor 400LS acquisition to minimize Hoechst bleed through in the PhenoVue Fluor 400LS channel.



Sequential acquisition

Acquisition cycles	Channels
1	**PhenoVue Hoechst 33342** Ex: 375 / Em: 435-480
2	**PhenoVue Fluor 488** (Ex: 488 / Em: 500-550)
3	**PhenoVue Fluor 555** (Ex: 561 / Em: 570-630)
4	**PhenoVue Fluor 647** (Ex: 640 / Em: 650-760)
5	**PhenoVue Fluor 400LS** (Ex: 425 / Em: 570-630)

Simultaneous acquisitions - Configuration 1

Acquisition cycles	Channel 1	Channel 2
1	**PhenoVue Hoechst 33342** Ex: 375 / Em: 435-480	**PhenoVue Fluor 488** (Ex: 488 / Em: 500-550)
2	**PhenoVue Fluor 555** (Ex: 561 / Em: 570-630)	**PhenoVue Fluor 647** (Ex: 640 / Em: 650-760)
3	**PhenoVue Fluor 400LS** (Ex: 425 / Em: 570-630)	

Simultaneous acquisitions - Configuration 2

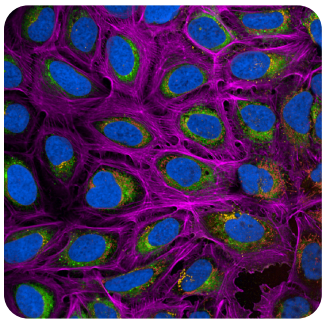
Acquisition cycles	Channel 1	Channel 2	Channel 3	Channel 4
1	**PhenoVue Hoechst 33342** Ex: 375 / Em: 435-480	**PhenoVue Fluor 488** (Ex: 488 / Em: 500-550)	**PhenoVue Fluor 555** (Ex: 561 / Em: 570-630)	**PhenoVue Fluor 647** (Ex: 640 / Em: 650-760) ⚠
2	**PhenoVue Fluor 400LS** (Ex: 425 / Em: 570-630			

Figure 8: U2OS cells stained with PhenoVue 641 mitochondrial stain (500 nM, mitochondria, red) then fixed, permeabilized and stained with mix of PhenoVue Hoechst 33342 (20 ng/mL, nuclei, blue), PhenoVue Fluor 400LS (165 nM, F-actin, fuchsia), PhenoVue Fluor 488 Concanavalin A (5 µg/mL, endoplasmic reticulum, green) and PhenoVue Fluor 555 WGA (1.5 µg/mL, Golgi apparatus, orange) for 30 min at RT. Acquisition on Opera Phenix Plus instrument with 63X water objective in confocal mode and sequential or simultaneous mode. Two configurations of simultaneous acquisition are displayed. The first one with PhenoVue Fluor 400LS and PhenoVue Fluor 555 channels, which are separated, allow good image quality with minimal crosstalk. The second one with PhenoVue Fluor 400LS and PhenoVue Fluor 555 channels appaired display PhenoVue Fluor 400LS - Phalloidin (F-actin) crosstalk in the PhenoVue Fluor 555 channel (Golgi apparatus).
Do not acquire Hoechst and PhenoVue Fluor 555 signals simultaneously when combined with PhenoVue Fluor 400LS.

Validation data

PhenoVue Fluor 400LS - Phalloidin is a long Stokes shift fluorophore designed to allow multiplexing up to five channels and probes while maintaining minimal crosstalk.

Example 1



U2OS cells stained with PhenoVue Hoechst 33342 (nucleus, blue), PhenoVue Fluor 400LS - Phalloidin (F-actin cytoskeleton, fuchsia), PhenoVue Fluor 488 Concanavalin A (endoplasmic reticulum (ER), green), PhenoVue Fluor 555 WGA (Golgi apparatus, plasma membranes, orange) and PhenoVue Fluor 641 mitochondrial stain (mitochondria, red) (see Figures 9 - 11) either alone or with a mixture of all. As shown in Figures 9 and 10, if the fluorophores are titrated properly, the five channels can be acquired with minimal crosstalk.

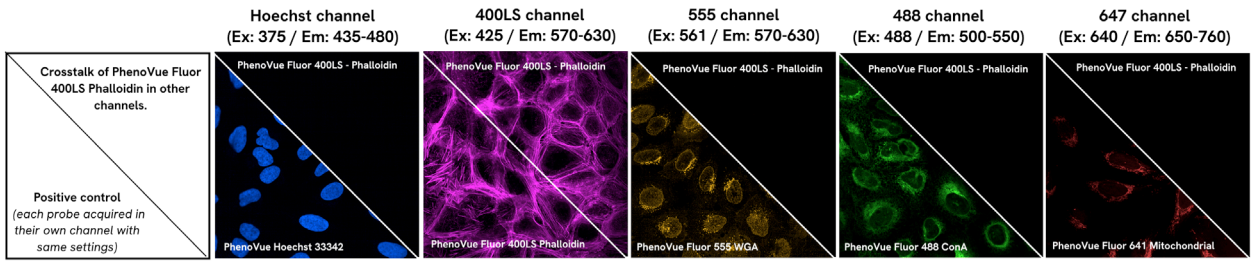


Figure 9: PhenoVue Fluor 400LS - Phalloidin does not bring significant crosstalk in other channels. U2OS cells were seeded, stained and acquired with same protocol used in Figure 11. Upper triangles display wells with cells stained with PhenoVue Fluor 400LS - Phalloidin only and lower triangle display positive control wells with cells stained with each probe alone. Upper and lower triangle images are shown with same acquisition settings (excitation filters, power and time; image contrast). PhenoVue Fluor 400LS - Phalloidin does not bring significant crosstalk in other channels as shown in upper triangles.

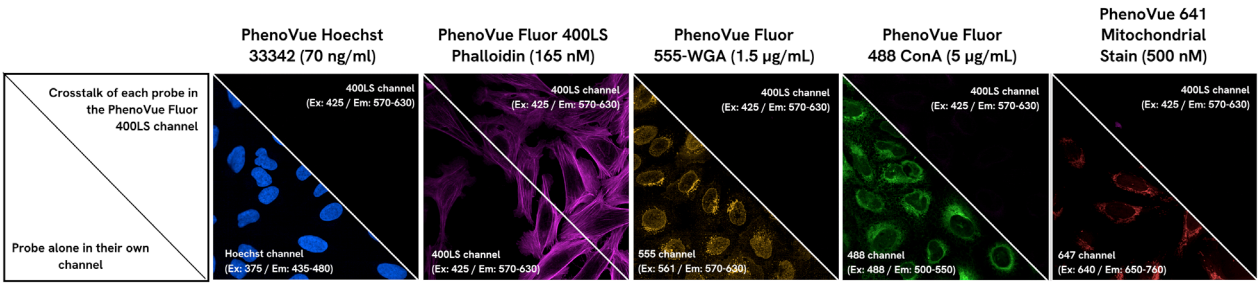


Figure 10: Other PhenoVue Fluor dyes do not bring significant crosstalk in the PhenoVue Fluor 400LS channel. U2OS cells were seeded, stained and acquired with same protocol than Figure 9 but with each probe alone in different wells (not mixed). Upper triangles display the PhenoVue Fluor 400LS channel (Ex: 425 / Em: 570-630) and the lower triangles display the same field of view in each probe proper channels. Standard PhenoVue Fluor dyes do not significantly bring crosstalk in the PhenoVue Fluor 400LS channel as shown in upper triangles.

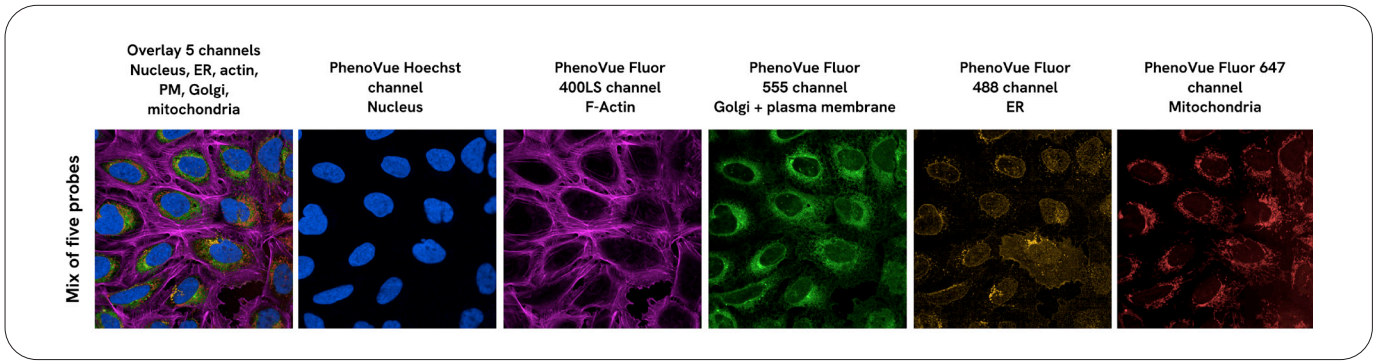
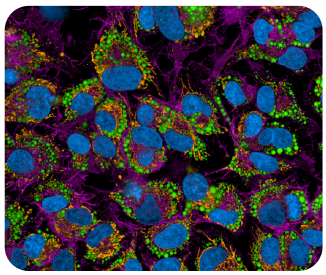


Figure 11: 5-plex experiment staining: nucleus, F-actin, Golgi apparatus, endoplasmic reticulum, mitochondria. U2OS cells were seeded into a PhenoPlate 96-well microplate (15,000 cells/well), cultured for 24h at 37 °C, 5% CO_2 and then stained with PhenoVue 641 mitochondrial stain (500 nM, 30 min at 37 °C, 5% CO_2, mitochondria, red). After fixation (PhenoVue paraformaldehyde 4% solution, 15 min, RT + PBS washing) and permeabilization (PhenoVue permeabilization 0.5% Triton X-100 solution, 15 min, RT + PBS washing) they were stained with a mix of PhenoVue Hoechst 33342 (70 ng/mL, nuclei, blue), PhenoVue Fluor 400LS (165 nM, F-actin, fuchsia), PhenoVue Fluor 488 Concanavalin A (5 µg/mL, endoplasmic reticulum, green) and PhenoVue Fluor 555 WGA (1.5 µg/mL, Golgi apparatus, orange) for 30 min at RT. Images were acquired on an Opera Phenix Plus equipped with 5 lasers using a 63X water immersion objective in confocal mode (see Table 1 for spectral acquisition settings).

Example 2



HepG2 were stained according to the protocol described in the PhenoVue multi organelle staining kit (PMOS11). Briefly cells were stained either with a mix of PhenoVue Hoechst 33342 (nucleus, blue), PhenoVue Fluor 400LS (F-actin, fuchsia), PhenoVue 493 lipid stain (lipid droplets, green), PhenoVue anti-HSP60 antibody + PhenoVue Fluor 555 - Goat anti-mouse highly cross-adsorbed (mitochondria-HSP60, orange) and PhenoVue anti-LAMP1 antibody + PhenoVue Fluor 647 - Goat anti-rat highly cross-adsorbed antibody (lysosome-LAMP1, red) or with the individual probes (Figure 12).

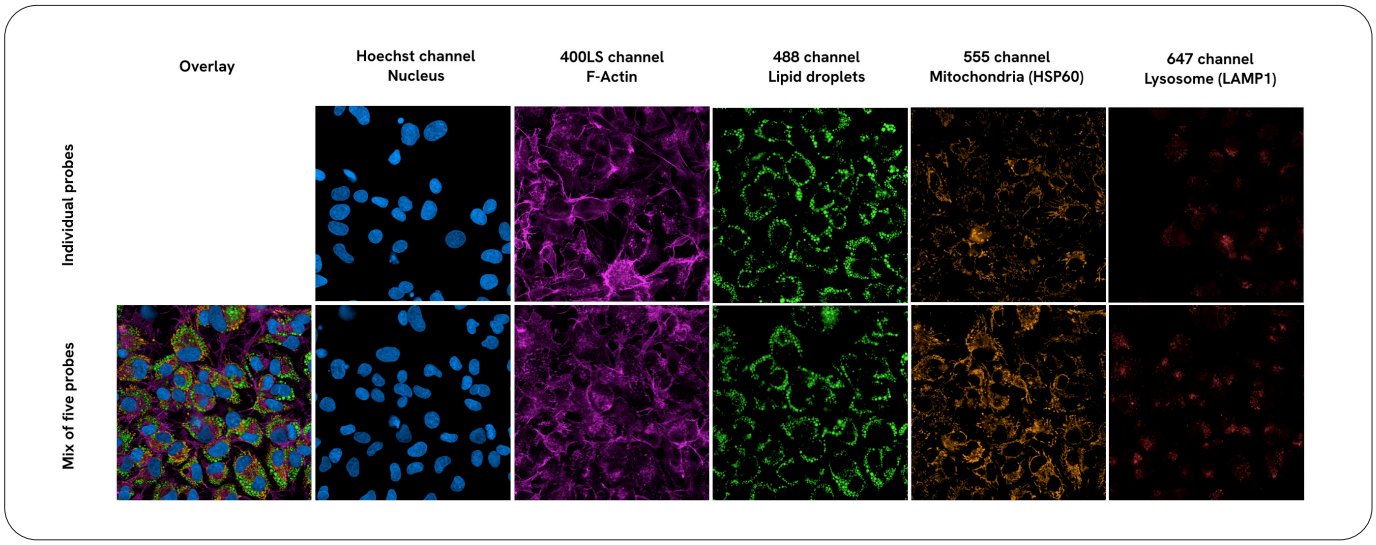


Figure 12: PhenoVue multi organelle staining kit – nucleus, F-actin, lipid droplets, mitochondria, lysosome. HepG2 cells seeded in PhenoPlate PDL coated 96-well (20,000 cells/well) 24h at 37 °C, 5% CO_2 then incubated with oleic acid (200 µM) for 24h at 37 °C, 5% CO_2. The cells were then fixed (PhenoVue paraformaldehyde 4% solution, 15 min, RT + PBS washing), permeabilized (PhenoVue permeabilization 0.1% Triton X-100 solution, 10 min, RT + PBS washing) followed by saturation step (PBS, 1%BSA, 1h, RT). The cells were then incubated with a mix of primary PhenoVue anti-HSP60 antibody and PhenoVue anti-LAMP1 antibody (overnight, 4 °C + PBS washing). Final staining with mix of PhenoVue Hoechst 33342 (nucleus, blue), PhenoVue Fluor 400LS (F-actin, fuchsia), PhenoVue 493 lipid stain (lipid droplets, green), PhenoVue Fluor 555 - Goat anti-mouse highly cross-adsorbed (mitochondria -HSP60, orange) and PhenoVue Fluor 647 - Goat anti-rat highly cross-adsorbed antibody (lysosome-LAMP1, red) for 1h, at RT. Acquisition on Operetta CLS (8LED -1600) instrument with 63X water immersion objective in confocal mode (see Table 1 for acquisition filters). Bottom panel shows that each probe alone or mix of the five probes display same staining for each cellular organelle.

Recommended PhenoVue Fluor Phalloidin dyes in multiplexing applications

Phalloidin stains	Up to 4-plex	5-plex
PhenoVue Fluor 400LS		▪
PhenoVue Fluor 488	▪	
PhenoVue Fluor 555	▪	
PhenoVue Fluor 568	▪	
PhenoVue Fluor 594	▪	
PhenoVue Fluor 647	▪	

Conclusion

PhenoVue Fluor 400LS - Phalloidin stain specifically binds F-actin and allows multiplexing applications with five channels or more. Here we provide guidelines for staining and image acquisition settings to minimize crosstalk and maximize fluorescent signal quality. Multiplexing PhenoVue Fluor 400LS - Phalloidin with other stains such as Hoechst and PhenoVue Fluor 488 conjugates may require adjusting reagent concentrations and exposure time, as well as sequence acquisition to capture the most significant biological relevance and understandings.

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