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Optimization of fluorescent labelling and imaging protocols for actin characterisation.
Optimització dels protocols de marcatge fluorescent i d'imatge per a la caracterització de l'actina.

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The greatest enemy of knowledge is not ignorance, it is the illusion of knowledge.

Stephen Hawking

Quiero agradecer a todas aquellas personas que han sido partícipes de que este trabajo haya salido adelante y han estado apoyándome durante el camino.

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REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

This study contributes to the Sustainable Development Goals (SDG), focused in particular on Goal 3: Good Health and Well-being. Out of the big topics, the 5 Ps (People, Planet, Prosperity, Peace and Partnership), this project helps "People" achieve a better understanding of how their body works.

This project focuses on the selection of the best methods for labelling actin using fluorescence microscopy. Actin is the most abundant protein in most eukaryotic cells. It is highly conserved and engages in more protein-protein interactions than any other known protein.[1] Therefore, it is reasonable to consider that actin malfunction could potentially be the root of future problems.

For instance, metastasis accounts for 90% of cancer-related deaths, making it the most critical issue associated with cancer. The movement of cancer cells is enabled by the formation of invasive actin-based structures, such as invadopodia, lamellipodia, and filopodia. These changes are driven by the reorganization of the actin cytoskeleton, a process regulated by actin-binding proteins (ABPs). Alterations in the expression or function of ABPs can disrupt actin dynamics, promoting cancer progression through increased cell motility.[2]

Due to the fact that actin also plays a crucial role, among others, on the immune system, the nervous system and the cardiovascular system, further complications, beyond cancer, are potentially to be related to the malfunction of actin organisation.[3] The objective 3.4 of ONU's SDG is the one which best describes a future objective of this study:

3.4 By 2030, reduce by one third premature mortality from non-communicable diseases through prevention and treatment and promote mental health and well-being.

Since actin plays a crucial role in many cellular functions, studying its organisation is essential to a better understanding of its dysfunction. Precise labelling of actin structures provides valuable insights into its dynamics, supporting the development of diagnostic tools and potential treatments.

This study focuses on the optimisation of actin labelling methods and the comparison of the affinity and specificity of the labelling agents towards actin. The best labelling methods were determined according to the labelling agent used. This information can be crucial for future researchers studying actin, as they can choose an adequate actin labelling method depending on their experimental constraints or labelling objectives.

Should this project be expanded into a larger study in the future, it could focus on investigating the role of actin in metastasis. By studying the mechanisms behind actin in metastatic cells, this research could contribute to the development of targeted therapies for disrupting actin reorganisation. If accomplished, it would help to limit cancer spread and significantly improve patient outcomes.

CONTENTS

1 SUMMARY

Since the invention of the microscope in the 17th century by pioneers such as Antonie van Leeuwenhoek and Robert Hooke, the study of cells has played a fundamental role in science. Cell labelling is a technique in which different cell structures or components are marked to ensure optimal visualization under the microscope. To carry out this labelling process, markers are used, such as fluorescent dyes, antibodies conjugated with fluorophores, and genetically encoded proteins. These markers specifically bind to their target, depending on the intrinsic characteristics of the marker. Researchers are not only able to observe the morphology and cellular composition, but also the biological processes taking place within the cell. This has led to advancements in the study of diseases caused by cellular dysfunction, such as, for example, aiding in diagnosis with the specific labelling of biomarkers in tumour cells.[4]

This project will focus on the study of actin, the protein that makes up the majority of the cellular cytoskeleton, in fixed HeLa cells. Since fixed cells will be used, the dynamics of actin will not be observed. To carry out the study, different actin markers will be tested, including fluorescent dyes and antibodies conjugated with fluorophores. Additionally, the effects that various cellular fixation processes may have on the preservation and labelling of actin will be studied.

To observe and study cellular components, imaging techniques are necessary to obtain detailed images and precise information about the elements of interest, such as in this case, actin. Because of the use of fluorescent markers, the technique of fluorescence spectroscopy is combined with confocal microscopy. The latter, due to its technical features, allows for high-resolution images, minimizing fluorescence interference from other cellular planes. In this study, three-dimensional (xyz) images will be obtained to determine the optimal cellular labelling conditions. Moreover, hyperspectral images (xyλ), with an emission spectrum per pixel, will be employed to compare the affinity and specificity of different markers with actin. When obtaining hyperspectral images with several fluorophores present, it is necessary to resort to chemometric tools to effectively separate the fluorescence signal from each fluorophore. In this study, the tool known as Multivariate Curve Resolution by Alternating Least Squares (MCR-ALS) was used to perform the chemometric analysis.

Based on the images obtained in xyz acquisition mode, it was determined that the most suitable fixation method for the fluorescent dyes (phalloidin, SiR-actin, SPY-555) was paraformaldehyde (PFA). Conversely, for the antibodies (anti-beta and anti-pan actin Ab), the fixation method combining PFA and methanol was selected.

Using hyperspectral images and subsequent chemometric analysis, it was possible to separate the fluorescent signals and compare two pairs of fluorophores. The pair comprising SPY-555 and phalloidin-568 exhibited regions of colocalization as well as areas where each fluorophore showed a preference for the different actin structures. On the other hand, the pair formed by phalloidin-568 and anti-pan actin Ab (ASTAR Orange) demonstrated very low colocalization. The actin filaments were clearly marked by phalloidin-568, while the antibody produced a more diffuse signal, predominantly localised in the cell's interior. Nevertheless, to obtain more consolidated results, a more extensive study would be needed, acquiring a greater number of images and ensuring that the studied cells are in the same physiological state.

Keywords: actin, fluorophore labelling, cell fixation, imaging techniques, chemometrics.

2 RESUMEN

Desde el invento del microscopio en el siglo XVII por pioneros como Antonie van Leeuwenhoek y Robert Hooke, el estudio de las células ha tenido un papel fundamental en la ciencia. La marcación celular es una técnica donde se marcan diferentes estructuras o componentes de la célula para asegurar una óptima visualización de ellas bajo el microscopio. Para llevar a cabo este proceso se utilizan marcadores como colorantes fluorescentes, anticuerpos conjugados con fluoróforos y proteínas codificadas genéticamente. Estos marcadores se unen específicamente a su objetivo, condicionado por las características intrínsecas del marcador. Los marcadores permiten observar la morfología y composición celular y también los procesos biológicos que se llevan a cabo en la célula. Gracias a ello, se ha podido avanzar en el estudio de enfermedades provocadas por un mal funcionamiento celular como, por ejemplo, en el diagnóstico basado en la marcación específica de biomarcadores de células tumorales.

Este proyecto se centrará en el estudio de la actina, la proteína que constituye la mayor parte del citoesqueleto celular, en células HeLa fijadas. Dado que se tratará de células fijadas, no se observará la dinámica de la actina. Para llevar a cabo el estudio, se experimentará con diferentes marcadores de actina, incluyendo colorantes fluorescentes y anticuerpos conjugados con fluoróforos. Además, se estudiarán los efectos que pueden tener diversos procesos de fijación celular en la conservación y en la marcación de la actina.

Para observar y estudiar los componentes celulares, son necesarias técnicas de imagen que permitan obtener imágenes detalladas e información precisa sobre los elementos de interés, como es en este caso, la actina. Como se usan marcadores fluorescentes, se emplea la técnica de espectroscopía de fluorescencia combinada con la microscopía confocal. Ésta última, debido a sus características técnicas, permite obtener imágenes de alta resolución, minimizando la interferencia de fluorescencia proveniente de otros planos celulares. En este estudio se obtendrán imágenes tridimensionales (xyz) para determinar las condiciones óptimas de marcaje celular, así como imágenes hiperespectrales (xyλ), en las que un espectro de emisión está asociado a cada píxel, que nos ayudarán a comparar la afinidad y especificidad de diversos marcadores con la actina. Al obtener imágenes hiperespectrales con varios fluoróforos presentes, es necesario recurrir a herramientas quimiométricas para separar de manera eficaz la señal de fluorescencia proveniente de cada uno. En este estudio se utiliza la herramienta denominada Resolución Multivariante de Curvas por Mínimos Cuadrados Alternantes (MCR-ALS) para llevar a cabo el análisis quimiométrico.

A partir de las imágenes obtenidas en el modo de adquisición xyz se determinó que el modo de fijación celular más idóneo para los colorantes fluorescentes (faloidina, SiR-actin, SPY-555) era el paraformaldehído (PFA). En cambio, para los anticuerpos (anti-beta y anti-pan actin Ab) el método de fijación que combina el PFA y el metanol fue el elegido.

A partir de las imágenes hiperspectrales, y después del análisis quimiométrico, se pudieron separar las señales fluorescentes y hacer la comparativa entre dos parejas de fluoróforos. La pareja compuesta por SPY-555 y faloidina-568 tuvo zonas donde había colocalización de los fluoróforos y otras en donde se podía ver una preferencia de los fluoróforos por las diferentes estructuras de la actina. Por otro lado, la pareja compuesta por faloidina-568 y anti-pan actin Ab (ASTAR Orange), demostraron tener una muy baja colocalización. Los filamentos de actina se vieron claramente marcados por la falodina-568 y el anticuerpo produjo una señal más difusa, predominantemente localizada en la parte interna de la célula. Aun así, para obtener resultados más consolidados, sería necesario realizar un estudio más extenso, obteniendo un mayor número de imágenes y asegurando que las células estudiadas se encuentren en el mismo estado fisiológico celular.

Palabras clave: actina, marcaje fluorescente, fijación celular, técnicas de imagen, quimiometría.

3 INTRODUCTION

3.1 CONTEXT OF THE WORK

Cell labelling is a process of tagging or marking cells to facilitate their identification and tracking under the microscope. It plays a vital role in biological research, enabling the visualisation of cellular structures, the tracking of dynamic cellular processes, and the study of molecular interactions.[5] Collectively, these techniques contribute significantly to advancements in disease treatment.

For instance, cell migration is essential for embryonic development, wound healing, immune response, and inflammation. It also influences neuronal targeting and cancer metastasis. The dysfunction of cell migration is linked to diseases such as vascular disorders and tumours.[3] Therefore, studying actin, the key protein involved in cell migration, could provide valuable insights into these malfunctions and aid in developing potential solutions.

However, cell labelling alone is not sufficient. Imaging techniques, which allow us to obtain visual representations of structures as small as cells (and even smaller), are essential for gathering the necessary information. Microscopy is the imaging technique typically used when working with cells as samples. For this study, fluorescence spectroscopy combined with confocal microscopy was employed. Since cell autofluorescence does not allow a proper study of cell structures, the use of fluorophores, molecules that absorb light and re-emit it at a longer wavelength, is required. Cell labelling is then accomplished through the conjugation of fluorophores with molecules that selectively bind to the target of interest (labelling agents).

This study focuses on investigating actin labelling by testing various labelling agents and media. Selecting the most suitable fluorophore is not always straightforward, due to the potential differences in labelling efficiency. Even though different fluorophores can provide a good fluorescence signal, it will also be of interest to understand the affinity of the labelling agents towards the different actin structures.

3.2 STRUCTURE OF ACTIN

Actin is a widely conserved protein family present in all eukaryotic organisms and represents the most abundant intracellular protein within eukaryotic cells.[3] The actin cytoskeleton, composed of actin filaments along with associated and regulatory proteins, serves as the cell's main machinery for generating force. Actin exists as filamentous actin (F-actin), which is in dynamic equilibrium with globular actin (G-actin), continuously undergoing cycles of polymerisation and depolymerisation.[6,7] The cell uses the energy of actin polymerization to generate protrusive forces and generates contractile forces through mutual sliding of actin and myosin filaments.[8]

In birds and mammals, actin has six isoforms, also named isoactins. However, only two of them are present in non-muscle cells, such as, used in this study, HeLa cells. Non-muscle β- and γ-actin play specific roles in many actin-controlled processes including cell–cell junction formation, axon development, cell division, and cell migration.[9]

These isoactins form F-actin that assemble into different structures to perform specific functions. The main types of actin structure are: lamellipodia, filopodia, stress fibres, cortical actin, actin comet tails, actin meshworks, podosomes and actin rings. However, HeLa cell isoactins are limited to the first four structures as illustrated in Figure 1.

Lamellipodia are major protrusions consisting of networks of actin filaments that are formed by migrating cells on flat surfaces. The formation of dense branched actin networks in lamellipodia, caused by cycles of nucleation, filament elongation, and capping, push the plasma membrane forward.[10,11] Filopodia, on the other hand, are slender, finger-like cytoplasmic extensions consisting of tightly packed actin filaments that protrude beyond the leading edge of lamellipodia. The filaments within filopodia are closely packed and aligned in the same direction, forming what are known as parallel bundles.[12] Both structures play a crucial role in facilitating cell motility.

The contractile actin cortex of eukaryotic cells is a thin layer, approximately 100 nm thick, that uniformly underlies the plasma membrane and plays a crucial role in regulating the cell's shape. This cortex consists of actin filaments organised into bundles, with myosin filaments distributed among them, forming a cross-linked gel-like network. The sliding motion of actin filaments against one another generates contractile forces essential for maintaining cellular tension and facilitating shape changes.[10,13]

Stress fibres are contractile bundles of actin and non-muscle myosin II filaments that generate tension within the cell. They are anchored to the plasma membrane through focal adhesions, which link them to the extracellular matrix. In migrating cells, these forces can either disrupt focal adhesions, leading to local retraction, or strengthen them, enabling later protrusion..[8]

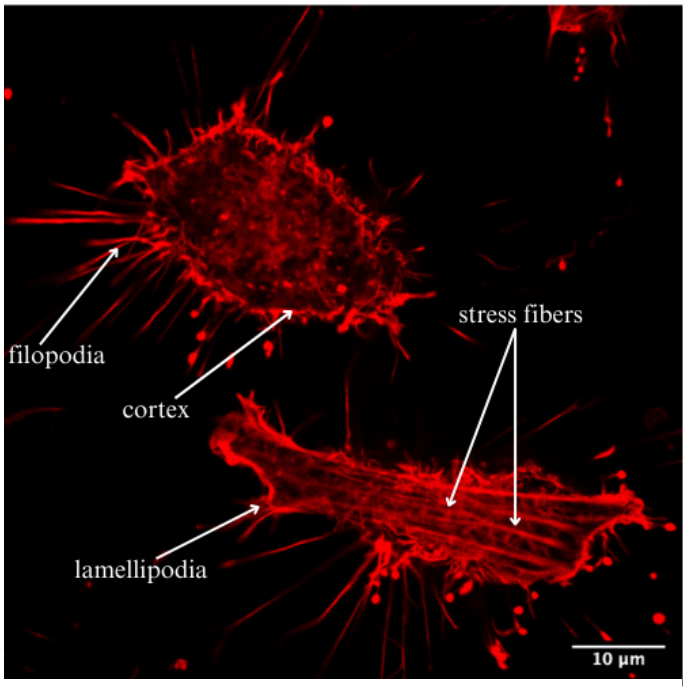


Figure 1: Cell structure with actin structures indicated. (image obtained through the study of images in xyz acquisition mode)

3.3 ROLE OF CELL FIXATION AND LABELLING AGENTS ON ACTIN CHARACTERIZATION

Cell fixation is a crucial step when experimenting with fixed cells. The objective of fixation is to achieve good morphological preservation, while stopping the degradative process of autolysis.[14,15]

The most common chemical fixatives are aldehyde-based and dehydrating fixatives.

Dehydrating or denaturing fixatives, like methanol, disrupt lipids and reduce the solubility of protein molecules, causing them to denature and precipitate in place. This denaturation can expose hidden epitopes, i.e., antigen molecules of the target protein, which can be beneficial for linking certain antibodies. Because the organic solvents remove lipids from the cell membrane, they can be used to fix and permeabilize cells at the same time. [14–16]

Aldehyde-based fixatives such as formaldehyde, are the best for preservation of tissue and protein structure. Aldehydes react with and crosslink cellular proteins, stabilizing and hardening the sample; they stabilize tissues by reacting primarily with basic amino acids to form methylene crosslinks. However, this also makes access for reagents more difficult and possibly masks some epitopes, i.e., antigen binding sites, making some proteins lose their antigenicity.[17] Alternatively, ethanol or methanol permeabilization can be carried out after fixation. This technique merges the fixation properties of crosslinking fixatives with partial denaturation, enhancing the signal for specific targets, especially those related to organelles or the cytoskeleton. Methanol permeabilization has been shown to boost the effectiveness of certain labelling antibodies. [15,17]

After cell fixation, the next step is the selection of an adequate labelling agent. A variety of labelling agents are used in this project, each one with a distinct binding method. Two primary groups of labelling agents were used; immunolabelling agents (antibodies) and chemical fluorescence probes.

Immunofluorescence allows detection and localization of a wide variety of antigens through antibody (Ab) labelling, with each antibody binding specifically to its target. The ones used in this project, anti-pan actin and anti-beta actin Ab, are supposed to recognize all mammal actin isoforms in the form of F- and G-actin. The antibody may be coupled to a fluorophore (direct fluorescence) or may be detected by a secondary antibody conjugated to a fluorophore (indirect fluorescence), as can be seen in Figure 2. As secondary

antibodies can recognize all primary antibodies derived from the same host species, multi-labelling can only occur if primary antibodies are raised in different species.[18,19] The indirect fluorescence is the one used in this experiment, since it ensures a higher fluorescence signal because of multiple secondary antibodies binding to each primary antibody. In this method, fixation is mandatory, particularly due to the need to permeabilise the cell, allowing the antibodies to enter and bind to actin. Fixation is also essential to stabilise the sample and protect it from the harming effects of the immunolabelling process.

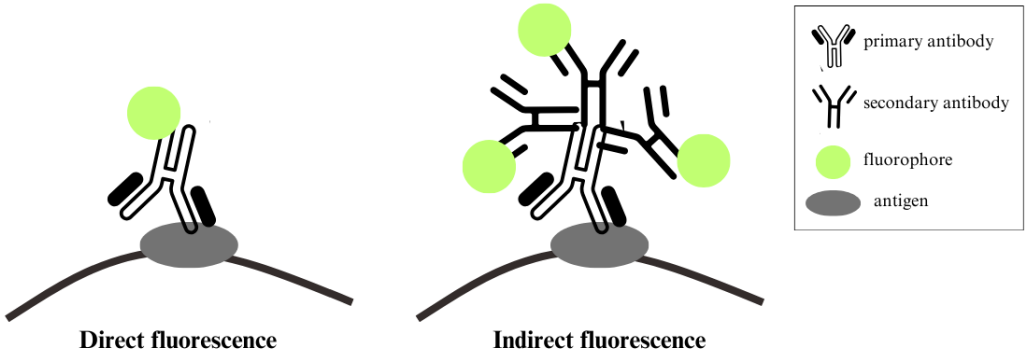


Figure 2: Diagram of the two types of immunolabelling: direct and indirect fluorescence.

In contrast, phalloidin, SiR-actin, and SPY-555 are chemical probes conjugated with fluorophores. These probes bind directly to actin, stabilising F-actin in the process. A shared characteristic of these actin-stabilising agents is their cyclic depsipeptide structure (Figures 3 and 4); a polymeric compound composed of both amino acids and hydroxy acids linked by peptide and ester bonds.

Phalloidin is a member of the group of phallotoxins produced by the mushroom *Amanita phalloides*, which binds to the quaternary structure of F-actin and prevents its depolymerization, resulting in cytotoxicity. Labelled phallotoxins have similar affinity for both large and small actin filaments and bind in a stoichiometric ratio of about one phallotoxin per actin subunit in both muscle and non-muscle cells. It has been reported that they do not bind to monomeric G-actin, in contrast to the antibodies used in this study.[20,21] Phalloidin can be conjugated to a broad range of fluorophores, i.e. the wide range of Alexa Fluor fluorophores.[22]

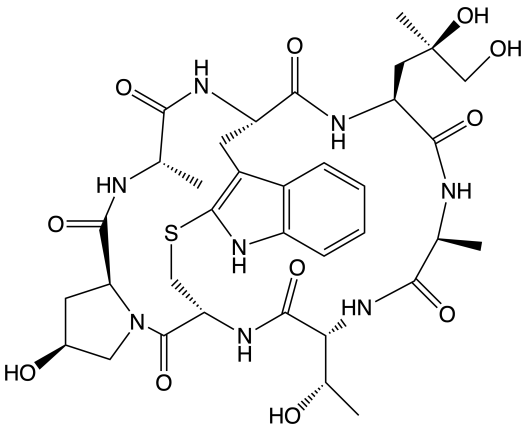


Figure 3: Phalloidin structure

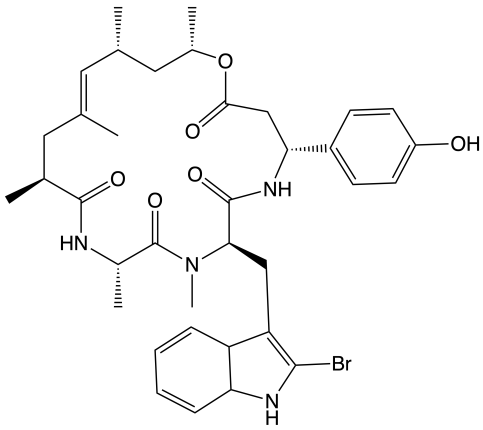


Figure 4: Jasplakinolide structure (SiR-actin and SPY-555's base molecule)

SiR-actin and SPY-555 are molecules derived from jasplakinolide, a macrocyclic peptide isolated from the marine sponge *Jaspis johnstoni*. Jasplakinolide is a potent inducer of actin polymerization by stimulating actin filament nucleation. SiR-actin is based on the fluorophore silicon rhodamine (SiR) and the actin binding natural product jasplakinolide, whereas SPY-555's structure is considered a trade secret due to intellectual property protections and market competition. The key features of SiR-actin and SPY-555 actin probes include their cell permeability, fluorogenic properties, minimal cytotoxicity, and photostability. For this reason, both probes do not require fixation and are compatible with live-cell imaging; however, in this study, only fixed cells were tested. Furthermore, SPY-555 represents an improved version of SiR-actin, enabling robust labelling at lower concentrations while reducing cytotoxicity and minimising perturbation of actin cytoskeletal dynamics.[17,23]

Despite the origin differences, phalloidin- and jasplokinoide-based fluorophores are expected to have similar interactions with F-actin.[24]

3.4 IMAGING AND CHEMOMETRIC TECHNIQUES FOR CELL STUDIES

Imaging techniques are methods used to create visual representations of objects, structures or processes. When analysing cell samples, microscopy helps us visualise cellular structures, offering varying levels of spatial resolution depending on the specific technique employed. The confocal microscope is particularly suitable to study labelled small samples, such as cells, at a very high spatial resolution of few nm per pixel.

As shown in Figure 5, laser scanning confocal microscope (LSCM) uses a pinhole placed in the imaging path to allow only light from the focal plane to reach the detector, eliminating out-of-focus fluorescence and therefore providing a clean, high-resolution image associated with a specific sample plane. A laser excites the fluorophore in a focused pixel spot, which is then scanned across the specimen using scanning mirrors. A hybrid detector (the combination of PMT and ADPs detectors) detects the resulting photons, and the image is digitally reconstructed pixel by pixel, generating a 3D image (xyz) when acquiring multiple planes of the same cell.[25,26] Additionally, LSCM allows the acquisition of images where a range of emission wavelengths is recorded at each spatial point (xyλ), known as hyperspectral images (HSIs).[27] Each image acquisition type provides valuable information tailored to specific study objectives.

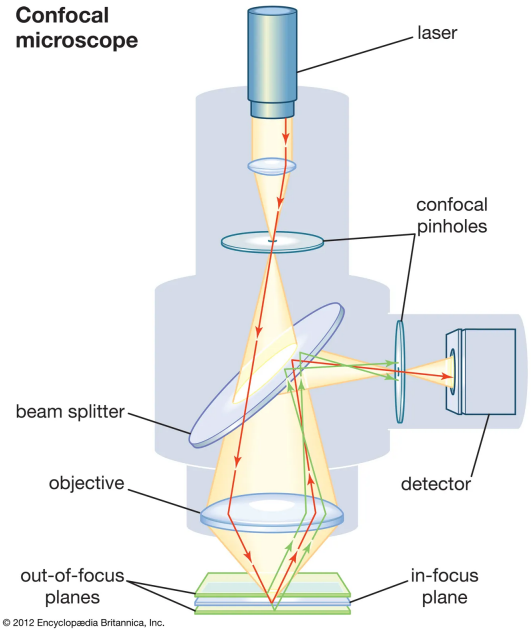


Figure 5: diagram of a confocal microscope (Ford, B. J., & Shannon, R. R. (2012), 26/12/2024 via *Encyclopaedia Britannica, Inc.)*

There are different image acquisition modes when working with a confocal microscope, as mentioned before. For the study of the labelling agents' affinity towards actin, the acquisition of HSIs followed by a chemometric analysis was considered the most effective approach since both the spatial resolution of the imaging technique and the use of the differences in the emission spectrum of the various labelling agents can contribute to a better understanding of the actin structures.

Hyperspectral imaging (HSI) generates a three-dimensional (3D) image cube of spectral and spatial information, obtained by collecting an ensemble of pixel spectra at across the sample plane. Each pixel has its characteristic emission spectrum, dependent on the contribution of the different fluorophores in that particular spatial spot.[27,28]

To interpret the large number of mixed spectra collected in a hyperspectral image, unmixing analysis techniques, such as Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS), are required. This chemometric tool helps to unmix the complex information in the initial image cube into the pure contributions associated with the different fluorophores present in the sample studied, as shown in Figure 6. Per each fluorophore, a distribution (or concentration) map and the related pure fluorophore emission spectrum

are obtained. MCR-ALS can analyse HSI data from multi-labelled samples and clarify differences in the cell structures labelled by all agents used.[29]

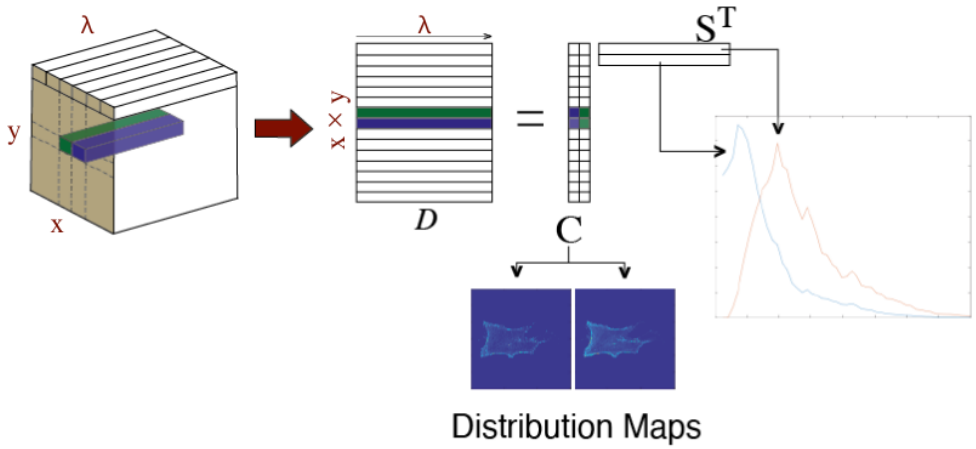


Figure 6: Description of the unmixing analysis carried out by MCR-ALS. **D** refers to the unfolded image cube into a matrix of pixel emission spectra. **C** are the concentration maps and $\mathbf{S}^T$ the pure emission spectra of the fluorophores used for labelling.

4 OBJECTIVES

The objectives of this study are focused on optimising the labelling and imaging of actin in HeLa cells using advanced fluorescence microscopy techniques to enable a detailed investigation of the affinity of different fluorophore typologies to the diverse actin structures. To achieve these general objectives, the following studies will be carried out:

- Evaluation and comparison of different fixation procedures, such as PFA, methanol, or a combination of PFA/methanol, to observe the effects on the binding effectiveness of the labelling agents to actin.
- Testing different labelling actin agents, such as phalloidin, SPY-555, SiR-actin, anti-beta actin Ab and anti-pan actin Ab, to investigate their potential and specificity on actin labelling for fluorescence microscopy.
- Utilization of a confocal microscope to obtain spatial and spectral information (xyz and xyλ modes), acquiring high-spatial resolution images and hyperspectral data as initial information for studying actin structures and fluorophore affinity.
- Performing chemometric analysis on the hyperspectral data to obtain crucial information, like concentration maps and emissions spectra of pure fluorophores, for a clear understanding of the affinity and specificity of different labelling agents towards actin.

5 EXPERIMENTAL SECTION

5.1 SAMPLE PREPARATION

5.1.1 Cell culture and cell fixation procedure

The samples used in this experiment were HeLa cells, provided by the biological laboratory at ICFO, which are referred to as immortal due to their unlimited capacity to proliferate. They were derived from a cervical carcinoma of a 31-year-old female, being the first human cell line maintained in continuous cell culture.[30]

To proceed with the cell culture, it is crucial to ensure that the work area, including the laminar flow hood and materials, is sanitised using UV light and ethanol. To begin with, a vial of cryopreserved cells is taken from liquid nitrogen and the cells transferred to a culture flask containing fresh medium. The cells are kept at an incubator at 37ºC and 5% CO_2 until cell fixation. The cells are allowed to defreeze for 24 hours and are afterwards rinsed twice with phosphate-buffered saline (PBS), a buffer solution (pH ~ 7.4), to remove dimethyl sulfoxide (DMSO), a cryoprotectant that is toxic to cells if left for extended periods. Trypsin is added and the flask is incubated for five minutes to detach the cells from the bottom. Subsequently, fresh medium is added and a preparation of 1:8 dilution is done in a new flask, adding additional medium. This process is repeated two or three times before fixing the cells.

The days and dilutions should be monitored carefully to achieve the desired level of confluence. For this experiment, we aimed for 50% confluence to capture the interaction of neighbouring cells without overcrowding. Finally, some of the cells were transferred to a cover-glass-bottom dish (type #1.5) to observe them under the microscope.

To fix the cells, three fixation procedures with paraformaldehyde (PFA), methanol and PFA/methanol were tested. Before fixation, it is required to remove the cell culture medium and rinse twice with PBS. For PFA fixation, a PFA 4% solution was used, the cells were left in the shaker for 10 minutes.[31] For methanol fixation, the cells were left in the shaker for 10 minutes with absolute methanol at -20ºC.[32] For PFA/methanol, the cells were fixed with a 4% PFA solution and left 10 minutes in the shaker, then rinsed twice with PBS and finally, the cells were left in the shaker with absolute methanol at 4ºC for 30 minutes.[33] To finalize with the fixation procedure, the cells were rinsed twice with PBS.

5.1.2 Labelling procedure

The labelling agents used for the experiments (along with the conjugated fluorophores), their fluorescence properties and their chemical typology are listed in Table 1.

Table 1: list of labelling agents used and relevant spectral and typology information about them

Labelling agents[(a)(b)]	λ excitation [nm][(c)]	λ emission range [nm][(d)]	Agent typology
Phalloidin			Chemical fluorescence probe (mycotoxin)
Alexa Fluor-488[34]	488	498-600	
Alexa Fluor-568[35]	568	578-700	
SiR-actin[36]			Chemical fluorescence probe (drug)
Silicon rhodamine	652	662-800	
SPY-555[37]	555	565-700	Chemical fluorescence probe (drug)
pan-actin Ab[33]			Antibody
ASTAR Orange[38]	589	598-750	
ASTAR Red[39]	638	648-800	
beta-actin Ab[40]			Antibody
ASTAR Orange[38]	589	598-750	
ASTAR Red[39]	638	648-800	

(a) Alexa Fluor and ASTAR (Abberior Star) are the conjugated fluorophores used in this experiment. Fluorophore present in commercial SPY-555 is not known for confidential reasons.
(b) Only the name of the primary antibody and the fluorophores are indicated, secondary antibody is the same, anti-mouse, for both.
(c) The single excitation wavelengths are those given by the laser source. When acquiring double-fluorophore images in xyλ mode, the lowest excitation wavelength of the two was used to detect all fluorescence emission of both.
(d) Emission range refers to the range capturing most of the fluorescence emission.

There are two different labelling procedures depending on the nature of the labelling agent used: one for antibodies and another for chemical fluorescence probes.

- *Labelling for chemical fluorescence probes*

When using phalloidin conjugated with a fluorophore, SiR-actin and SPY-555, the same labelling procedure takes place. After the cell fixation, a fresh solution of 1,5% bovine serum albumin (BSA) and 0,05% Triton X-100/PBS was prepared for blocking and permeabilization of the cells. All potential nonspecific binding sites in the tissue sample must be blocked to prevent nonspecific binding.[41] The permeabilization helps the probes to enter inside the cell, as mentioned before, SiR-actin and SPY-555 are able to permeabilize the cells on their own, however, phalloidin cannot. The solution is added to the cover-glass and left in the shaker for one hour and afterwards rinsed twice with PBS. The phalloidin (1:500), SiR-actin (1:1000) or/and SPY-555 (1:1000) are added following the recommended dilutions on the Safety Data Sheet (SDS) and is left with no agitation for one hour. To finalize, three washes with PBS are carried out.[31,36,37]

- *Labelling for antibodies*

In this experiment the primary antibodies used are either anti-pan actin Ab or anti-beta actin Ab. Both are mouse monoclonal Ab, meaning that the antibodies are generated from immune cells coming from mice. Because of this, the secondary antibody is anti-mouse and specifically binds towards mouse monoclonal antibodies.

After fixation, a fresh solution of 1,5% BSA for blocking and 0,05% Triton X-100/PBS for permeabilizing were used to cover the cells and leave them in the shaker for 1 hour. Afterwards, the cells were incubated with the primary antibody (either anti-pan actin Ab or anti-beta actin Ab) at a final concentration of 8 µg/mL and left in the shaker for two hours. Once the cells were washed twice with PBS, the secondary antibody conjugated with the suitable fluorophore was added. The secondary antibody was diluted with the previous permeabilizing and blocking solution to a final concentration of 8 µg/mL and was left without agitation for one hour. It was observed that permeabilizing the cell when adding the secondary antibody increased the fluorescence signal. To finalize, three washes with PBS were carried out.[33]

As it will be described in more detail in the results (section 7.1.), the labelling procedure when several labelling agents are used on the same sample needs to be slightly modified according to the nature of the combination of agents used.

5.2 IMAGE ACQUISITION

The images were obtained with a super-resolution and confocal fluorescence microscope, LEICA TCS SP8 STED 3X microscope (LEICA MICROSISTEMS, Mannheim, Germany), placed at ICFO.[42] In this project, two types of images were acquired using xyz and xyλ acquisition modes. The xyz acquisition mode allows capturing images at different planes of the cell and reconstructing a three-dimensional image. The signal obtained in each pixel corresponds to a single value, integrating the fluorescence in the full emission range selected. The xyλ acquisition mode provides images in a single two-dimensional plane. However, the detector collects fluorescence emission values at different wavelength channels. In this case, every pixel is associated with a full fluorescence emission spectrum.

To obtain all images, an oil immersion objective was used (HC PL APO CS2 100x/1.40 OIL). The process began by applying oil to the lens. After placing the cover-glass and focusing on the cells, the next step was to select a single cell for measurement.

5.2.1 xyz acquisition mode

These types of images were obtained to conduct a preliminary analytical study to investigate the efficiency of the labelling agents and the effect of the fixation medium and other reagents used on the fluorescence intensity emitted by the cell sample.

In the xyz acquisition, a stack of images covering the entire cell width was acquired. After locating the cell, a z-stack was done from the beginning to the end of the cell volume, with a z-step size of 0,30µm. The zoom factor was consistently set to 1.59 for all measurements, along with the 1024x1024 format, resulting in a pixel size of 71.53 x 71.53 nm. The acquisition speed was set at 400 Hz. Collectively, these settings enabled the acquisition of an image with a high spatial resolution. The type of laser and its intensity, the frame accumulations and the detector emission range varied depending on the sample and the labelling agent used. The laser

excitation wavelength depends on the maximum absorption of the fluorophore, as shown in Table 1. The intensity of the laser and the frame accumulations depend on the sample brightness; however, it is important to consider the photobleaching effect, as it can lead to the degradation of the fluorophore. The emission range starts 10 nm above the laser wavelength to avoid detecting the excitation light, which could interfere with the fluorescence signal from the sample, and extends until the emission intensity decreases, as indicated in Table 1. All the fluorescence intensity collected at the full emission range is integrated in a single value per pixel. Images where later processed with ImageJ, a Java-based image processing program, to adjust brightness and select a single and representative plane for the study.

5.2.2 xyλ acquisition mode

The xyλ images were obtained to study the affinity of different typologies of fluorophores with actin. To do so, single labelling and double labelling of cells were carried out. The acquisition of the full emission spectra in these images allows, with subsequent data analysis, to separate the signal coming from fluorophores with two closely overlapping emission spectra and determine the spatial location of them in the cell sample.

Without a z-stack, the z-position was determined by selecting the clearest and most interesting focal plane in the cell. The pixel size was set to approximately 100x100 nm in all images. Therefore, the variable parameters in these images are the spatial resolution and the zoom factor. The acquisition speed remained set at 400 Hz. The emission range also started at 10 nm above the laser wavelength and was extended until the emission intensity decreased.

Two double-labelling combinations, with overlapping fluorophores, were selected for the affinity study: the first consisting of SPY-555 and phalloidin-568, to study the affinity of two labelling agents of similar typology (both chemical fluorescence probes), and the second containing phalloidin-568 and anti-pan actin Ab conjugated with Abberior Star Orange, to study the combination of labelling agents of different nature (antibody and chemical fluorescence probe). The emission spectra of these fluorophores can be found in the appendices.

For the images detecting phalloidin-568 and SPY-555, the laser was set at a 555 nm, and the emission range went from 565 nm to 720 nm, in a λ step-size of 3 nm. For the images detecting phalloidin-568 and pan-actin Ab (conjugated with Abberior Star Orange), the laser was set at 568 nm excitation wavelength, and the emission range went from 578 nm to 721 nm, in a λ step-size of 3 nm.

6 DATA TREATMENT

Data treatment is an essential aspect of this project and a mandatory task to extract detailed information from xyλ images, which are organized in the form of a hyperspectral cube. This cube contains two dimensions of spatial information (x and y pixel coordinate axes) and, in the third dimension, the spectra related to each pixel (λ), forming a hyperspectral cube [27,28], as seen in Figure 7.

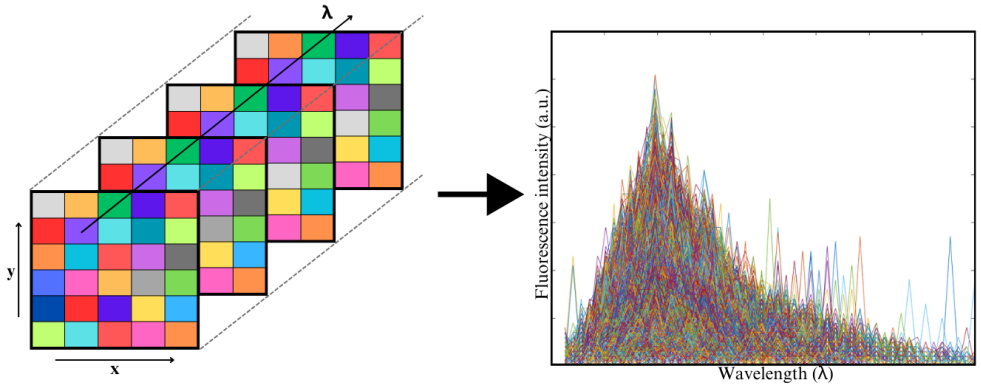


Figure 7: On the left, a representation of a hyperspectral cube with the two spatial directions (x and y pixel coordinates) and the spectral direction (λ). On the right, the emission spectra corresponding to each pixel on a real cell image labelled with phalloidin).

6.1 PREPROCESSING OF IMAGES

To begin with, a preprocessing of the spectra and spatial data was performed.

To improve the signal-to-noise ratio of the spectra, spatial binning was performed by summing the spectra of adjacent pixels to create larger pixels, each one associated with a higher-intensity signal and a single spectrum.[43] For single-labelled fluorophore images, a 5x5 binning factor (25 pixels merged into 1) was used to get better defined spectra (see Figure 8), while for double-labelled fluorophore images, a 2x2 binning factor was applied to keep better spatial resolution and, hence, distinguish more accurately the differences in the labelling behaviour of the two agents used.

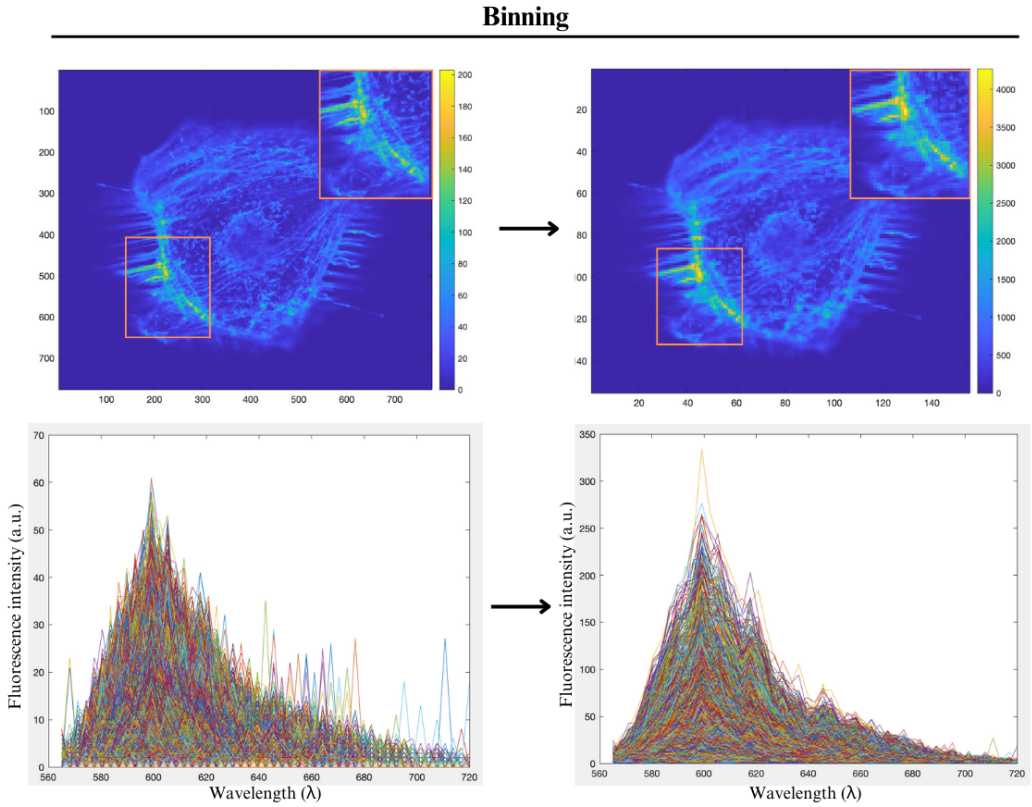


Figure 8: On the left, raw image and related emission spectra. On the right, image and emission spectra after a binning of 5x5. The improvement of the signal-to-noise ratio of the spectra can be observed.

6.2 MULTIVARIATE CURVE RESOLUTION – ALTERNATING LEAST SQUARES (MCR-ALS)

The hyperspectral cube contains extensive and complex data, requiring specialized tools to extract meaningful information. For this purpose, chemometric analysis using Multivariate Curve Resolution – Alternating Least Squares (MCR-ALS) was conducted with a MATLAB program.[44]

In fluorescence studies, MCR-ALS proves highly effective in distinguishing closely overlapping emission spectra. The detector collects the combined signal, and through this analysis, it becomes possible to identify which fluorophores are contributing to the signal.[29]

A hyperspectral image (HSI) is represented as a data cube, where the x and y axes correspond to pixel coordinates, and the λ axis represents the spectral dimension.[27] Each pixel in this cube is associated with a complete spectrum. When unfolded, the HSI cube becomes a matrix (D), where rows contain the spectra of individual pixels. Unmixing methods use a bilinear model, similar to Lambert-Beer's law, to decompose the total spectral signal into contributions from individual components within the image.[43]

The observed spectrum in each pixel (d_i) can be expressed as the sum of signal contributions from all components (N), each weighted by its concentration ($c_{i,n}$) and its pure spectrum (s_n^T) (Equation 1). The typical bilinear model for unmixing methods is represented compactly, where matrix S^T contains the pure spectral profiles of the components in the image, and matrix C holds the corresponding concentration profiles (Equation 2). The residuals of the model are expressed by e_i and E.[43]

$$d_i = \sum_{n=1}^{N} c_{i,n}\, s_n^T + e_i \quad [43] \qquad \text{Equation 1}$$

$$D = CS^T + E \quad [43] \qquad \text{Equation 2}$$

Using MCR-ALS, the raw information in hyperspectral images can be described by the concentration maps and emission spectra profiles of the fluorophores in the sample. This approach facilitates the identification and spatial distribution of different fluorophores, providing valuable insights into the sample composition.

MCR-ALS is an iterative unmixing method that works optimizing matrices $\boldsymbol{C}$ and $\boldsymbol{S^T}$ following an alternating least-squares calculation under constraints. In this case, the constraints applied were non-negativity for both concentrations and spectra, as well as the correspondence of species, which indicates the absence or presence of a fluorophore in a sample, when several of them are analysed simultaneously. The use of constraints provides more accurate results and better-defined concentration profiles and spectra.[29,43]

The method starts by doing an estimation of the number of components present in the original data set $\boldsymbol{D}$ by Principal Component Analysis (PCA)[45] or taking advantage of previous knowledge of the sample. Since the samples of this work were prepared in the laboratory, the number of fluorophores used was known. Thereafter, an initial estimate of matrix $\boldsymbol{S^T}$ was generated using an algorithm based on the Simple-to-use Interactive Self-modelling Mixture Analysis (SIMPLISMA)[46] method. This method selects a number of pixel spectra equal to the number of components required by the model and the chosen spectra are the most dissimilar of the image under study. This $\boldsymbol{S^T}$ initial estimate, along with the matrix $\boldsymbol{D}$, was used to initiate the least-squares alternating optimization of $\boldsymbol{C}$ and $\boldsymbol{S^T}$, under the constraints defined above, until convergence was achieved. The convergence criterion was defined based on a value related to the difference in fit improvement between consecutive iterations.[43]

To help in the separation of the fluorophore contributions present in a double-labelled HSI, this image was analysed together with HSIs of their individual fluorophore-labelled images, building a single data structure, called multiset. This multiset is built by putting one HSI below the other, resulting in a column-wise augmented multiset $\boldsymbol{D_{aug}}$. The images must have identical spectral range and consistent wavelength step-size. In this project, all multisets were built by putting the single-labelled images in the first and second image blocks, and the double-labelled image in the last row, as shown in Figure 9.

After application of MCR-ALS to the multiset, the resulting bilinear model was formed by a single $\boldsymbol{S^T}$ matrix with the two emission spectra of the fluorophores used, and an augmented $\boldsymbol{C_{aug}}$ matrix containing the concentration maps of each fluorophore in each of the images analysed.[43]

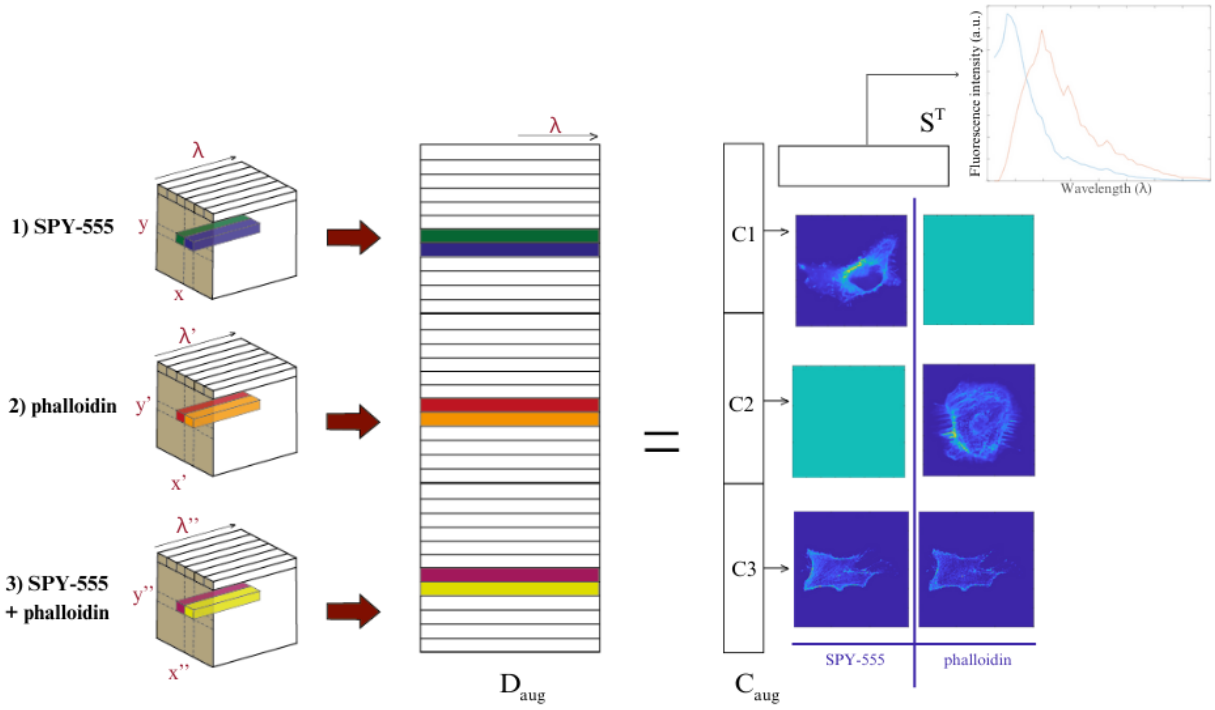


Figure 9: : representation of the MCR-ALS descomposition of a multiset using non-negativity and correspondence of constraints. In sample 1) it is set the presence of SPY-555, in sample 2) the presence of phalloidin and in sample 3) the presence of both fluorophores. Green maps are null maps because the related fluorophore is absent in the image.

All preprocessing operations were done with in-house routines under MATLAB environment. A MATLAB coded Graphical User Interface (GUI) was used to perform the MCR-ALS analyses.[44]

7 RESULTS AND DISCUSSION

7.1 SELECTION OF FLUOROPHORES AND EFFECT OF FIXATION MEDIUM. STUDY OF XYZ IMAGES.

The aim of this analytical study was to identify the optimal methods for single and double labelling of actin. The study of double labelling of actin is interesting for the affinity study, where a comparison between the different fluorophores' behaviours would be performed. To conduct this study, several techniques for labelling each fluorophore were tested, as illustrated in Figure 10. Although these images were acquired as a z-stack, the images presented here are shown in two dimensions (xy) for the sake of clarity.

Phalloidin was the first labelling agent tested. PFA fixation provided high-quality results, whereas methanol and PFA/methanol fixation produced very poor-quality images. SPY-555 and SiR-actin, labelling agents of similar typology to phalloidin, exhibited the same behaviour and gave the best fluorescence signal using PFA fixation. In the research group where this project was conducted at ICFO, SPY-555 had not been tested previously, making it particularly intriguing to evaluate its functionality for actin labelling. Subsequently, anti-pan actin Ab and anti-beta actin Ab were tested using the same three fixation methods, with PFA/methanol fixation yielding the best results.

To justify the experimental results obtained with single labelling, section 3.3 has to be taken into consideration. Indeed, phalloidin, SPY-555 and SiR-actin bind specifically to the quaternary structure of F-actin, stabilising the filaments. For these probes, alcohols like methanol denature the native protein conformation, leading to labelling incompatibility. However, as mentioned earlier, cross-linking (via PFA) can mask antigens when working with antibodies. Due to their larger size compared to fluorescent probes, antibodies face greater challenges in reaching every corner. For antibodies, the combination of PFA fixation followed by methanol permeabilization helps preserve the actin cytoskeleton structure while introducing partial denaturation that aids in unmasking antigens.

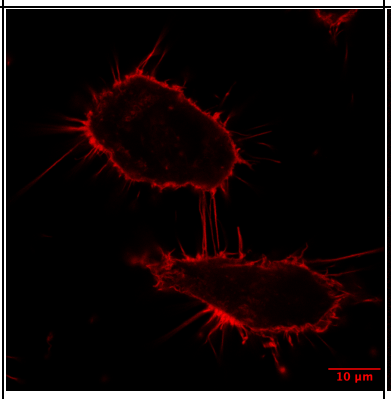
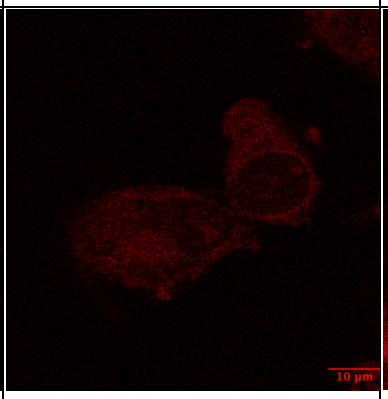
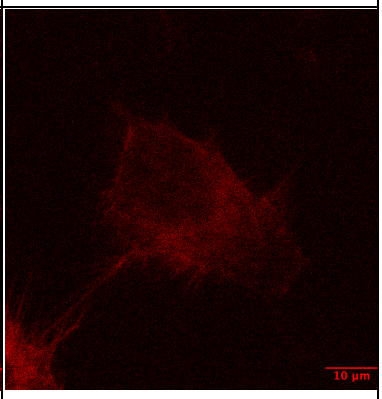
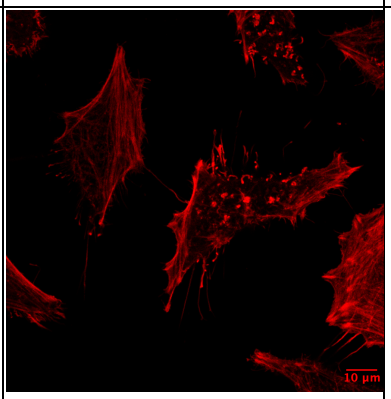
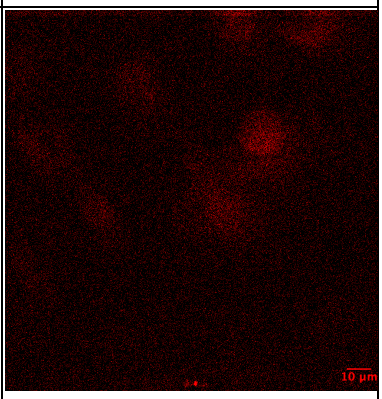
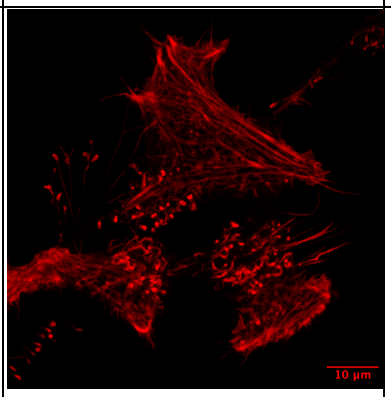
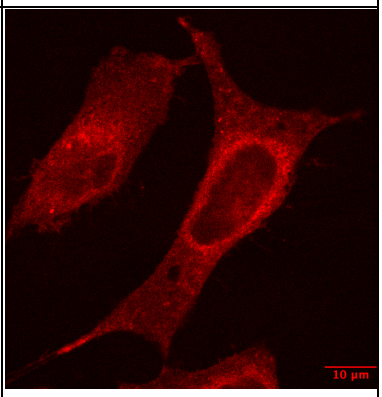
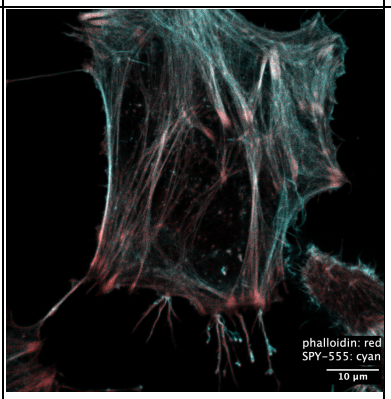
When trying to do double labelling, some considerations need to be taken into account about the possible labelling agent combinations. For instance, double labelling with anti-pan actin Ab and anti-beta actin Ab is unfeasible. As noted earlier, both antibodies are derived from mouse immune cells. Consequently, the secondary antibody is anti-mouse, making it impossible to perform specific double labelling with anti-pan actin Ab and anti-beta actin Ab, since the secondary antibody, carrying the fluorophore, would bind non-specifically to both primary antibodies.

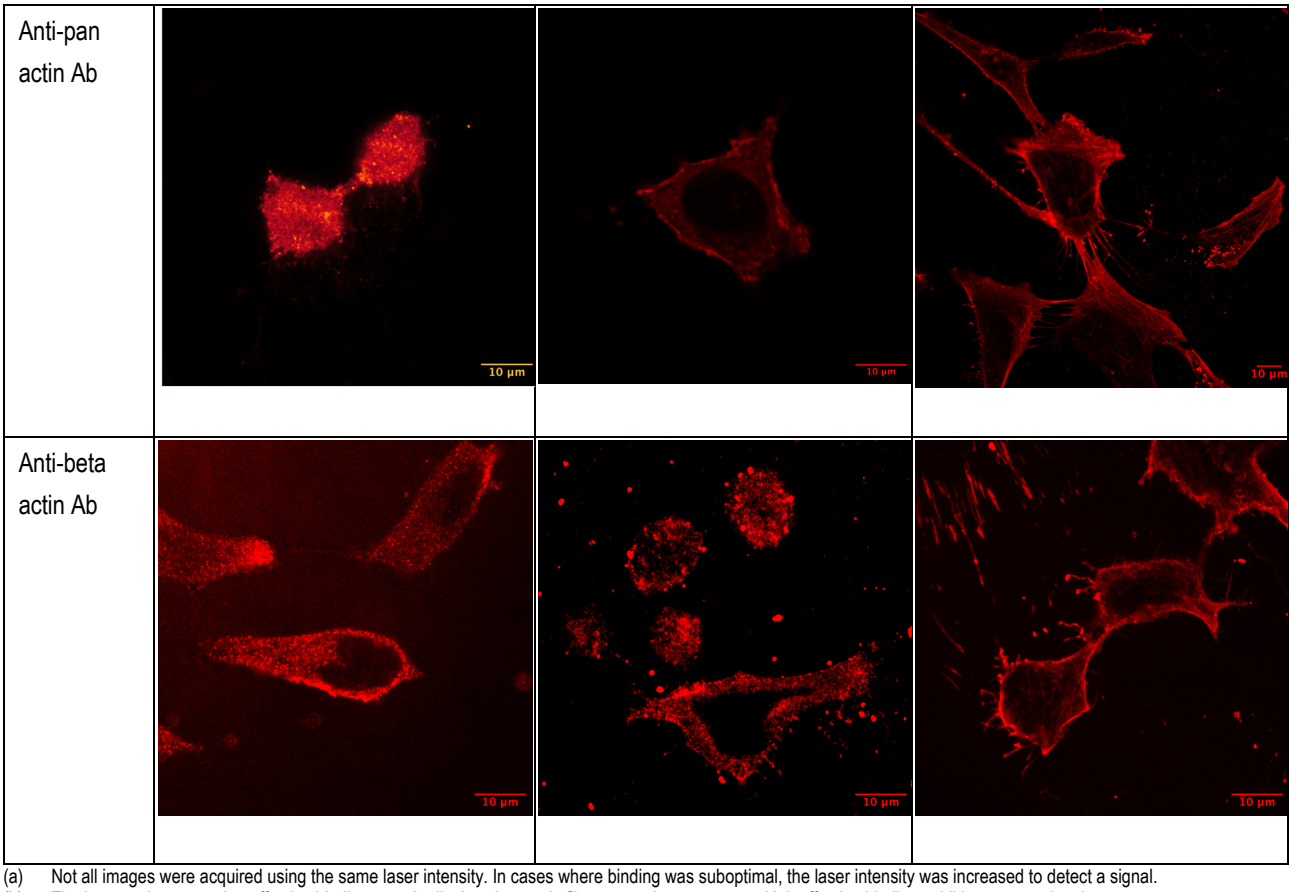
As the chemical fluorescent probes (SiR-actin, SPY-555, and phalloidin) provided high-quality results with the same cell fixation method, PFA fixation, double or even triple labelling was made possible. A double labelling was conducted using phalloidin-488 and SPY-555 to confirm the effectiveness of PFA fixation, particularly in preparation for the future affinity study with xyλ images. Furthermore, a triple labelling consisting of SiR-actin, SPY-555 and phalloidin was conducted to make a superficial comparison of the three fluorophores, especially interesting for the lab group at ICFO. The results showed that SPY-555 provided the highest signal intensity, whereas phalloidin exhibited the lowest one. Therefore, if the aim is to minimise laser exposure to the sample, SPY-555 and SiR-actin would be the optimal choice. The triple labelling image is not presented, as the laser intensity for each fluorophore was adjusted to achieve similar fluorescence intensities, resulting in an image that is not representative of the previously explained results.

Looking at the results obtained with single labelling of chemical fluorescent probes and antibodies, it can be concluded that none of the fixation tested media works simultaneously for both families. In an attempt to establish an effective method for double labelling using an antibody and a chemical fluorescent probe, a sequential approach was tested. This involved first labelling with phalloidin using PFA fixation, followed by anti-pan actin Ab labelling with the PFA/methanol method. The sequential method successfully demonstrated both fluorophores labelling actin, as will be seen in the next section, where work with xyλ images will be presented.

As a summary of the study, the sequential method was determined to be the best method to label an anti-actin and a chemical fluorescence probe at the same time, while the PFA fixation method showed the best results for labelling multiple chemical fluorescent probes.

Figure 10: Images obtained combining different labelling agents (rows) and fixation media (columns) in xyz acquistion mode.

	PFA	Methanol	PFA/methanol
phalloidin			
SiR-actin			
SPY-555			
Phalloidin + SPY-555			



(a) Not all images were acquired using the same laser intensity. In cases where binding was suboptimal, the laser intensity was increased to detect a signal.
(b) The images demonstrating effective binding to actin display clear actin filaments, whereas areas with ineffective binding exhibit punctate signals.

7.2 INTERPRETATION OF AFFINITY OF LABELLING AGENTS ON ACTIN. STUDY OF XYλ IMAGES.

A second study was conducted to investigate the affinity of the different fluorophores for actin. For this study, hyperspectral fluorescence images were analysed using MCR-ALS. Thanks to the data analysis, samples containing fluorophores with overlapping emission spectra yielded images that clearly distinguished the labelling of each fluorophore.

This study demonstrates the possibility to distinguish two overlapping fluorophores, combining images acquired in xyλ and chemometrics with the ultimate goal of investigating whether different labelling agents mark the same actin structures.

Two double-labelling combinations were selected for this affinity study: the first consisting of SPY-555 and phalloidin-568, with a simultaneous labelling performed in PFA medium, and the second one comprising phalloidin-568 and anti-pan actin Ab conjugated with Abberior Star Orange, using the sequential labelling method that starts with PFA for phalloidin-568 and continues with PFA/methanol to label anti-pan actin Ab.

To analyse each of the two double-labelling systems mentioned above, MCR-ALS was applied to a multiset with the structure shown in Figure 7, formed by three blocks of image spectra: two single-labelled images with each of the fluorophores used in the double-labelling combination and the third image formed by the double-labelled sample. As described in section 6.2, the results obtained are a single $\boldsymbol{S}^{\boldsymbol{T}}$ matrix with the two pure emission spectra of the fluorophores used, and an augmented $\boldsymbol{C}_{\boldsymbol{aug}}$ matrix, containing the concentration maps of each fluorophore in each of the images analysed. In the next subsection 7.2.1, some analysis will be made on the resolved pure emission spectra by comparison with the information of related standard spectra. Subsection 7.2.2. will display the related concentration maps that provide insights into the distribution of the fluorophores on actin, paying special attention to the maps related to the double-labelled samples.

7.2.1 Pure emission spectra

For the multiset related to the system containing phalloidin and SPY-555, the emission maximum of phalloidin-568 (in orange in Figure 11) was observed at 599 nm, which is close to its standard emission peak of 603 nm.[35] The emission maximum of SPY-555 (in blue in the same figure) was detected at approximately 574–578 nm, compared to its standard emission peak of 580 nm.[37]

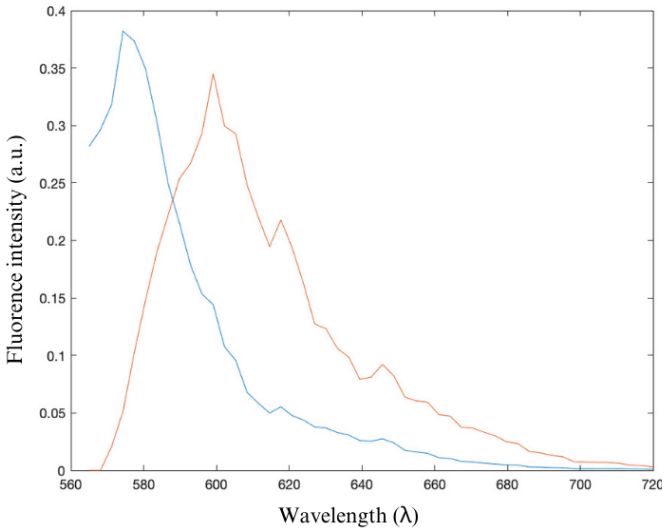


Figure 11: Emission spectra of phalloidin (orange) and SPY-555 (blue) obtained by MCR-ALS multiset analysis. The x axis corresponds to wavelenghts (λ) and the y axis to fluorescence intensity (in a.u.).

For the multiset related to the system containing phalloidin and anti-pan actin Ab, the emission maximum of phalloidin-568 (in orange in Figure 12) was observed at approximately 594 nm, which is close to its standard emission peak of 603 nm.[35] The emission maximum of Abberior Star Orange, i.e. the fluorophore for anti-pan actin Ab (in blue in the same Figure), was detected around 615 nm, compared to its standard emission maximum of 616 nm.[38]

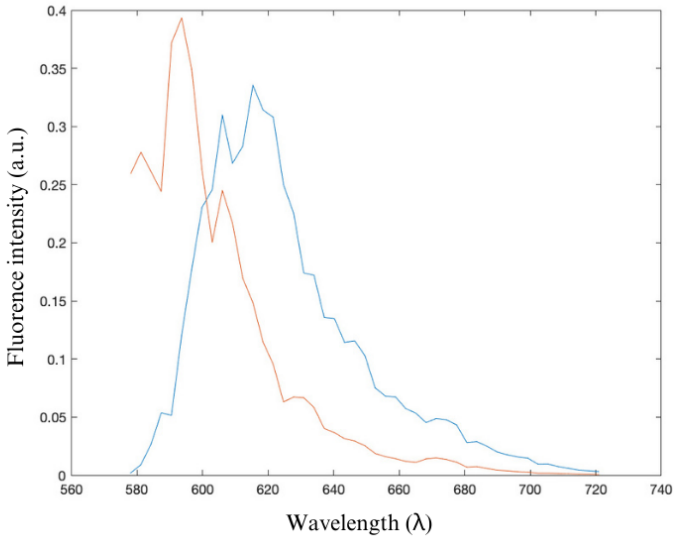


Figure 12: Emission spectra of phalloidin (orange) and anti-pan actin Ab (conjugated to ASTAR Or.) (blue) obtained by MCR-ALS multiset analysis. The x axis corresponds to wavelenghts (λ) and the y axis to fluorescence intensity (in a.u.).

Through the study of the emission spectra, the presence and identity of the two expected fluorophores in each sample is confirmed, due to their resemblance to the standard spectra. High-quality spectra are essential for obtaining accurate and reliable distribution maps.

7.2.2 Distribution maps

As mentioned before, MCR-ALS provides the distribution maps that give a precise localization of each of the fluorophores in the sample; a crucial information to understand the fluorophores affinity towards actin.

Figure 13 below shows the maps for the system combining phalloidin and pan-actin. Columns from left to right show the maps of the pan-actin single-labelled cell, phalloidin single-labelled cell and the double-labelled cell. The first row corresponds to pan-actin maps and the second row to phalloidin maps. Green maps relate to the absent fluorophore in the single-labelled images.

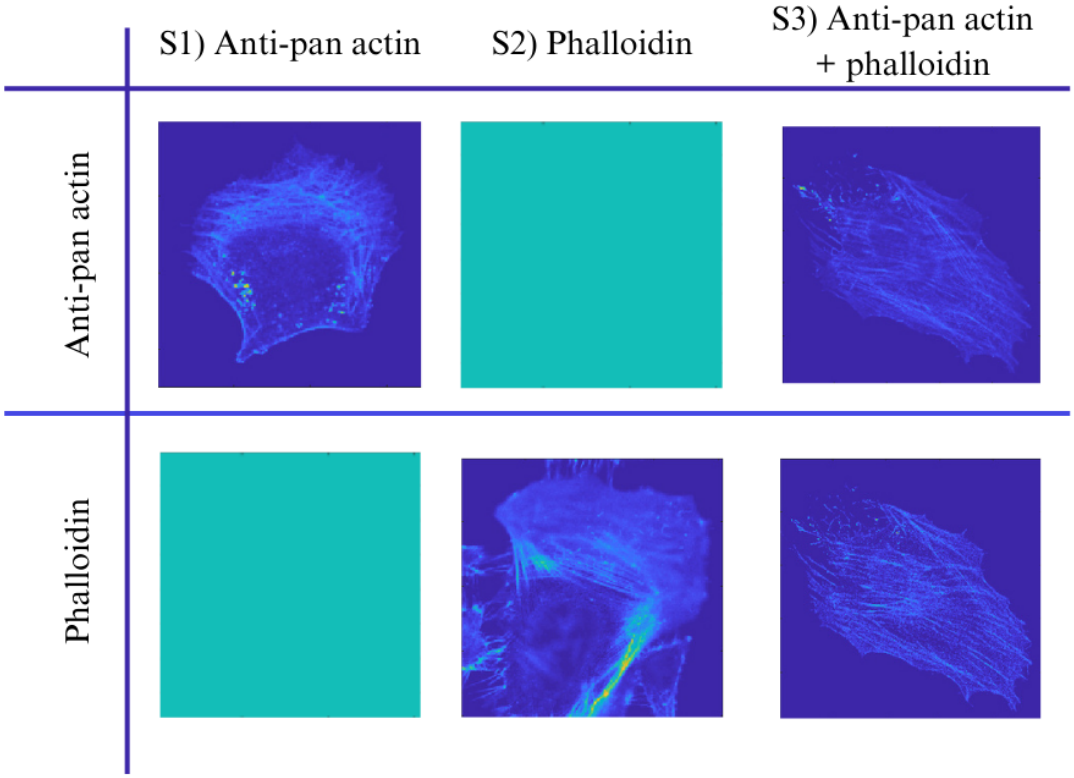


Figure 13: Concentration maps of phalloidin and anti-pan actin Ab from the multiset combining both labelling agents..Green maps represent null presence of a fluorophore in one image

Likewise, Figure 14 shows the analogous maps for the system combining phalloidin and SPY-555, being the first and second row related to SPY-555 and phalloidin, respectively.

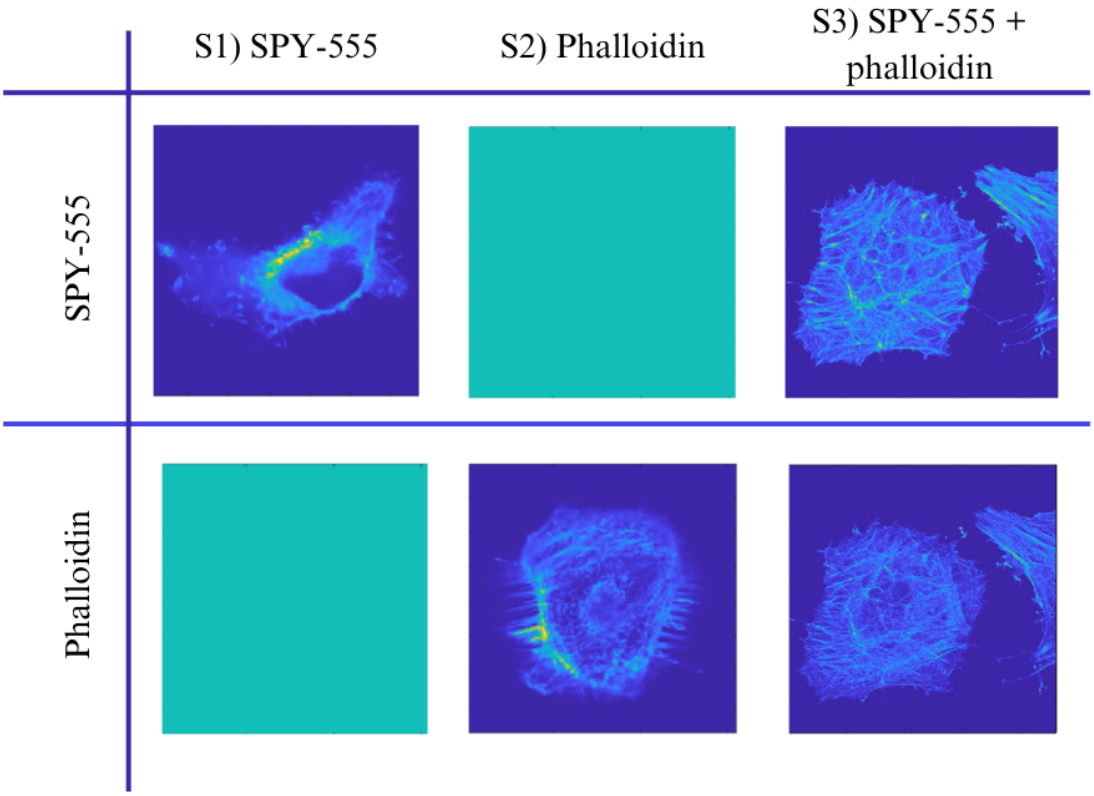


Figure 14: Concentration maps of phalloidin and SPY-555 from the multiset combining both labelling agents..Green maps represent null presence of a fluorophore in one image

In order to study the localization of both fluorophores present in the same sample, the distribution maps of each fluorophore in the double-labelling sample were overlaid. Each fluorophore is represented by a distinct colour, and when both coexist, the mixed colour is displayed.

When analysing the distribution maps of the double labelling for SPY-555 and phalloidin, distinct differences can be observed. Both label well-defined F-filamentous actin and their differences are set on the parts of the filaments labelled by each fluorophore, as seen in Figure 15. SPY-555 (in red) appears to predominantly mark lamellipodia, the branched dendritic network involved in cell migration, and cortex, a thin isotropic network, which is associated with regulation of cell shape. Instead, phalloidin (in green) shows stronger labelling of filopodia, the thin protrusions that contribute to sensing the environment and guiding cellular movement. Finally, both, but with a clear predominance of phalloidin, mark the stress fibres; the thick, contractile bundles of actin filaments that provide structural support and mechanical tension to the cell. [8,11–13,47] As can be seen in the figure, although clearly red and green zones can be found, there are also yellowish zones, which indicate that both dyes coexist in certain actin regions.

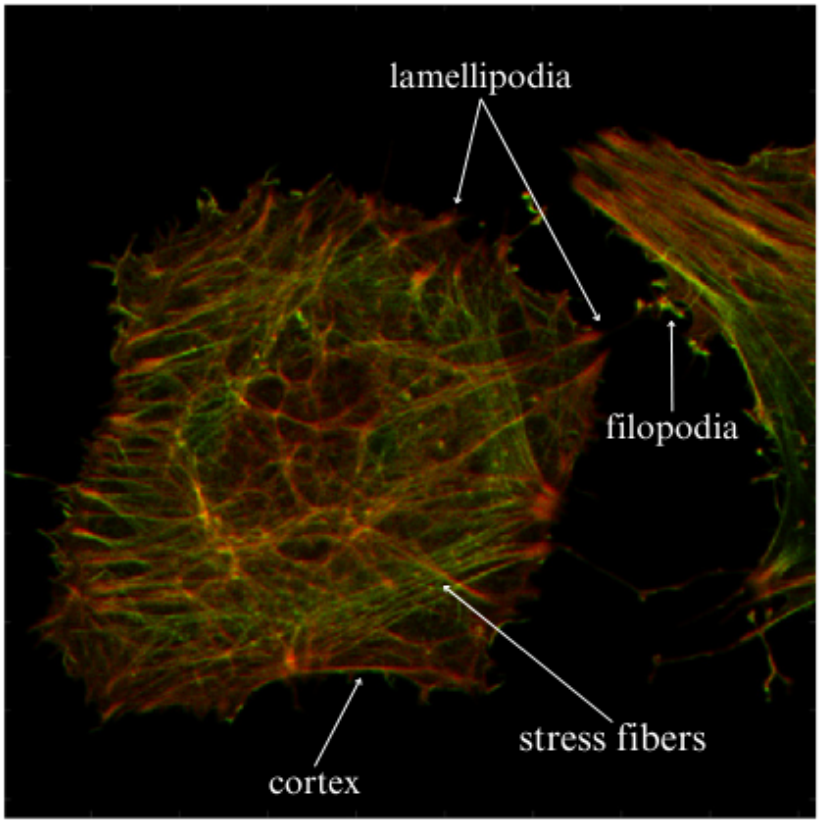


Figure 15: The phalloidin signal is shown in green, the SPY-555 in red and the overlap of both signals in yellow/orange. The different actin structures are indicated on the image.

When analysing the distribution maps of the double labelling for anti-pan actin Ab and phalloidin, distinct differences can be observed (Figure 16). Phalloidin specifically labels long, filamentous actin structures (F-actin), highlighting well-defined cytoskeletal filaments, such as stress fibres and the cortex. In contrast, anti-pan actin Ab produces a more diffuse labelling pattern, focusing on the internal part of the cell and some filopodia as well.

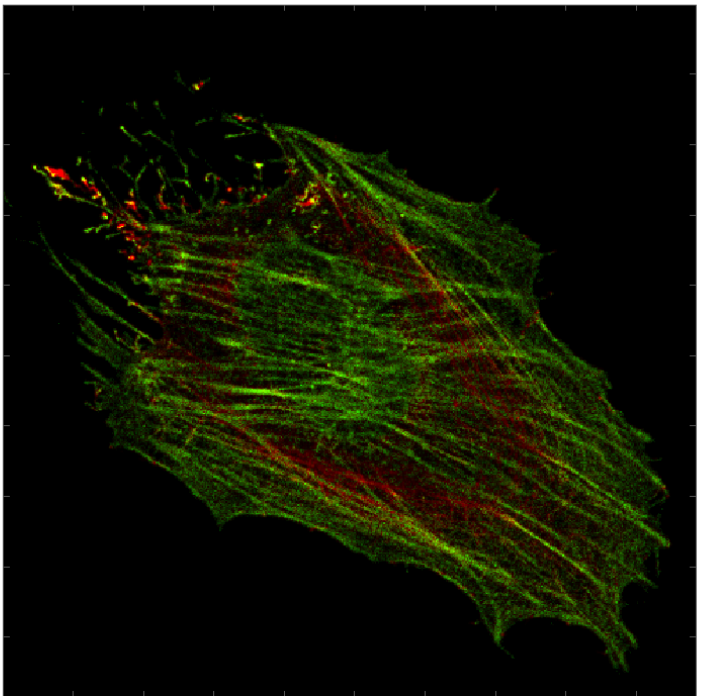


Figure 16: The phalloidin signal is shown in green, the anti-pan actin Ab in red and the overlap of both signals in yellow.

Therefore, when comparing the two double-labelled cells in Figures 15 and 16, notable differences can be observed.

In the image where SPY-555 and phalloidin are used (Figure 15), the predominance of the orange/yellow colour is predominant; the result of the fluorophores' colocalization. This observation aligns with the expected behaviour of the fluorophores, since both specifically label the same actin structure: filamentous actin (F-actin). However, a slight difference in the binding to specific F-actin structures is observed. SPY-555 mainly marks the external part of the cell: lamellipodia and the cortex, while phalloidin shows a predominance on the stress fibres and the filopodia.

In contrast, the image where the labelling agents are the antibody and the phalloidin (Figure 16), shows minimal yellow areas, reflecting a lower degree of colocalization. This difference can be attributed to the distinct labelling specificities of the markers. While phalloidin exclusively labels F-actin, the antibody marks both filamentous and globular actin, resulting in a broader and less structured distribution that reduces overlap with the phalloidin signal. Additionally, the size of the two molecules must be taken into consideration, since the antibody has an approximate molecular weight of 43kDa[33] and phalloidin of 800 Da[21]. This may allow phalloidin to bind more easily to F-actin in tightly packed or structurally complex areas. In contrast, the larger antibody may encounter steric barriers, limiting its ability to bind to actin epitopes in less accessible regions of the cell.

This affinity study shows quite promising results, but further investigation is required to reach more consolidated conclusions. A promising approach would involve fixing the cells at the same cell physiological state, ensuring that the cellular structures remain as comparable as possible, which would enhance the reliability of the sample comparisons. Additionally, a significantly higher number of images would be required to confirm that the observations presented are consistent labelling agent behaviour trends.

8 CONCLUSIONS

In this study, different actin markers were evaluated in fixed HeLa cells using confocal microscopy techniques and hyperspectral imaging combined with the chemometric analysis by MCR-ALS. After determining the best fixation and labelling protocols for single and double actin labelling, the results showed that the used labelling agents exhibited significant differences in their affinity and specificity for actin.

The objective of fixation is to achieve good morphological preservation, while stopping the degradative process of autolysis. An aldehyde-based fixation, PFA, was found the best option for labelling chemical fluorescence probes, such as phalloidin, SPY-555 and SiR-actin, since PFA crosslinks cellular proteins, helping to stabilize and conserve the protein structure. However, this process can limit access to certain epitopes and may even partially reduce antigenicity. This explains why the selected antibodies, anti-pan actin Ab and anti-beta actin Ab, did not perform well when cells were fixed solely with PFA. Nevertheless, combining PFA fixation with methanol treatment, which induces partial protein denaturation, yielded the best results for antibodies. Methanol effectively unmasked antigens, enhancing the signal and improving the quality of labelling. Since a common fixation method for simultaneously labelling actin with a chemical fluorescence probe and an antibody was not identified, a sequential approach was tested and proved to be effective. In this method, phalloidin labelling was performed first using PFA fixation, followed by anti-pan actin Ab labelling using the PFA/methanol fixation method, all within the same sample.

For the comparison of affinity and specificity studies of various labelling agents towards actin, hyperspectral imaging (HSI) combined with MCR-ALS analysis was performed. Two pairs of labelling agents were tested: one comprising two chemical fluorescence probes (phalloidin and SPY-555) and the other combining a chemical fluorescence probe and an antibody (phalloidin and anti-pan actin Ab). As expected, phalloidin and anti-pan actin Ab exhibited the most distinct signal localizations. Unlike phalloidin, which specifically labels F-actin, anti-pan actin Ab can label both G-actin and F-actin. This results in a broader and less structured distribution, leading to reduced overlap with the phalloidin signal. Additionally, differences in their binding mechanisms and molecular sizes may further explain the observed differences. On the other hand, phalloidin and SPY-555 showed slightly different structural affinity towards actin. SPY-555 mainly marks the external part of the cell: lamellipodia and the cortex, while phalloidin shows a predominance on the stress fibres and the filopodia. However, an additional study with a broader dataset of images and the fixation of cells at the same cellular stage would be necessary to draw more accurate conclusions.

Finally, the MCR-ALS analysis proved to be an effective method for analysing HSIs, facilitating the separation of overlapping fluorescence signals and providing related concentration maps that offered valuable insights into the spatial distribution and relative abundance and affinity of labelling agents for the different actin structures.

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10 ACRONYMS

Ab: antibody

BSA: bovine serum albumin

DMSO: dimethyl sulfoxide

F-actin: filamentous actin

G-actin: globular actin

HSI: hyperspectral imaging or hyperspectral image

ICFO: Institut de Ciències Fotòniques i Òptiques

LSCM: laser scanning confocal microscope

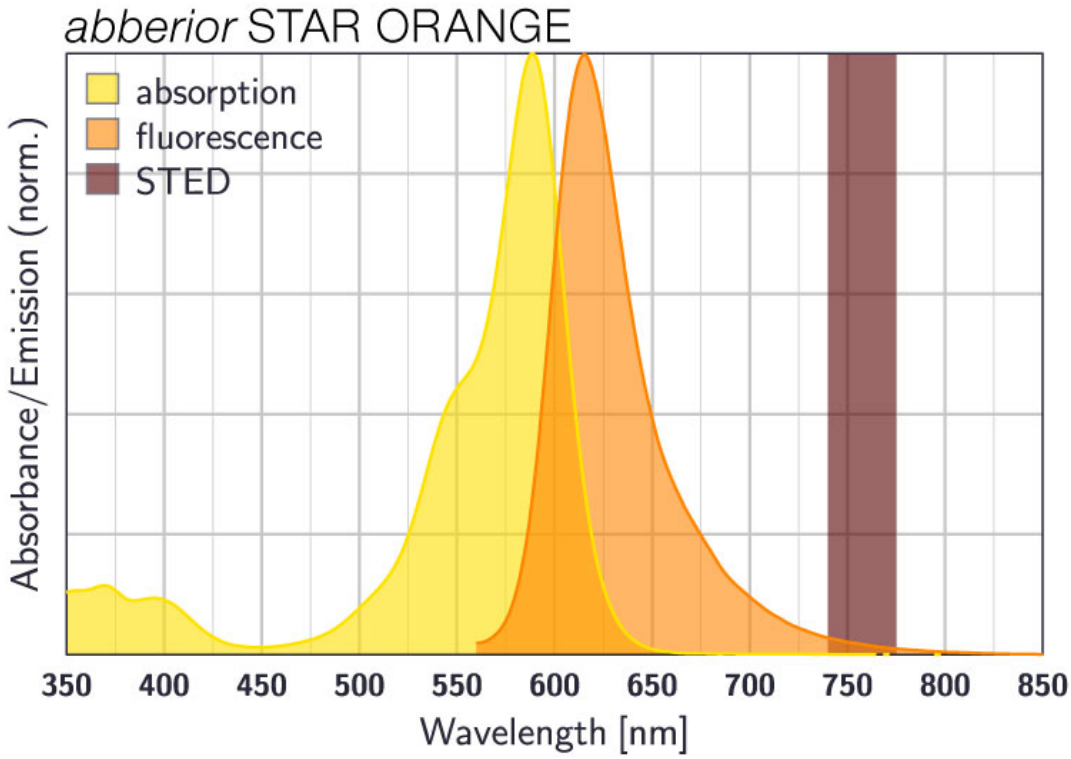
MCR-ALS: Multivariate Curve Resolution-Alternating Least Squares

PBS: phosphate-buffered saline

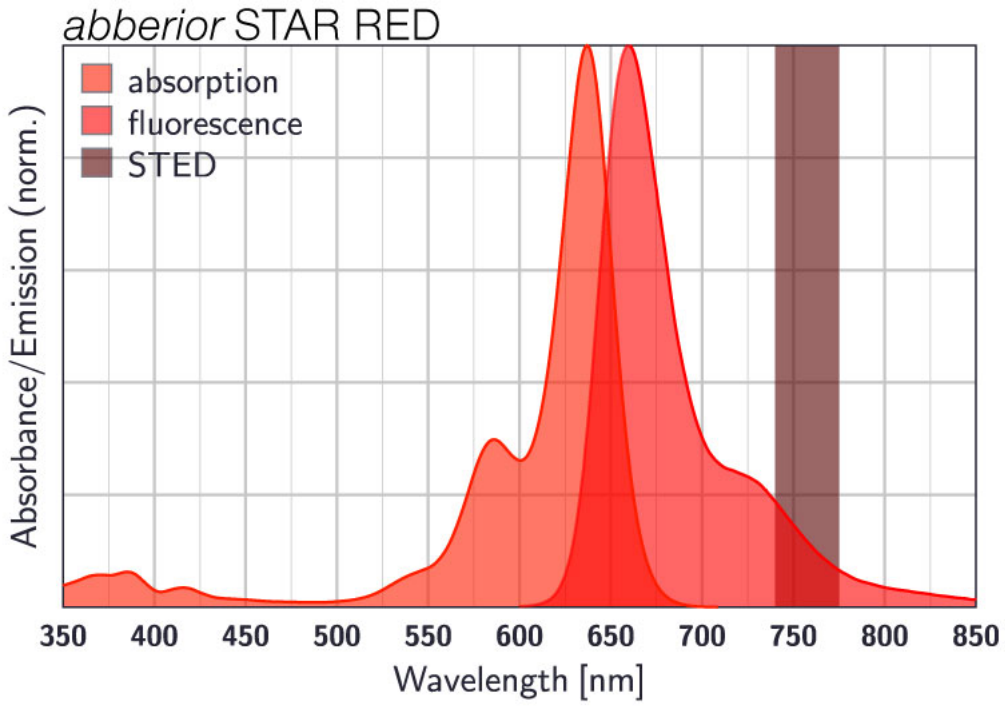
PFA: paraformaldehyde

APPENDICES

11 APPENDIX 1: SPECTRA OF ANTIBODIES' FLUOROPHORES (ASTAR RED AND ORANGE)



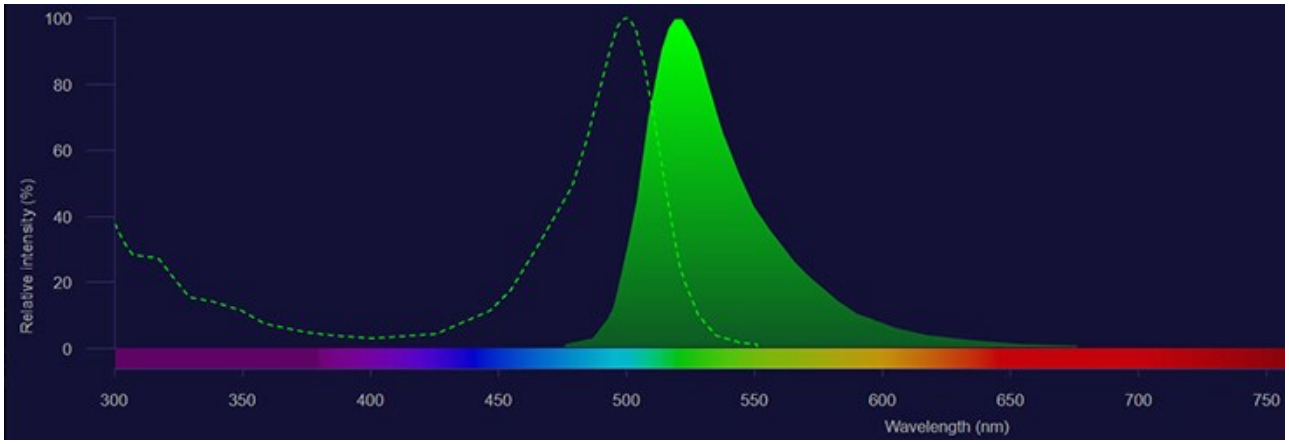
[Ref: 38]



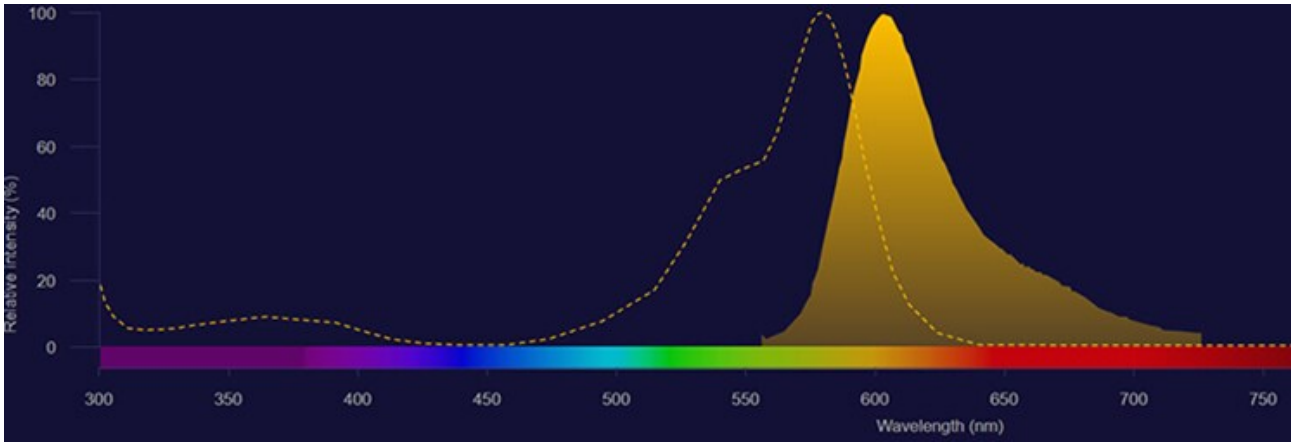
[Ref: 39]

12 APPENDIX 2: SPECTRA OF PHALLOIDIN'S FLUOROPHORES (ALEXA-488 AND ALEXA-568)

ALEXA-488[34] (dotted line: excitation spectrum, continuous line: emission spectrum):

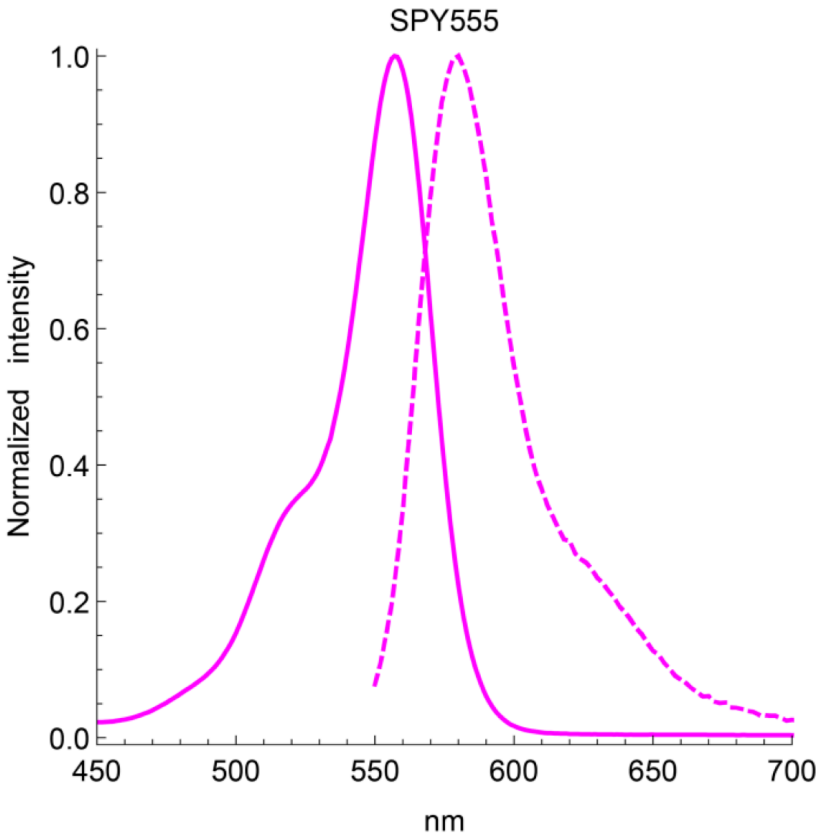


ALEXA-568[35] (dotted line: excitation spectrum, continuous line: emission spectrum):

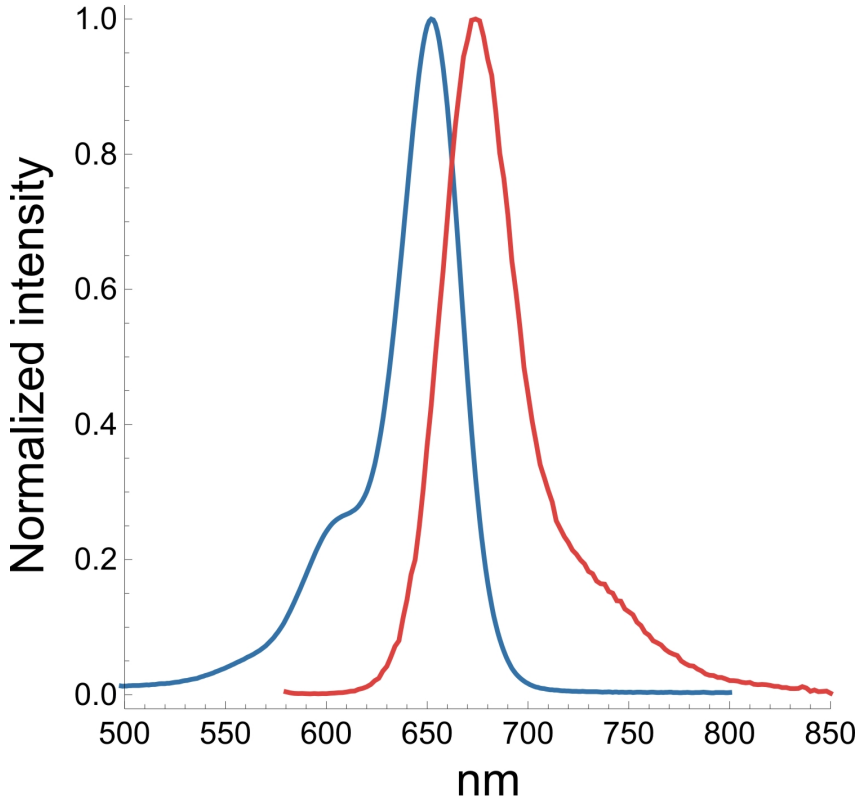


13 APPENDIX 3: SPECTRA OF SPY-555 AND SIR-ACTIN

SPY-555[37] (continuous line: excitation spectrum, dotted line: emission spectrum):

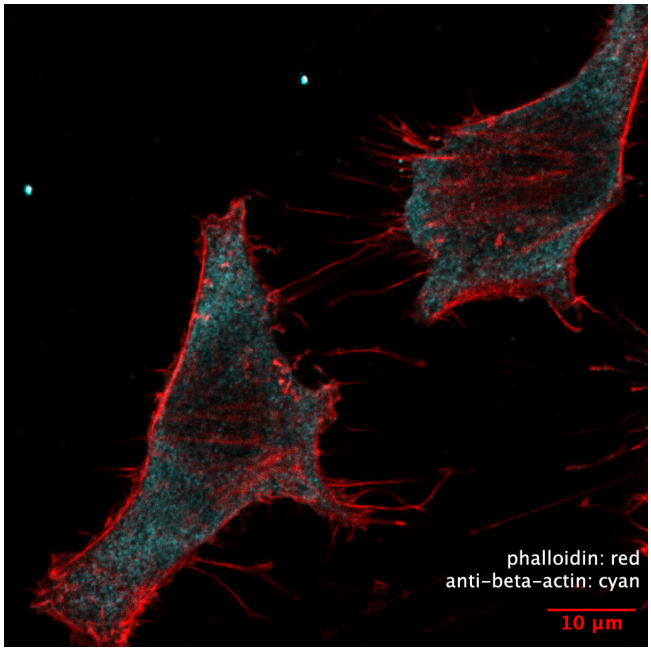


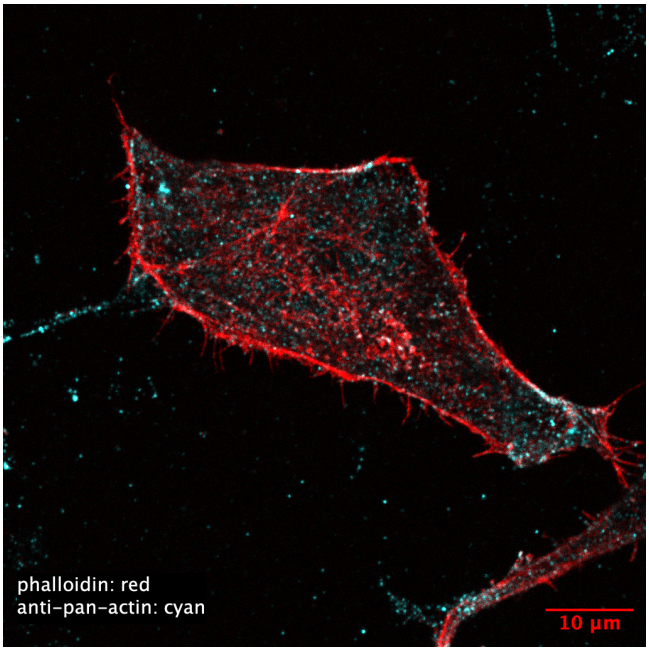
SIR-ACTIN[36] (continuous line: excitation spectrum, dotted line: emission spectrum):



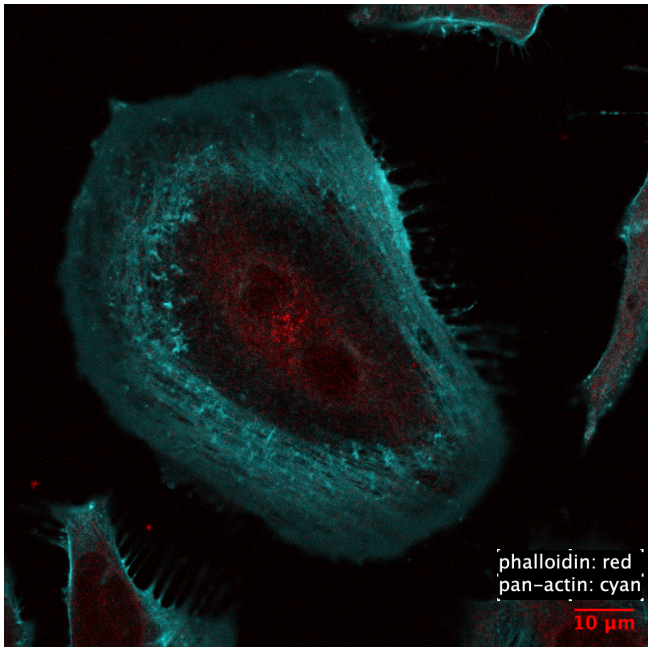
14 Appendix 4: Images xyz, Double labelling (phalloidin + Ab)

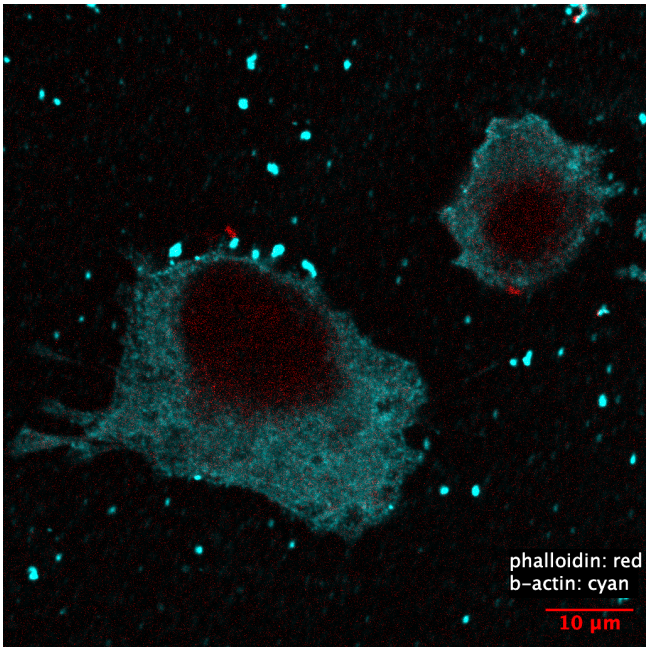
PFA fixation



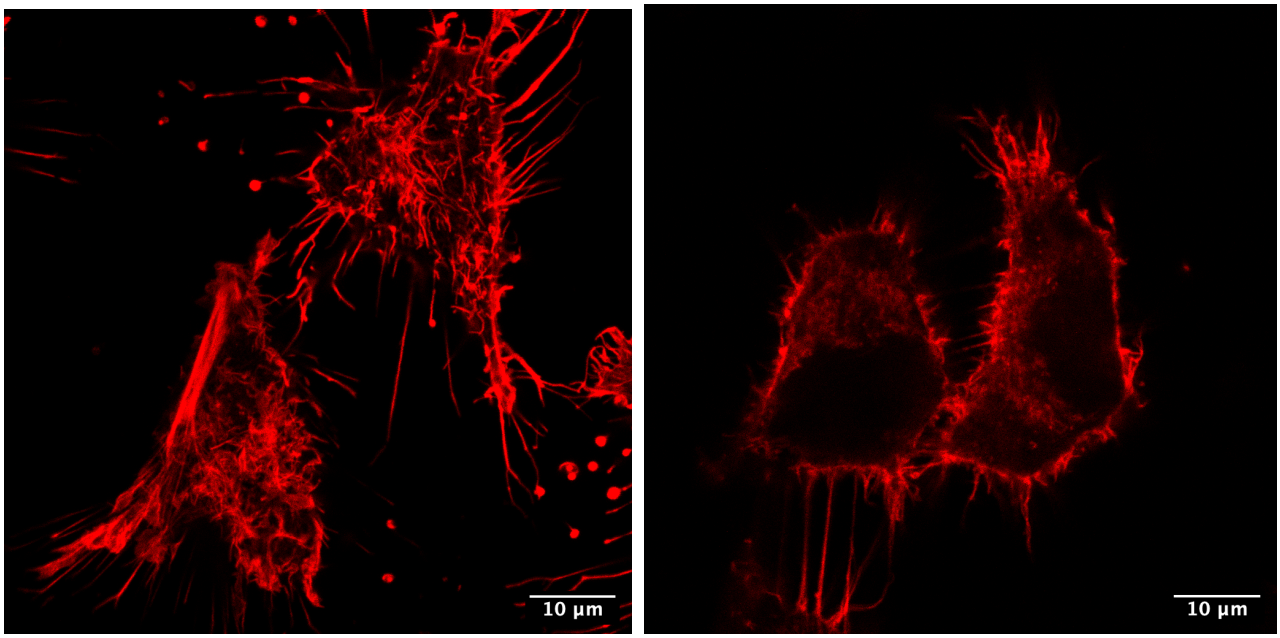


Methanol fixation

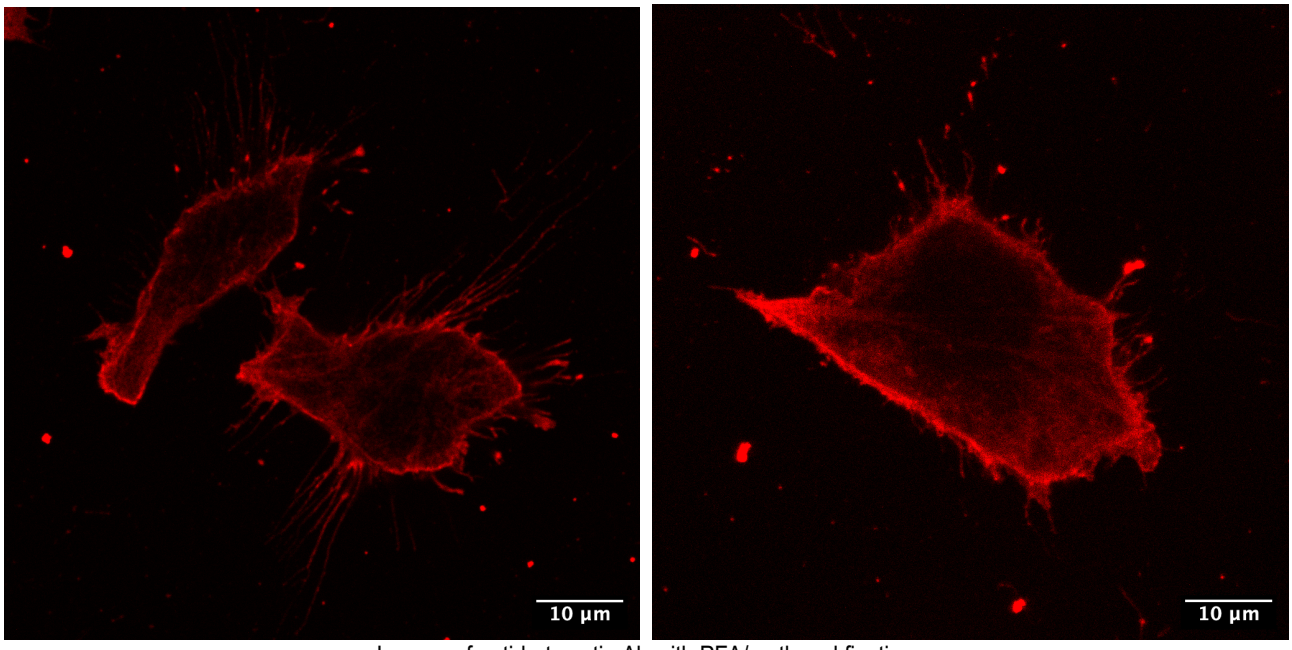




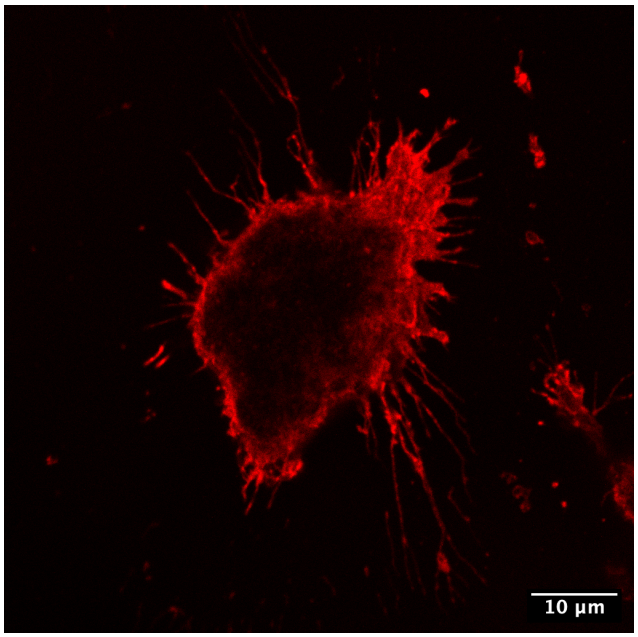
15 APPENDIX 5: IMAGES XYZ, SINGLE LABELLING (POSITIVE LABELLING)

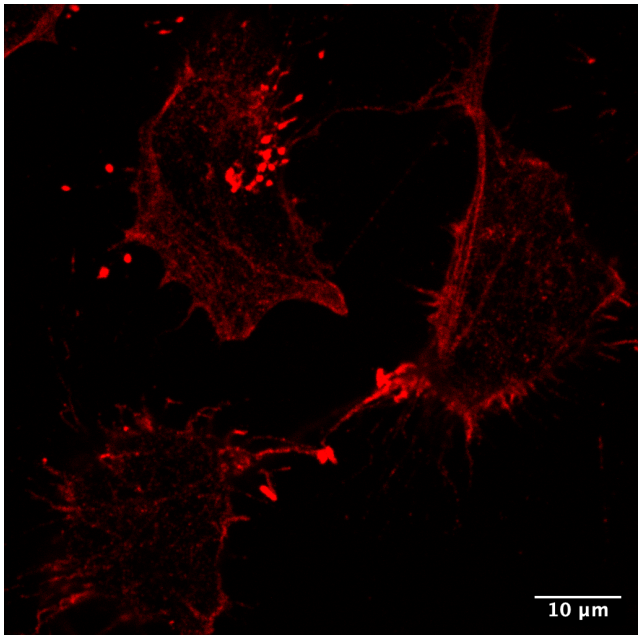


Images of phalloidin with a PFA fixation



Images of anti-beta actin Ab with PFA/methanol fixation





Images of anti-pan actin Ab with PFA/methanol fixation

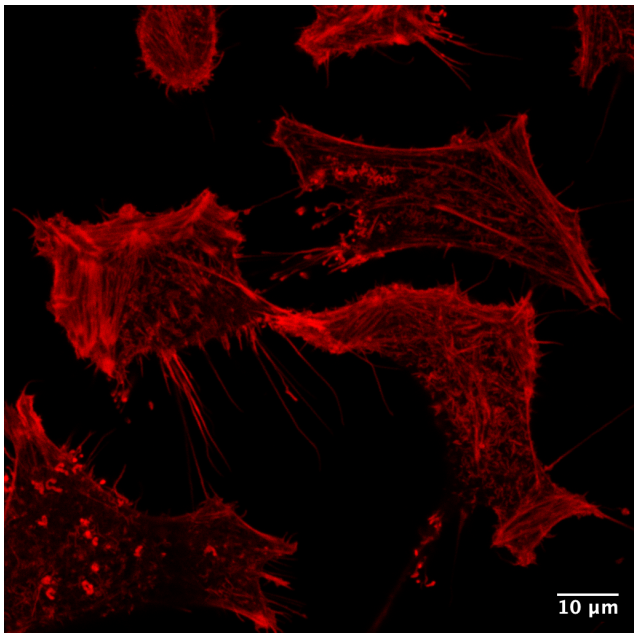


Image of SiR-actin with PFA fixation

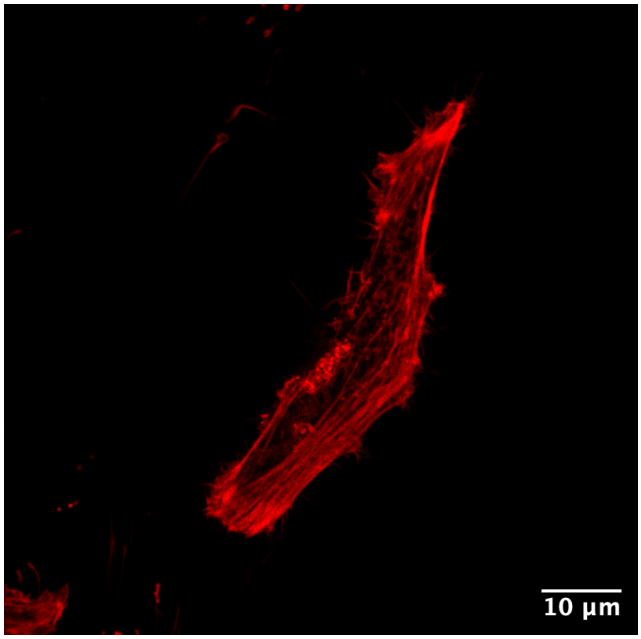


Image of SPY-555 with PFA fixation

16 APPENDIX 5: IMAGES XYλ OF SPY-555 + PHALLOIDIN