



Investigation of encapsulins as multimodular imaging tools

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Abstract

Microscopy is an essential tool for investigation of many vital biological processes. Research in the biological field requires a deep knowledge both at the single-cell level and tissue as well as the organism level. Hence, microscopy techniques are vital to any investigation, allowing the visualization of cell structures, surface mapping, tumor imaging and much more. Specifically, light microscopy can provide functional data on a cell's environment and dynamic in real-time while electron microscopy gives detailed data on the ultrastructure organization of cells. Hence, a combination of both types of microscopy - correlative light electron microscopy - is required to fully understand biological events.

Along with microscopy, imaging reporters are also essential to the study of proteins, their interaction and its role in the cell. Fluorescent reporters like GFP, mCherry and other fluorescent proteins have enabled great achievements in this field however, reporters for electron microscopy imaging are scarce and limited. In addition, the previously mentioned fluorescent reporters have very limited applications in electron microscopy and correlative-light electron microscopy given the complex sample preparation protocols that damage the fluorescent proteins and create artifacts that can destroy the native ultrastructure.

In this dissertation, encapsulins are proposed as a new genetically encoded reporters for fluorescence microscopy (FM), electron microscopy (EM) and correlative light-electron microscopy (CLEM). Encapsulins are a class of spherical protein nanocompartments responsible for oxidative stress regulation, detoxification and homeostasis in bacteria and archaea. To fulfil the proposed goal, encapsulins were engineered to possess fluorescent tags, targeting moieties, metal-binding tags and other specific tags, capable to generate signal in different types of microscopy.

For imaging in fluorescence microscopy, the fluorescent reporter eUnaG was fused to the C-terminal of Qt encapsulins. Targeting was achieved by fusing an intrabody against mCherry to the outer surface of the encapsulin and expressing mCherry in the cellular membrane. Other method was the use of sfCherry2, split between strands 1-10 (exposed on the surface of a Qt encapsulin) and strand 11 (expressed on the cellular membrane). The complementation of both strands showed fluorescence across all membrane. To evaluate encapsulins ability to measure calcium levels in a cell, calcium indicators: Twitch2b and CaM-RS20 complex, were co-transfected with Qt encapsulins and evaluated under confocal microscopy with calcium stimulation. Encapsulins successfully presented themselves differently by enhancing the fluorescence signal and by forming clusters, respectively. For electron microscopy, MT3 was used as a metal binding tag to enhance contrast. Additional MT3 domains were fused to either the N- and C-terminal of Qt encapsulins. This resulted in different patterns of contrast being obtained - "barcodes". In addition, all experiments performed in FM were then imaged under EM for correlation of obtained data. All experiments were performed using HEK293T cells and all results were achieved without any damage on emitted fluorescence and disruption of the encapsulins' assembly as proved by obtained biochemistry data.

The work performed here, proved that encapsulins can serve as genetically encoded reporters with a potential physiological role given their targeting and sensing capacities in multiple imaging tools.

Keywords: microscopy, genetically encoded reporters, encapsulins, contrast.

Resumo

A microscopia é uma ferramenta essencial para a investigação de muitos processos biológicos vitais. Investigação em Biologia requer um conhecimento profundo tanto ao nível da célula e tecido como ao nível do organismo. Assim, as técnicas de microscopia são vitais para qualquer investigação, permitindo a visualização de estruturas celulares, mapeamento de superfície, imagiologia de tumores e muito mais. Especificamente, a microscopia de fluorescência pode fornecer dados funcionais sobre o ambiente e a dinâmica de uma célula em tempo real, enquanto a microscopia eletrónica fornece dados detalhados sobre a organização ultraestrutural das células. Portanto, uma combinação de ambos os tipos de microscopia - microscopia correlativa eletrónica e óptica - é necessária para entender totalmente eventos biológicos.

Em conjunto com a microscopia, marcadores de imagiologia também são essenciais para o estudo das proteínas, sua interação e seu papel na célula. Marcadores fluorescentes como GFP, mCherry e outras proteínas fluorescentes permitiram grandes descobertas neste campo, no entanto, os marcadores para microscopia eletrónica são escassos e limitados. Além disso, os repórteres fluorescentes mencionados anteriormente têm aplicações muito limitadas em microscopia eletrónica e microscopia correlativa eletrónica e óptica, devido aos complexos protocolos de preparação de amostras que danificam as proteínas fluorescentes e criam artefactos que podem destruir a ultraestrutura nativa.

Nesta dissertação, encapsulinas são propostas como novos repórteres geneticamente codificados para microscopia de fluorescência (FM), microscopia eletrónica (EM) e microscopia correlativa eletrónica e óptica (CLEM). Encapsulinas são uma classe de nanocompartimentos proteicos esféricos responsáveis pela regulação do stress oxidativo, desintoxicação e homeostasia em bactérias e archaea. De forma a cumprir o objetivo proposto, as encapsulinas foram geneticamente modificadas para possuir marcadores fluorescentes, ligandos para alvos moleculares, domínios de ligação a metais e outros marcadores específicos, capazes de gerar sinal em diferentes tipos de microscopia.

Para geração de imagens em microscopia de fluorescência, o marcador fluorescente eUnaG foi expresso no C-terminal de encapsulinas Qt. Ao expressar um intracorpo contra mCherry à superfície externa da encapsulina e expressando mCherry na membrana celular foi possível marcar a membrana celular com sucesso. Com o mesmo propósito, outra técnica foi o uso de sfCherry2, dividido entre as cadeias 1-10 (expostas na superfície de uma encapsulina Qt) e cadeia 11 (expressa na membrana celular). A complementação das cadeias levou à emissão de fluorescência em toda a membrana. Para avaliar a capacidade das encapsulinas de medir os níveis de cálcio numa célula, os indicadores de cálcio: Twitch2b e o complexo CaM-RS20, foram co-expressos com encapsulinas Qt e observados sob microscopia confocal com estimulação de cálcio. Encapsulinas apresentaram-se de forma diferente, aumentando a intensidade do sinal de fluorescência e formando clusters, respectivamente. Para microscopia eletrónica, MT3 foi usado como domínio de ligação a metais para aumentar o contraste. Os domínios MT3 extra foram fundidos ao N- e C-terminal de encapsulinas Qt resultando na obtenção de diferentes padrões de contraste - "códigos de barras". Além disso, todas as experiências realizadas em FM foram, também, observados sob EM para correlação dos dados obtidos. Por fim, todas as experiências foram realizadas usando células HEK293T e todos os resultados foram alcançados sem nenhum dano na fluorescência emitida e disrupção na formação das encapsulinas, conforme comprovado pelos dados bioquímicos obtidos.

O trabalho realizado neste projeto provou que as encapsulinas podem servir como repórteres geneticamente codificados com potencial para aplicações clínicas, devido à sua capacidade de “targeting” e de resposta a estímulos fisiológicos em vários tipos de microscopia.

Palavras-chave: microscopia, marcadores geneticamente codificantes, encapsulinas, contraste.

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Abbreviations

APX	Ascorbate Peroxidase
BR	Bilirubin
BSE	Backscattered Electrons
CaM	Calmodulin
CFP	Cyan Fluorescent Protein
CLEM	Correlative-Light-Electron Microscopy
CLSM	Confocal Laser Scanning Microscopy
CM	Confocal Microscopy
CT	Computed Tomography
DhaA	Haloalkane Dehalogenase
DNA	Deoxyribonucleic Acid
eAPEX	Enhanced Ascorbate Peroxidase
eGFP	Enhanced Green Fluorescent Protein
EM	Electron microscopy
ER	Endoplasmatic Reticulum
FISH	Fluorescence in situ Hybridization
FITC	Fluorescein Isothiocyanate
FP	Fluorescent Protein
FPALM	Fluorescence Photoactivated Localization Microscopy
FM	Fluorescence Microscopy
FRAP	Fluorescence Recovery After Photobleaching
FRET	Förster Resonance Energy Transfer
GECI	Genetically Encoded Calcium Indicator
GFP	Green Fluorescent Protein
GPCR	G Protein-Coupled Receptors
HA	Hemagglutinin
HRP	Horseradish Peroxidase
I	Light Wavelength
LM	Light microscopy
LY	Lucifer Yellow
M	Magnification
MCS	Membrane Charge Sensor
mNG	Membrane Neon Green
MRI	Magnetic Resonance Imaging
MT	Methallothielinein

NA	Numerical Aperture
PALM	Photoactivated Localization Microscopy
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PMT	Photomultiplier Tube
POI	Protein of Interest
PSF	Point-Spread Function
QD	Quantum Dot
R	Resolution
RESOLFT	Reversible Saturable Optical Fluorescence Transitions
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Room Temperature
SEM	Scanning Electron Microscopy
SMLM	Single Molecule Super Resolution Microscopy
SRM	Super Resolution Microscopy
SSIM	Saturated and Structured Illumination Microscopy
STED	Stimulated Emission Depletion
STEM	Scanning Transmission Electron Microscopy
STORM	Stochastic Optical Reconstruction Microscopy
TEM	Transmission Electron Microscopy
YFP	Yellow Fluorescent Protein
UV	Ultraviolet

Purpose of the work

1.1.Context and Motivation

Imaging tools are the core to all research fields and to the understanding of all biological, chemical and physical events that surround us. In addition, humans are mostly a visual species making visualization techniques of even greater importance [1]. Biological systems have multiple levels of complexity, from atoms to proteins, tissues or entire organisms. A deep knowledge of structure and function of these biological systems is required not only for clinical practice and its development but also to answer fundamental questions in the scientific world. Current imaging techniques as magnetic resonance imaging (MRI), positron emission tomography (PET), computed tomography (CT) and many others are used for the study of three-dimensional structures in a non-invasive way [2]. This allowed to efficiently diagnose and study the progression of many diseases. Biological imaging has quickly evolved with the development of microscopes with sub-diffraction resolution, allowing the study of single molecules, proteins, organelles, etc. [3], [4]. Furthermore, new findings on the fields of molecular biology and nanotechnology have allowed the development of new areas as molecular imaging. Molecular imaging focus on the study of cellular and subcellular processes and expand those findings to the understanding of full organisms and cell networks. While functional data can be achieved by fluorescence microscopy - possible to image cells in real-time at three dimensions [5], [6], structural data is obtained by electron microscopy - highest resolution enables imaging at a nanometric level [7].

In molecular imaging, the use of reporters is essential. Ideally, reporters that can efficiently tag proteins without disruption of their native structure and function while serving as sensors that translate the physiological state of a cell or as targets for specific receptors or organelles. In addition, genetically encoded reporters are preferred due to their ability to be expressed dependent on the used promoter which can be cell specific, pH specific or others [8]. Fluorescent reporters are abundant and have already showed great results when applied to the study of gene and protein expression, tracking of proteins and even as calcium indicators in neurological studies [9], [10]. However, when these reporters are imaged, all non-tagged molecules aren't visible and provide no data. That being, there is a lack of structural information which, consequently, limits the amount of functional information that can be attained. In addition, when using these reporters in electron microscopy, their fluorescence is damaged because of the complex protocols used in sample preparation [11]. So, in spite of having ultrastructure data, the lack of fluorescence unable any take on function of the studied protein. That being, there is a need for reporters that can show fluorescence but also be visible in EM to obtain the desired functional and structural data, either simultaneously or later correlated using complex software and machinery. EM reporters are mainly based on the immunolabeling (using antibodies linked to gold nanoparticles or quantum dots) or in the

polymerization of 3,3'-Diaminobenzidine (DAB) to enhance the observed contrast. However, these present limitations due to size constrictions, in case of antibodies use, and addition of exogenous compounds in sample preparation, in case of DAB polymerization [12].

Motivated by this lack of reporters capable of providing reliable and well-rounded data, comes this Master Thesis that looks into the development of a new genetically encoded reporter for FM, EM and CLEM.

1.2.Aim

This dissertation was developed at the Institute of Synthetic Biomedicine at the Helmholtz Center under the “EMcapsulins” project which aims to use encapsulins, a new genetically encoded reporter, to visualize the molecular dynamics of neurons in electron and fluorescence microscopy. In the future, this would allow to obtain functional maps of neurons for applications in neurological disorders.

As a contribution to this project, the developed work focus on the engineering of encapsulins to study their potential to act as genetically encoded reporters in CLEM. Particularly, the main goals are:

- Observation of encapsulins in FM by direct fusion of fluorescent proteins to their N- or C-terminal.
- Observation of encapsulins in EM by direct fusion of metal-binding domains to their N- and/or C-terminal - obtaining different “barcodes”.
- Targeting of organelles by direct fusion of a specific intrabody to the outer surface of encapsulins or by the split fluorescence mechanism. Visible both in EM and FM
- Physiological sensing by entrapment of a calcium sensor to the lumen of encapsulins or by the RS20-CaM complex mechanism. Visible both in EM and FM.
- Observation of encapsulins in CLEM by direct fusion of chemical tags to the C-terminal of encapsulins.

1.3.Document Structure

This thesis is structured in a logical and sequential way.

The introduction begins with a review on microscopy, its story and evolution until the microscopes of nowadays. Particularly, it focuses on LM and its variants - FM, CM and SRM; EM and its variants - TEM and SEM - and CLEM, mentioning its constitution, work of principle, advantages/disadvantages and applications. Then, the current reporters for FM, EM and CLEM are mentioned and characterized, along with GECIs. Lastly, the proposed genetically-encoded reporters - encapsulins - are reported with its history, characteristics and its current and future applications.

Next, the used methods are mentioned regarding cell culture, molecular biology, gel electrophoresis, microscopy and data analysis.

Then, the obtained results along with a description of the performed experiments are described and discussed accordingly. Finally, the main conclusions are mentioned with remarks on possible improvements of this work along with future challenges and advances on this topic.

Introduction

2.1. Microscopy

Most of the fundamental processes that occur in nature are at a microscopic level and can't be seen at the naked eye. That being, new technologies that allow humans to go beyond their visual limits can provide vital information that help to understand many different biological, chemical and physical processes that were unattainable to comprehend before. Imaging tools allow us to visualize and obtain data in multiple dimensions (x, y, z, t) and analyze multiple parameters. Through these techniques, we can measure surface properties [13], elemental composition [14] while providing a temporal understanding of the cell's dynamics [15].

From the 4th century, the concept of an optical lens was already commonly discussed, and by the 13th century, glass lenses were already used to improve people's eyesight and for magnifying common objects [16]. In 1609, Galileu Galilei was known for the invention of the first microscope based on an objective lens that collected light and a secondary lens that magnified the obtained image [17]. However, it was only in 1683 that such instrument was used for scientific purposes by Robert Hooke and Antonj van Leeuwenhoek [18]. They are said to be the pioneers of microscopy and published many studies regarding the identification and discovery of many biological structures as bones, skin, nerves and some microorganisms as parasitic worms, some protists and bacteria [1]. From simple microscopes that only use light to image their samples on two dimensions to highly sophisticated microscopes that require sample preparation and advanced expertise to image their samples on higher dimensions along with the development of great software and machinery, the evolution of this technology has risen above all expectations [1], [19]. The current resolution achieved by the most complex microscopes allow us to evaluate not only what happens at a molecular, cellular or tissue level, but also at an atomic level, reaching sub nanometer resolution - 0.02nm [20]. Furthermore, not only is it possible to evaluate samples from a 2D point of view but, now, 3D images are attainable which provides a full-rounded analysis of each cell and its components. Currently, microscopy and its different applications have proof to be a vital technique for many areas of scientific discovery, including biology, materials, nanotechnology, and many others [1].

Along with the development of microscopy, there were new advances in molecular and cell biology that allowed for the progression of this field. The use of transgenic animal models and the development of molecular probes are examples of these [21]. Also benefiting this, improvements on image processing techniques allowed for an easier handling and analysis of all the obtained data. Lastly, the manner that medicine is perceived also evolved. The molecular knowledge on diseases' origin and progression is vast and it has taken a pivotal role in the definition of its therapies and clinical practice. There are more personalized therapies being offered and more research studies show that molecules are often disrupted before any physical symptoms are noticeable. That being, any molecular information that can be

attainable is vital to improve the current medical practice [22]. All of these factors, helped the development of new noninvasive in vivo imaging methods that show specific cellular and molecular processes [23]-[25]; the monitoring of multiple molecular events in real time [5], [26], [27]; the tracking of multiple cells, proteins or molecules [28]-[30]; the monitoring of the molecular and cellular response to drugs [31], [32] and the evaluation of disease diagnostics and its progression [33]-[35].

The ultimate goal would be to develop imaging tools that can evaluate these molecular actuators such that the obtained data is presented in a reproducible, quick, multidimensional (x, y, z, t) and quantitative way providing pivotal information on diagnosis and therapies for patients [21].

There are many different types of microscopy. Each with their own characteristics and advantages/disadvantages depending on the desired application. With higher relevance to this Master Thesis there are: Light Microscopy (LM), Electron Microscopy (EM) and Correlative-Light-Electron Microscopy (CLEM), which are explained below according to their constitution and principle of work, strengths/weaknesses and main applications.

2.1.1.Light Microscopy

Fundamentally, LM is based on the principle that light is emitted and goes through cells suffering diffraction, reflection or scattering which is then captured by magnifying lenses obtaining an image. A light microscope is essentially based on an eyepiece through which the operator observes its samples (usually with a 10X magnification), one or more objectives lens that collect light and possess different magnifications, focus wheels that allow to move the stage vertically and are responsible for focus, a light source which is usually and internal incandescent tungsten halogen lamp [36], a condenser that focus the light into the sample and can have filters and a diaphragm to control which area of the sample is imaged and under which angles the light interacts with the object and a stage that is the platform where the sample is located and visualized [16]. In this system there are often apertures and optical filters to define which of the scattered light from the sample will contribute to the resulting image, according to the regions of interest. The optical components and pathway of a conventional light microscope are presented in Figure 1.

The most important component are the objectives, which define most of the key properties of the microscope: magnification (M), numerical aperture (NA) and resolution (R). More specifically, the objective depends on its M and NA [37]. Magnification stands for the relative enlargement of the image over the real object, meaning that a 10X magnification will show an image 10 times larger than the sample [38]. The NA of an imaging system is defined by two parameters: half angle of the objective's collection cone and the index of refraction of the immersion medium [39]. In more simple terms, NA is the amount of light that is collected by the objective lens so a higher NA means the objective will collect more light [38]. A combination of these 2 parameters will determine the resolution in which the image is obtained.

As this technique is based on visible light alone, the cells have no detrimental reaction, allowing a long time of exposure. Besides, LM also allows the analysis of its samples on real time capturing the dynamics of the cells and their surrounding environment. On the other hand, LM also presents many optical challenges that limit their resolution and make it one of the least powerful imaging techniques. The main problems are to find an optimal focal plane, obtain contrast against the background and get high resolution images. For light microscopy the

highest resolution obtained is of several hundred nanometers which makes it impossible to image subcellular structures and organelles [40]. This is due to Abbe's diffraction limit which explains that when light goes through the lenses it does not converge to a single point of focus but instead, it "spreads out" through the imaged sample making it blurrier than intended and limiting the obtained resolution. These are called point-spread function (PSF). Essentially, when two points are closer than the defined diffraction limit, they blend in appearing as one single and unresolvable entity. The width of this spot is defined by: $0.5\lambda/NA$, being λ the light wavelength and NA the numerical aperture of the lens. Given this equation, it was thought that this diffraction limit was impossible to overcome [41]. That being, researchers focused on the preparation of the samples for LM as fixation and cutting to improve its stability and quality of imaging. Although presenting some better results, these techniques would unable the real time analysis of samples and, consequently, the understanding of the complex and dynamic environment of cells [41]. New technology developments and improved knowledge on Abbe's diffraction limit allowed to surpass these limitations and invent new imaging techniques with higher resolution. Examples are: fluorescence microscopy (FM), confocal microscopy (CM) and super resolution microscopy (SRM).

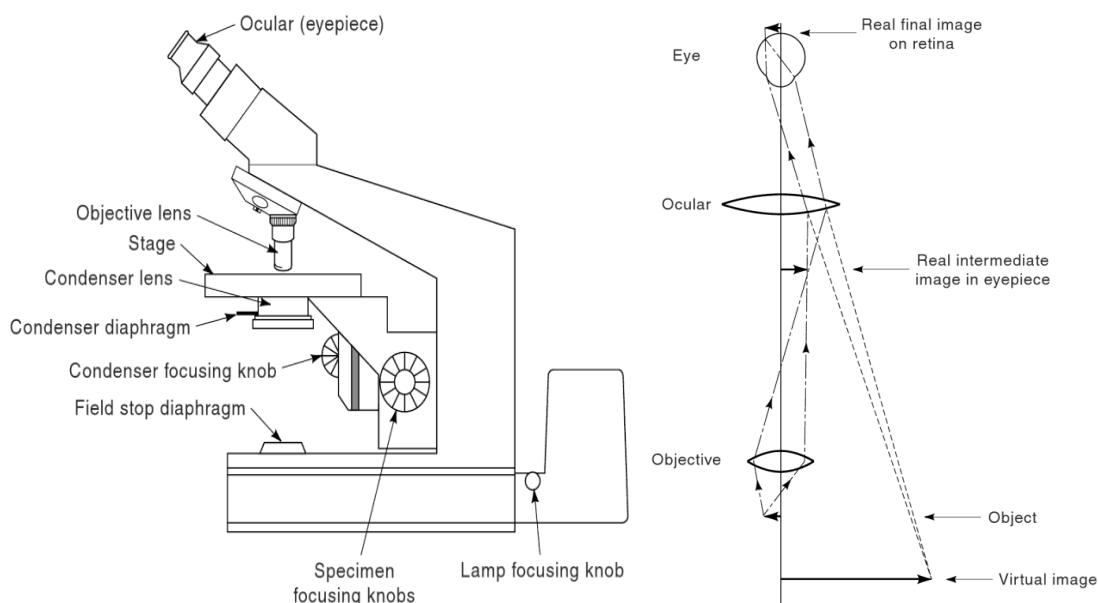


Figure 1 - Optical components of the light microscope. To the left, the constitution of a compound light microscope. On the right the optical pathway in a microscope. Image obtained via [65]

2.1.1.1. Fluorescence Microscopy (FM)

Within light microscopy there is fluorescence microscopy. FM is one of the most commonly used and resourceful imaging techniques currently available. It was developed by companies Carl Zeiss and Carl Reichert in the beginning of the 20th century after Herschel first observed fluorescence from a quinine solution when subjected to UV light [42].

As the name states, FM is based on fluorescence which means that when a sample is illuminated by light of a specific wavelength, the fluorophores absorb this light and enter an excited electronic singlet state. Here, the fluorophore will go under conformation changes leading to a dissipation of energy that brings the fluorophore back to a ground state with emission of fluorescence on a longer wavelength [43]. This difference in the wavelength of

excitation and emission is called the Stokes shift and it is fundamental for fluorescence analysis. This shift allows for isolation of the emission wavelength from the excitation one with the use of specific spectrum filters. These filters are particularly helpful when multilabeling a sample with different fluorophores. In this case, changing the spectral filters allows for the visualization of every single fluorophore without any interfering from the other fluorophores [44]. Fluorophores are typically polyaromatic hydrocarbons or heterocycles and will be later discussed with more detail on section 2.1.1.4-”Labeling tools for LM”.

As similar as LM, its constitution is also based on an illumination system and an imaging system. Given that FM is based on fluorescence, the main difference between these two microscopes is the light source. FM requires for a xenon-arc-lamp, high-power LEDs or lasers that have the required intensity to excite the samples opposed to the light bulbs used in LM. Besides, other components of FM are excitation and emission filters and a dichroic mirror [45]. These vary according to the application in question and the specific fluorophores in use. Figure 2 shows a scheme of the optical principles behind FM.

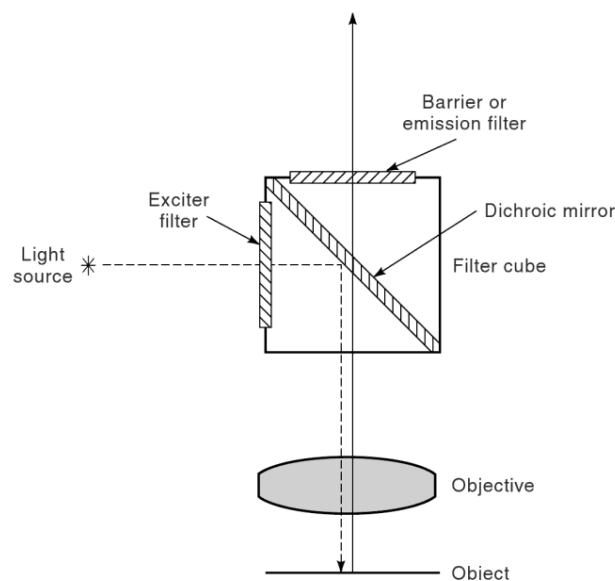


Figure 2 - Optical principle of a fluorescence microscope. The beam of light passes through the exciter filter and is reflected by the dichroic mirror towards the object (dotted line). The resulting emitted fluorescence passes through the dichroic mirror and emission filter reaching the eye or camera. Image obtained via [65]

As mentioned before, one of the main issues with LM is the lack of contrast between the sample and the background. This is not the case for FM. Despite the fact that cells possess some level of natural fluorescence, it is not enough to be visible at the microscope. However, by tagging specific proteins with the mentioned fluorophores, a molecule-specific contrast can be attained while maintaining the live-cell imaging *in vivo* component that LM is known for. On the other hand, this can also present some disadvantages since no other information is attained and there is no structural information on the tagged proteins. Other limitation is the fact that FPs can interfere with the normal behavior of the cell and disrupt its structure [46]. Another concern to be considered is photobleaching. This refers to all processes that interfere with fluorescence resulting in its depletion. Despite the fact that a fluorophore, in theory, can go through infinite cycles between excited and basal states, the production of reactive oxygen species (ROS) by excited fluorophores limit this ability [47]. This restricts the time of exposure under the microscope. Alternatives to diminish this effect are the use of more robust

fluorophores or reducing the amount of light exposure to the sample [48]. However, some techniques focus on this photobleaching phenomenon to better understand cells dynamics in vivo [49]. One of these techniques is fluorescence recovery after photobleaching (FRAP) which excites a particular region of interest, causing the depletion of fluorescence in this area. Then, FP molecules still fluorescing will diffuse into this new “dark area” which allows for the study of diffusion of these FP-labelled molecules [50].

Despite the mentioned limitations, FM has had a great impact in the current existing knowledge in biology and many research fields. For example, techniques as fluorescence in situ hybridization (FISH) allow for the detection of DNA and RNA using specific probes [51]. In 2007, Lécuyer et al. used this technique to localize thousands of mRNA molecules in the genome of embryo, ovary and larval tissues of *Drosophila* [52]. Another example is Immunofluorescence which allows the labelling of specific proteins when tagged with a fluorescent protein and an antibody. Due to this, Blot et al. were able to follow the trajectory of GLUT4 (glucose transporter in the membrane which is regulated by insulin levels) by fusing GLUT4 with a GFP molecule on its C-terminus and a hemagglutinin (HA) epitope on the N-terminal [53]. Another FM technique is Förster resonance energy transfer (FRET), this allows to study the dynamics of spatiotemporal protein interactions in situ and real-time at a molecular resolution [54]. FRET is based on the transfer of non-radioactive energy from an excited state donor to a ground state acceptor via dipole-dipole interactions. In the choosing of both fluorophores there are some requirements to be considered, as a fluorescence spectral overlap and distance (around 10nm between the fluorophores) [55]. Using FRET, Ma et al. successfully developed a sensor able to detect changes in the membrane charge of a cell - membrane charge sensor (MCS) [56]. In addition, FM can be used in flow cytometry for cell sorting and identification [57], and in immunoassays as ELISA and immunohistochemistry for specific protein localization and quantification [58], [59].

2.1.1.2. Confocal Microscopy (CM)

Confocal microscopy, commonly known as confocal laser scanning microscopy (CLSM) was initially invented by Minsky in 1957 but only in 1971, its theory was translated into the development of the first confocal microscope by Davidovits and Egger [60], [61]. CLSM, as FM, uses fluorescence as a source of light and it is based on the same principle of excitation and emission of fluorophores to obtain its images. However, contrary to FM, where the all sample is illuminated with no restrictions, in CLSM, the laser beam is only focused on a particular area of interest. This is possible due to the existence of a pinhole inside the optical system of this microscope that efficiently restricts the detection of emitted fluorescence avoiding the out-of-focus plane of the sample. Another characteristic of this microscopy is that it illuminates the sample in a specific level of depth allowing to take several images on different z-planes (z-stack) by the use of oscillating mirrors [62]. Along with the appropriate software it can create 3D images of the imaged specimens [63]-[64]. The optical pathway of CM is shown in Figure 3.

That being, CLSM is able to overcome one of the main limitations of LM - increased background signal and low-contrast images [65] - by using a pinhole that diminishes the given numerical aperture and avoids background fluorescence from cells that are of no interest, obtaining higher resolution images [66]. Another great advantage of this imaging technique is the 3D imaging capacity while maintaining the ability for analysis of multicolor labelling when using the appropriate lasers and emission/excitation filters [67]. On the other hand, there are

some limitations. Given that a part of the emitted fluorescence is blocked by the pinhole, it also means that the intensity of signal is decreased so a balanced trade-off between these two variables should be considered [68]. To decrease the impact of this limitation, a more sensitive fluorescence detector can be used, as a photomultiplier tube (PMT) [69]. Another limitation of this technique is that given that the source of light is a laser, it can only emit specific intervals of wavelength instead of the full range of wavelengths that a conventional fluorescence microscope can reach [70].

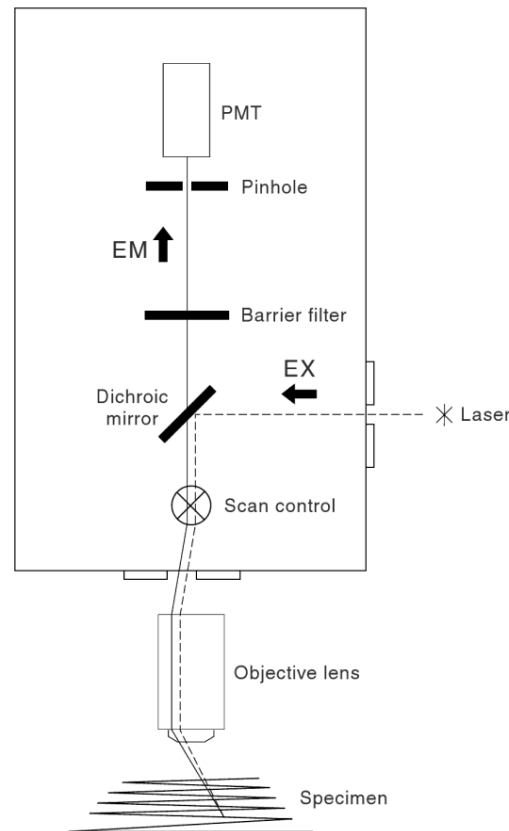


Figure 3 - Optical principle of confocal microscopy. The laser beam is reflected by the dichroic mirror passing through the scan control back and forth across the specimen (dotted line). The objective lens collects fluorescent light which is descanned at the scan control, passes through the dichroic mirror and emission filter. Finally, it goes through the pinhole into a photomultiplier tube (full line). Image obtained via [65]

CM has multiple applications and has highly contributed to great discoveries in biology. It can be used for determining the cellular localization of ions, organelles and macromolecules, for tracking specific proteins or cells and to obtain 3D projections of a cell or structure [70]. Some examples are, Domer et al. which labeled alpha-synuclein (aggregates associated with the development and progression of Parkinson's disease) with a FP and successfully observed its transfer between neurons and its localization in the lysosomal system using CM [71]. Peranzoni et al. who fluorescently labeled CD8 T cells infiltrating in human lung tumor slices and successfully observed their migration within the tumor microenvironment using CM [72]. Moreover, Sugawara et al. reconstructed the full morphology of osteocytes from chick calvarial in 3D obtaining information on their cellular processes and their native architecture [6].

2.1.1.3. Super resolution microscopy (SRM)

Super resolution microscopy was a microscopy technique developed to go beyond the established Abbe's diffraction limit of LM. As mentioned before, when light passes through the sample in LM, a single point of light will appear blurry, diminishing the image resolution [73]. There are different techniques used in SRM that can overcome this diffraction limit and present higher resolutions than LM, FM and CM. The first ever developed was in 1986 by Okhonin and it was based on stimulation emission depletion [74].

These techniques can be near-field microscopy or far-field microscopy. While near-field has proved to achieve higher resolution, below 100 nm [75], [76], it still presents some limitations since it requires that the sample and light source to be physically close from each other. This limits the range of view and difficult the analysis of cells and tissues as a whole. On the other hand, the latter relates to a slight improvement on resolution by taking advantage of the physical properties of fluorophores to generate images with no diffraction restrictions. In this case, there are two main groups. The first is based on the use of ensemble imaging techniques that recognize illumination patterns from the fluorophores and spatially modulates their behavior so that they do not emit fluorescence simultaneously and sub-diffraction limit resolution is achieved [73]. Within this class, there are techniques as stimulated emission depletion (STED) [77], [78], RESOLFT [79] technology and saturated structured illumination microscopy (SSIM) [80]. The second group is considered stochastic super resolution as it explores the chemical complexity of fluorophores using photo switching and photo reversibility to randomly activate single molecules at different timepoints. Then, using an advance software the images of the individual fluorophores and their positions are reconstructed. The most well-known techniques in this category are stochastic optical reconstruction microscopy (STORM) [81], photoactivated localization microscopy (PALM) [82], and fluorescence photoactivation localization microscopy (FPALM) [83]. The work of principle of STED and STORM techniques are presented in Figure 4 and 5, respectively.

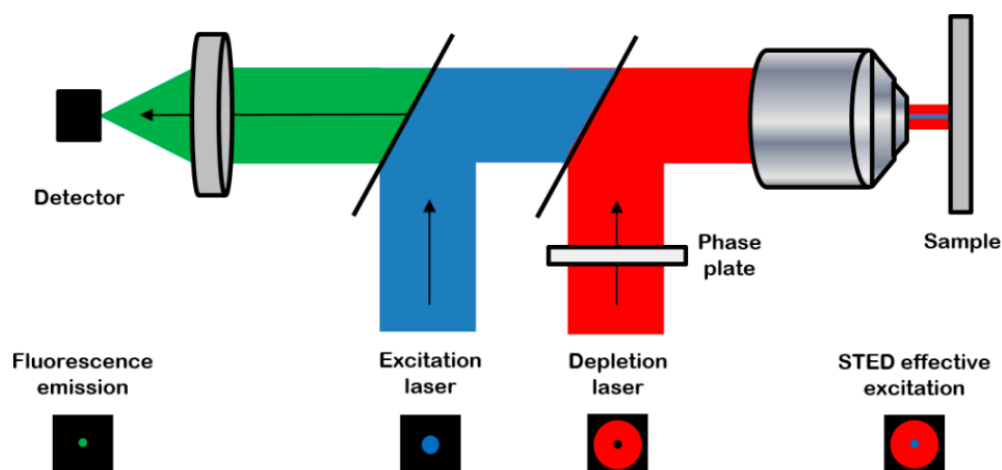


Figure 4 - Optical principle of stimulated emission depletion (STED) microscopy. There is combination of the excitation laser (blue) with the depletion laser (red) passing through a phase plate which models the beam to present a “doughnut-shape” in the focal plane. The resulting beam is an overlap of the two lasers, leading to a high-resolution probe scanning the sample. Then, the detected fluorescence emission (green) is collected with a detector. Image obtained via [286]

Due to the innovative physical and chemical properties of SRM a new and deeper understanding of biology, able to provide new information regarding cellular and molecular processes that were unattainable with the techniques previously referred to, were

accomplished. Thus, Eric Betzig, W.E. Moerner and Stefan Hell were awarded the Nobel Prize in Chemistry in 2014 [84]. Also, its use has been broadened to not only biology but to many different areas as biomaterials, supramolecular chemistry, plasmonics, etc. [85]. As FM and CM, SRM also has been extremely helpful in the localization and tracking of molecules in a cell [86], [87]. More specifically, using STORM, Conley et al. studied stack of cells from the bacteria *Caulobacter crescentus* by labelling the surface of the cells with the heterodimer Cy3-Cy5 [88]. This heterodimer is based on cyanine dyes (Cy) and functions as an “optical switch”. Essentially, Cy5 would turn on, emitting fluorescence when excited by a green laser (532 nm) and turn off, returning to a dark state when excited by a red laser (638 nm). Despite the mechanism by which Cy3 contributes to the switching behavior of Cy5 remains unclear, it is known that it is vital for this mechanism [89]. Also using STORM, Huang et al. obtained 3D-images of the complete mitochondrial network and studied the interaction of mitochondria and microtubules in monkey kidney BS-C-1 cells. Such interactions that were previously unseen in microscopes of lower resolution [90]. On another note, STED also shows promising results. In 2012, Berning et al. imaged dendritic spines of mouse and their dynamics in vivo using this type of super resolution technique with a resolution as low as 70 nm [91]. Velasco et al. successfully obtained 3D-images of neurons of mouse brains reaching a depth of 164 μm in fixed brain tissue and 76 μm in the brain of a living mouse [92].

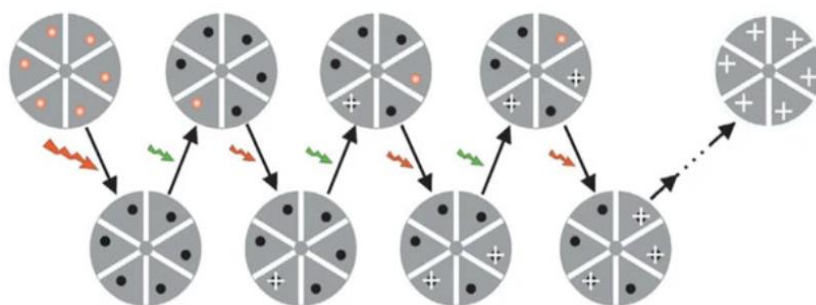


Figure 5 - Optical principle of a stochastic optical reconstruction microscopy (STORM). A STORM imaging sequence of a hexameric sample labeled with a photo-switchable RFP. All fluorophores are initially switched off by being exposed to a red laser. Then, a fraction of the fluorophores are exposed to a green laser, emitting fluorescence which allows to determine their localization. Then, in the next cycle, another set of fluorophores is excited and their position determined. The resulting image is reconstructed based on the fluorophores positions of the multiple images obtained. Image obtained via [81]

2.1.1.4. Labeling Tools for LM

Microscopy markers are the base of the current biological imaging techniques. They allow for the imaging and consequent analysis of most cellular events. Using these markers it is possible to label vital proteins and understand their function, location and interactions with the different subcellular compartments and other endogenous/exogenous proteins in living cells and organisms [93]. Antigens, specific ligands as antibodies, hormones or others are responsible for the targeting which coupled with a suitable marker, allows the visualization over the microscope with high temporal and spatial resolution [94].

One possibility to label proteins or cells for visualization under FM is the use of fluorescent dyes as fluorescein isothiocyanate (FITC), lucifer yellow (LY), rhodamine or Alexa dyes [95], [96]. These are small molecules with great brightness and photostability along with a narrow bandwidth of excitation and emission fluorescence spectrum. They can be chemically modified to present different characteristics according to the desired application [97]. The main

challenges to the use of these fluorescent dyes are to achieve molecular specificity and cell permeability [97].

On the other hand, there is the use of fluorescent proteins (FPs) to label molecules for FM visualization. These proteins are a class of proteins with a barrel-like structure and a chromophore in its center. FP reporters are composed of a fluorophore and a linker which binds to the protein of interest (POI). Fluorophores are usually composed of aromatic amino acids which are responsible for the UV absorbance effect and they can either occur naturally or be synthetically created [43]. Their main characteristic is their emission and excitation spectrums which define at what light wavelengths they emit or excite light. To prevent any interference between these different radiation wavelengths, filters are added in this type of microscopy [98]. To successfully label proteins or molecules, it is necessary to ensure that both the FP and POI are correctly folded and fully functional while assuring the correct length and sequence of the used linker [99]. The main advantage of FPs is the fact that they are genetically encodable. This means that FP reporters can be fused to a gene or protein of interest and then constitutively expressed by cells, allowing its localization when observing under FM and its use in a much wider set of potential applications of new FPs for in vivo imaging of organisms. In addition, this ability allows to surpass the previously mentioned limitations of fluorescent dyes.

Fluorescent markers have shown to be useful in the tracking of individual or multiple cells [100], [101]; in the study of protein-protein interactions [102], [103] and as biosensors to report on cellular processes [104], [105].

Below, the reporters used for the development of this Master Thesis are mentioned, with GFP reporters: GFP, eYFP and mCherry being characterized with higher detail.

2.1.4.1. GFP and eYFP

The most common FM reporter and one of the greatest discoveries in Biology is the green fluorescent protein (GFP), found in the jellyfish *Aequorea aequorea*. As the name states, this protein emits green light with a spectrum that goes from an emission maximum of 507 nm wavelength and an excitation maximum wavelength of 488 nm [106]. GFP requires no cofactors or substrates to emit fluorescence and their fusion to proteins doesn't interfere with their conformation, hence maintaining their original function, location and mobility. Besides, it shows great stability, heat and photobleaching resistance and it represents no toxic threat to cells [107]. Its discovery was made in 1962 by Shimomura et al., and later, the same research group has described its chromophore. Since then, others have characterized its sequence [108] and protein structure [107]. GFP consists of an eleven β -sheet barrel structure with an α -helix that diagonally crosses the barrel [109]. The chromophore is located in the center of the barrel, protected from any damaging species which most likely contributes to GFP's high stability. The chromophore formation is based on the autocyclization of 65Ser-Tyr-Gly67 and requires the folding of GFP into the 11-strand [110], [111]. Besides, the chromophore autocyclization is oxygen-dependent. Given this, this process can be quite slow as GFP's fluorescence can take from 90min to 4h to be observed after the synthesis of the protein [112].

The extensive characterization of GFP has allowed to unravel many mysteries to biology. In 1994, Chalfie et al. were the first to successfully use GFP as a marker for gene expression on *C. elegans*. In this case, GFP was fused to the gene *mec-7* which encodes for p-tubulin, a highly expressed protein in "six touch receptors of neurons" of *C. elegans*. As expected, the GFP pattern was similar to the *mec-7* antibody [9]. Later, in 1999, Sun et al. showed that GFP

allowed the dynamic visualization of the nervous system in *Drosophila* by engineering transgenic cell lines promoted by neuron-specific promoters. GFP fluorescence was visible in all stages of development of *Drosophila*, highlighting new discoveries in this fundamental events [113]. Besides its great ability to act as a gene and protein marker, GFP is also known to be used for FRET analysis to recognize intermolecular interactions [114].

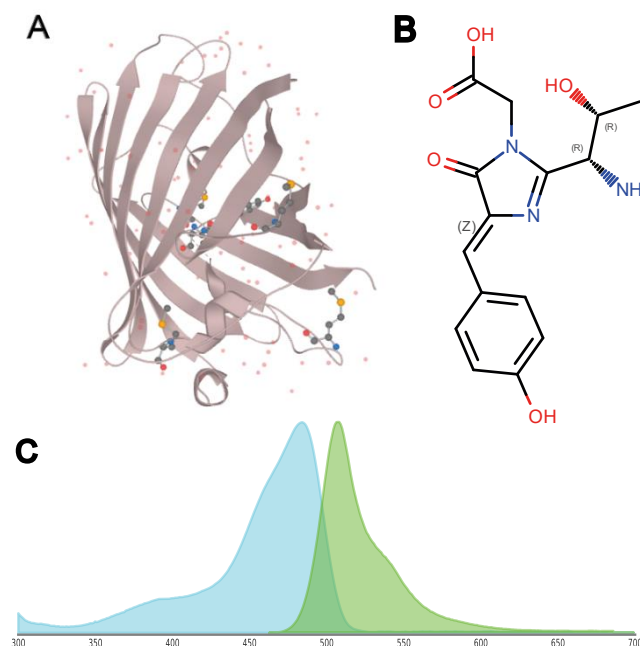


Figure 6 - eGFP. A) Structure. B) Chromophore. C) Fluorescence spectrum. Image obtained via <https://www.fpbases.org/>

Since the discovery of GFP, many have tried to enhance its characteristics by genetically engineering GFP to achieve higher stabilization, higher fluorescence and photoactivatable properties [115], [116]. Currently, there are many well characterized GFP mutants, being the most well-known and commonly used, the enhanced GFP (eGFP) [117]. eGFP possesses chromophore mutations (Ser65 to Thr and Phe64 to Leu) that enhance its brightness by 35-fold when compared to wild type GFP and allow for higher solubility. Besides eGFP was modified to have codons of highly expressed protein in humans as replacement for the wild-type codons which enable higher expression in mammalian cells [112]. Despite great improvements being made, GFP and their variants still have limitations that restrict their use in multiple experiments. As mentioned before, their chromophore formation implies post-translational steps that are time-consuming and are dependent on oxygen availability [107], [118]. In addition, GFP sensitivity is reasonably low which can make it not distinguishable from the background fluorescence when not expressed in high levels, hence less visible when expressed in mammalian cells [119]. The structure, chromophore and fluorescence spectrum of eGFP is shown in Figure 6.

2.1.4.2.UnaG and eUnaG

Given these disadvantages, many have tried to find other fluorescent proteins that could be alternatives for GFP. Within the green spectrum, there is UnaG, a fluorescent protein with an excitation and emission spectrum maximum of 498 nm and 527 nm, respectively. This protein was isolated from *Anguilla japonica* in 2013 by Kumagai et al. [120]. UnaG is part of the fatty-acids-binding-proteins (FABP) and, consequently, its structure is similar to these, consisting of ten antiparallel β -strands capped by two short α - helices [120]. Unlike GFP, this protein requires a cofactor to emit fluorescence: bilirubin (BR), an endogenous heme metabolite and a biomarker for liver or renal diseases. BR binds with high-affinity and specificity to UnaG via hydrogen bonds within the central cavity of the barrel. Having a co-factor allows UnaG to be used not only as a fluorescent marker for gene or protein expression but also shows great potential as a clinical marker and allows for a photo-inducible reporter in free-bilirubin cells [121]. Besides, UnaG fluorescence is oxygen-independent allowing for its use in hypoxic and anaerobic conditions [120], a most instantaneous fluorophore formation making it much faster to show fluorescence and has a smaller size which can be useful when tagging proteins or structures which possess size constrictions [122], [123]. Regarding brightness, UnaG was shown to be comparable to eGFP when transfected to mammalian (HeLa) cells [120]. The structure, chromophore and fluorescence spectrum of UnaG is shown in Figure 7.

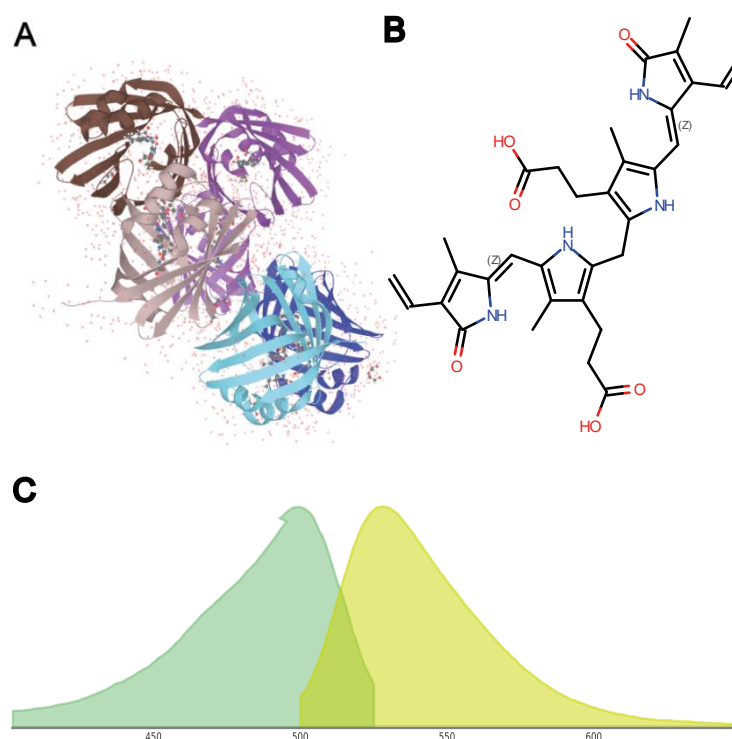


Figure 7 - UnaG. A) Structure. B) Chromophore chemical structure. C) Fluorescence spectrum. Image obtained via <https://www.fpbases.org/>

In 2017, Shitashima et al. showed that UnaG when bound to BR (holoUnaG) goes through two different fluorescent states: holoUnaG1 and holoUnaG2. It was described that holoUnaG1 would first form and then, by a reversible intra-molecular reaction, a brighter holoUnaG2 would form [123]. This discovery opened the possibility for the engineering of a brighter UnaG variant. This happened when Ko et al. engineered an enhanced UnaG (eUnaG) by a V2L mutation on UnaG [124], [125]. This resulted in a FP with higher stability and two times brighter than UnaG. In addition, it showed great potential as a fluorescent marker for single molecule super

resolution microscopy (SMSR), labelling its targets without disruption of proteins' native conformation while maintaining the photo-switchable fluorescence [124]. Another study, also showed that eUnaG can successfully detect the flow of organic compounds in ABC and SLC reporters, responsible for the excretion of BR [126].

2.1.4.3.mNeonGreen

Another FP recently discovered was mNeonGreen (mNG). This is a monomeric yellow-green FP derived from a tetrameric FP from *Branchiostoma lanceolatum* [127]. It was isolated in 2013 by Shaner et al. and its spectrum has the values of maximum excitation and emission of 506 nm and 517 nm, respectively (between standard GFP and YFP wavelength) - see Figure 8. mNG, as GFP, presents an 11-strand β -barrel structure with the chromophore located in the center of the barrel.

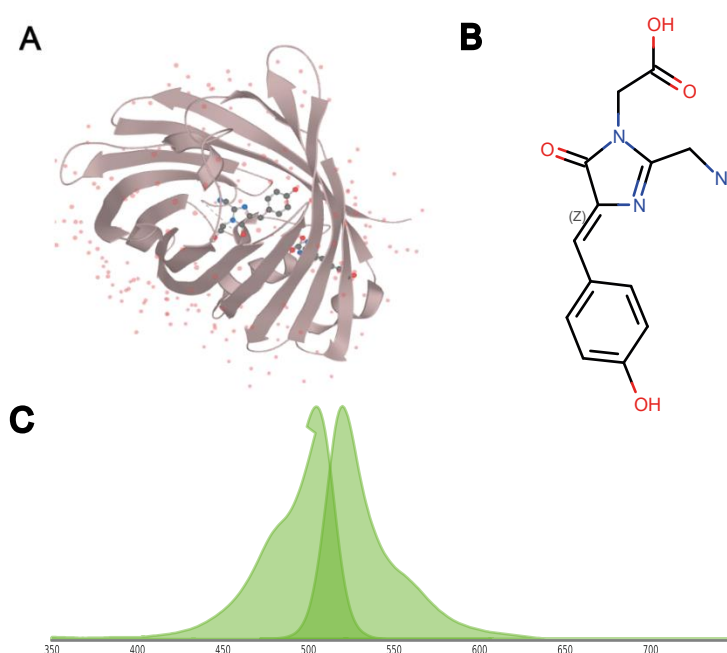


Figure 8 - mNeonGreen. A) Structure. B) Chromophore chemical structure. C) Fluorescence spectrum. Image obtained via <https://www.fpbases.org/>

mNG is mentioned as the brightest monomeric FP given that it shows a brightness of 1.5 to 3 times higher than regular GFPs and YFPs. mNG also has shown great results in labeling proteins in single-molecule super resolution microscopy (SMSR), by showing higher brightness than fusions with Clover, mEGFP, and mEmerald [128]. mNG was also tested as a new potential FRET acceptor from cyan FPs and potential donor for red FPs, being that the new tested pair mTurquoise-mNeonGreen showed higher FRET-efficiency than known FRET-pairs [129]. When tested in living animals, *C. elegans*, mNG was 3-5 times brighter than GFP and despite showing similar expression patterns to those of GFP, only mNG was visible in low expression tissues. Besides, successful targeting of subcellular structures and tracing of endogenous proteins was achieved in this study [127].

Moreover, mNG has also been studied as a reporter for split fluorescence. Split fluorescence is based on the use of self-complementing split FPs. These are FPs that have their DNA split in two fragments and can complement each other to their native conformation emitting fluorescence with no interference from other proteins [130]. In 2017, Feng et al. have

successfully engineered mNG2, a mutated version of mNG split between strands one to ten, forming a 213 amino acids fragment (mNG1-10) and strand eleven, forming a 16 amino acid fragment (mNG11) [130]. This split FP proved to have higher ratio of complemented signal against the background of FP1-10 expressing cells when compared to GFP split version - GFP1-10/11. The use of this FP allowed for the visualization of endoplasmic reticulum (ER) tubules. Possible applications rely on the tagging of proteins with low expression while allowing for the study of protein oligomerization.

2.1.4.4.Others

By now, many other FPs of different color spectrums have been discovered and characterized. Known red fluorescent proteins (RFPs) are mCherry and mScarlet. Also, there are yellow fluorescent proteins (YFPs) as mVenus, cyan fluorescent proteins (CFP) as mCerulean, and many others, as seen in Figure 9.

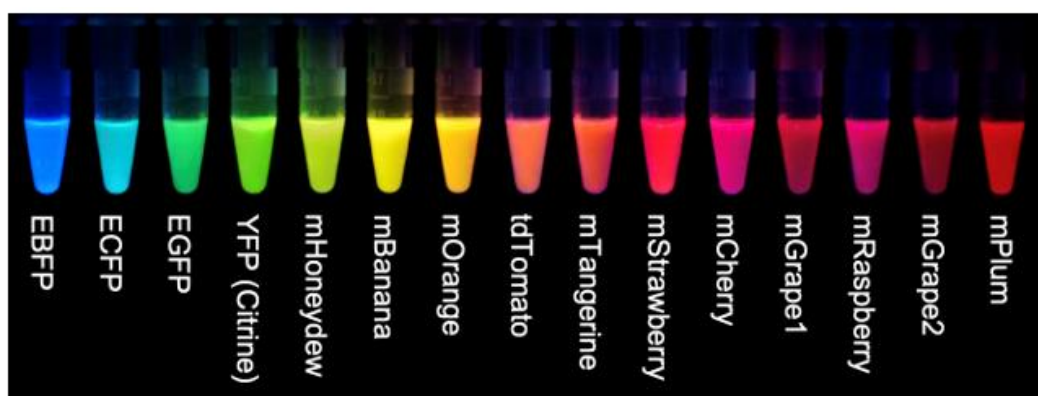


Figure 9 - Existing Fluorescent Proteins across the Visible Spectrum. Image obtained via [287]

mCherry is a monomeric RFPs (mRFPs) and it was isolated from the tetrameric RFP DsRed of *Discosoma sp.* Its fluorescence spectrum presents a excitation wavelength of 587 nm and emission wavelength of 610 nm [131]. Its structure is based on a 13-beta barrel. Some advantages rely on its high photostability, fast maturation and pH resistance while being independent from any cofactor [132]. mCherry has been proven successful in the localization and targeting of specific proteins, the study of protein interactions as oligomerization, for FRET applications and others [129], [133], [134]. Another variant from mCherry is superfolder Cherry (sfCherry), a RFP with 6 mutations developed by Nguyen et al. [135]. As mNG, Feng et al. developed sfCherry2, by adding 3 extra mutations, and split it between strands 1-10 and 11 for its use in split fluorescence [130]. This split FP showed a 10-fold brightness compared to sfCherry similarly split [130].

mScarlet, as mCherry is also a mRFP, however it is a synthetic protein not naturally occurring in nature. It is excited by 569 nm radiation and emits light on a 594 nm [136]. This FP has a beta barrel structure with the chromophore right in the center. Comparing to mCherry, the chromophore is only differently placed on the two conjugated rings. For this reason, this FP has the highest brightness among all other mRFPs (3.5 fold higher than mCherry), since it provides a much higher rigidity to mScarlet structure. Besides, its maturation time is comparable to other mRFPs and it is stable on acidic environments [136]. This protein has proven to be of great use for FRET and super resolution applications while also being used for the trafficking and localization of proteins, successfully.

On another hand, mVenus is a monomeric yellow fluorescent protein (mYFP) isolated from *Aequora victoria*'s YFP - Venus - and it is genetically modified to present higher brightness. It presents a spectrum with an excitation maximum of 515 nm and emission maximum of 527 nm [137]. The main application for mVenus is as a FRET acceptor.

Finally, mCerulean3 is a monomeric cyan fluorescent protein (mCFP) originated from *Aequora victoria*'s CFP - Cerulean. Its structure, similar to GFP-like proteins is characterized by a 11-strand beta barrel with its chromophore located in the center [138]. It has a fluorescence spectrum with maximums of excitation and emission of 433 nm and 475 nm, respectively [139]. This protein has various mutations to increase its brightness, fluorescence lifetime and photostability. As mVenus, mCerulean3 is great for FRET applications, in particular as a FRET donor, when paired with mVenus, it shows great FRET ratios compared to other well-known pairs [139].

2.1.4.5. Labeling tools for Calcium Imaging

Another important type of reporters to consider are calcium indicators, which, as the name states, refer to the sensing of calcium levels within a cell. Calcium (Ca^{2+}) is one of the most important messengers in many vital biology processes. It regulates transcription, metabolism, apoptosis, proliferation, and many others. That being, for research purposes and to fully understand what happens at a cellular level it is important to regulate and quantify calcium levels in distinct cells at different environments/conditions and timepoints.

One type of Ca^{2+} sensors are chemical indicators. For example, BAPTA-derived indicators. BAPTA is an aminopolycarboxylic acid with four carboxylic acid groups, which allows the binding of two Ca^{2+} ions with high affinity. When used as a Ca^{2+} sensor, BAPTA contains a fluorophore on the left aromatic group to allow its visualization on FM [140]. These can be either permeable, when coupled with an acetoxymethyl ester group (AM ester), or impermeable, when in the free salt form, which means they have to be incubated or injected for cell expression, respectively [141]. The main limitations of these indicators are the lack of control over their cellular localization and tendency to compartmentalize in long exposure periods [141]. Other examples of well-known chemical indicators are Fura-2 and Fluo-4, ratiometric Ca^{2+} indicators. These are fluorescent dyes with well-characterized excitation/emission spectrum and allow to measure the Ca^{2+} concentration as a function of the shift in their spectrum when it is bound to Ca^{2+} [142].

More recently, another type of Ca^{2+} sensors are genetically encoded reporters (GECIs), which has the genetically encoded reporters mentioned above, present many advantages and flexibility in its applications. Several GECIs have already been discovered and characterized. The most well-known are GCaMPs, a fluorescence-intensity and single-wavelength based GECI. These were first developed by Nakai et al. in 2001 and are characterized by a central circularly permuted EGFP (cpEGFP) fused N-terminally to the M13 fragment, a 26 residue target peptide for Calmodulin (CaM) derived from the skeletal myosin light chain kinase (sk-MLCK) [143], and fused at the C-terminal to CaM [144]. As an alternative for M13, it is possible to use RS20, a synthetic peptide that binds noncovalently to calmodulin allowing for Ca^{2+} binding and consequent, conformational change [145], [146]. CaM is part of a family of low relative molecular mass acidic calcium-binding protein, shown in Figure 10. This molecule is composed of 148 amino acids polypeptide chain with two domains, each containing two Ca^{2+} binding sites. It is mainly studied for its ability to regulate intracellular Ca^{2+} levels [147]-[149]. At the basal

level, the two domains of CaM surround the M13 fragment in an alpha-helice conformation causing the chromophore to be in a protonated state. However, when Ca^{2+} binds to CaM, there are conformational changes in this complex - CaM binds to M13 - restoring the deprotonated state of the fluorophore that, consequently, results in fluorescence being emitted [144], [150]. Since the discovery of this Ca^{2+} probe, many others have developed different GCamPs. In 2006, Tallini et al. reported the development of GCamP2, which successfully measured the Ca^{2+} transients of myocytes in mouse hearts during four weeks, improving in terms of brightness and temperature stability compared to that of GCamP1 [151]. In 2009, GCamP3 was developed to study the neuronal activity of different cell populations in *Caenorhabditis elegans*, *Drosophila melanogaster* and mouse. This GCamP showed higher fluorescence, improved dynamic range and Ca^{2+} affinity compared to previous GCamPs [152]. Newly developed GCamPs as GCamP5, GCamP6, GCamP7 and many of their variants have aim to enhance the signal to noise ratio, dynamic range and sensitivity to Ca^{2+} transients [153], [154]. In 2013, Chen et al. developed the most well-known and widely used in research GCamP, GCamP6. This is an ultrasensitive GECI capable to detect single action potentials in neuronal stomata and synaptic calcium transients in individual dendritic spines [155]. That being this type of GECIs have allowed to image and analyze multiple neuronal processes which provided a much higher understanding of the brain dynamics in vivo [10], [156]-[159]. Some limitations related to these single-wavelength Ca^{2+} sensors are related to high buffering for long-term GECIs exposures and cytotoxicity.



Figure 10 - The Structure of the Calmodulin RS20 Peptide Complex. Green - Calmodulin. Red - RS20 Peptide. Image obtained via <https://www.rcsb.org/>

Other type of GECIs are FRET-based Ca^{2+} sensors as yellow cameleon 3.60 (YC3.60) [160], D3cpv [161], yellow cameleon Nano [162] and TN-XXL [163]. These are mainly based in CFP and YFPs or GFPs and RFPs as FRET pairs given their have a spectral overlap that allows for the transfer of energy between the donor and acceptor FPs [164]. Compared to single-wavelength sensors, FRET sensors are less influenced by modifications in the optical path length, excitation light intensity and tissue growth and development. Moreover, they present higher brightness so they can be more suitable for calcium imaging, especially on long-term functional studies while having higher sensitivity that allows distinguishing between different basal and active levels of Ca^{2+} on several neurons [165]. In 2014, Thestrup et al. developed a new FRET-based GECI called

“Twitch”, as presented in Figure 11 [165]. These Ca^{2+} sensors are based on the C-terminal domain of troponin C (TnC), a sequence composed of 67 amino acids, isolated from swim bladder and white muscle of *Opsanus tau* with linkers on both ends to the respective donor and acceptor FPs. TnC is similar to CaM in its structure but with different calcium binding processes and biological functions, possessing two globular domains with two Ca^{2+} binding sites in each [166], [167]. In this study, specifically the C-terminal domain was isolated hence there are only two binding sites for Ca^{2+} in total highly reducing the size of the sensor. This can help reduce cytotoxicity and improve the kinetics of the sensor’s response since it implies conformational changes. Several variants of “Twitch” were engineered, specifically, “Twitch2b”, a FRET sensor based on mCerulean3 and cpVenusCD as donor and acceptor FPs, respectively. Twitch2b proved to have a higher signal-to-noise ratio and higher brightness than other Twitch variants. This sensor was successfully used for long term imaging of juxtaglomerular neurons and allowed visualization of different basal levels in neurons [165], [168].

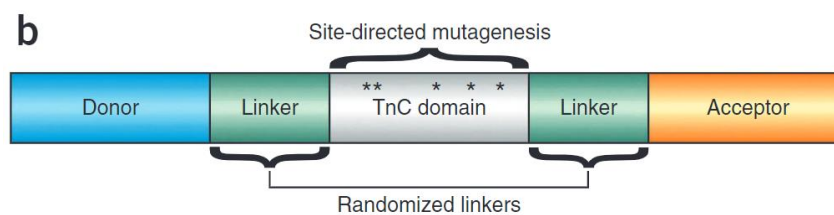


Figure 11 -Structure of Twitch presenting a donor and acceptor domain and a linker in between. Image obtained via [166]

2.1.2. Electron microscopy (EM)

Electron Microscopy is an imaging technique that uses electrons as the source opposed to photons in light microscopy. These possess a much shorter wavelength than photons (100.000-fold shorter) so the resolution obtained is much higher [169]. Initially it was only associated with the analysis of inorganic material, but soon biologists captured the great potential of this technique and applied it to the visualization of the highly detailed structure of organelles, cells and tissues [170].

The microscope consists of an electron beam, a series of electromagnetic and electrostatic lenses to control the trajectory of the electrons and apertures with filters to define which electrons will contribute to the obtained images. All these components are inside a chamber under high vacuum. Essentially, the electrons are gunned towards the chamber, either by heating a tungsten filament or by generating a strong electrical field removing electrons from their atoms. Then, the electrons are directed into the chamber by the application of a high voltage (kV range) in a vacuum environment, they react with the sample and are captured by a CCD camera forming an image of high resolution [171]. When interacting with the sample, there are two different kinds of possible interactions: elastic and inelastic. The first is the product of the deflection of the electron beam by the sample’s atomic nucleus or by the outer shell electrons with the same energy. This cause energy loss and trajectory changes of the scattered electrons. In this case, electrons which are scattered in an angle of more than 90 degrees are called backscattered electrons (BSE). These are electrons which have been through multiple scattering events and have high energy (above 50 eV). The number of BSE is dependent on the atomic number of the sample given that a higher atomic number relates to more positive charges on the nucleus and more backscattering of electrons. Due to its high energy, they reach

higher depth in the sample and can provide information beyond the surface. That being, they are mainly used when gathering topographical and compositional information. Regarding inelastic interactions, these are caused by a series of reactions between the incident beam of electrons and the electrons and atoms of the specimen resulting in ionization of the atoms. This leads to electrons being released from the sample which are called secondary electrons. These are low energy electrons (3-5 eV) and usually remain within a few nanometers of the specimen in analysis [172]. That being they provide very accurate information on the topography of the surface with good resolution. Moreover, when the electron beam interacts with the sample, X-rays are emitted (chemical information) along with Auger electrons (surface's chemical information) and cathodoluminescence [7]. A scheme of the interaction between electrons and a sample in EM is shown in Figure 12.

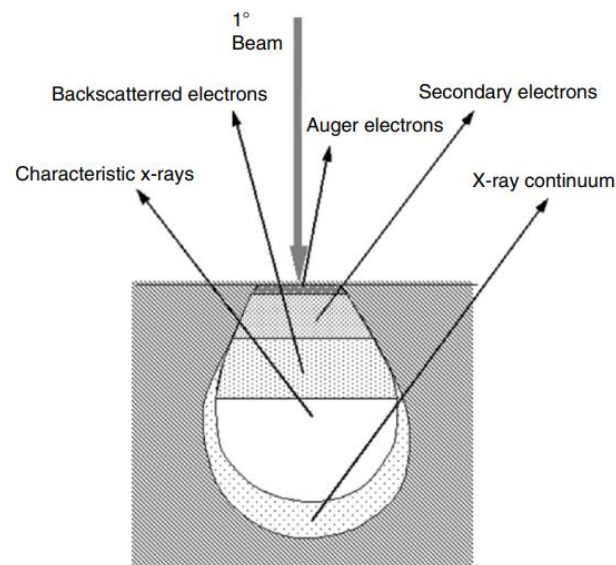


Figure 12 - Electrons Interaction with a Sample in an Electron Microscope. Image obtained via [288]

Given that in EM, electrons are the source of radiation, the obtained image will only depend on their interaction with the sample, their trajectory and energy level. For this reason, in EM images there is no distinction between colors but on gray values and their level of contrast. Contrast is generated when electrons have a high level of interference and scattering with the sample, either by absorption or deflection. The areas of the sample with higher amount of scattered electrons are seen as darker pixels, which contrast with the brighter background. Being that biological components are not electron opaque and usually composed of atoms of low atomic number, the electron beam passes through it very easily, not generating any contrast [170]. Besides, this samples are subjected to vacuum and high temperature conditions in the microscope so there is the need for very complex and time-consuming protocols of preparation for EM observation [173], [174].

In conventional protocols, samples go through fixation of all its components (proteins, lipids, nucleic acids), dehydration, resin embedding, sectioning and post staining. For fixation of proteins, formaldehyde and/or glutaraldehyde are used while for lipids, the standard is osmium tetroxide (OsO_4). This solution is used to preserve the membrane's phospholipids and, consequently, its structure. Given that osmium is a heavy metal, during this process, OsO_4 generates a black insoluble precipitate enhancing the created contrast [175], [176]. Uranyl acetate, a heavy metal salt, can also be used in fixation to bind to proteins, lipids and nucleic

acids while enhancing the obtained contrast. Dehydration is usually performed with ethanol or other solvents. Then, for resin infiltration, epoxy resins are used since they provide a level of hardness which allows cutting of thinner sections compared to LM [177]. For sectioning, a Porter Bloom microtome is commonly used since it allows for cutting of samples thinner than 100 μm in an automatic manner. Another important aspect is the placing of the samples in grids since the electron beam isn't able to penetrate glass. Finally, and again, to enhance contrast, samples are poststained with lead citrate. This is a heavy metal salt and, as osmium tetroxide and uranyl acetate, it binds to cell components enhancing its contrast by interfering with the electrons passing through the sample. According to the application in question and the information of interest to be obtained, different steps of the mentioned sample preparation can be made.

That being, EM is a great improvement to the resolution of images obtained compared to LM and can provide very useful information on a sample's ultrastructure providing the necessary context when studying many biological processes. On the other hand, its complex protocols of sample preparation, particularly the fixation steps, make it difficult to follow the dynamics of the cell's environment, lacking the temporal component. Also, some of the used solutions can interfere with the biological function of the proteins, specifically, FPs which are used for labelling in FM.

Within EM there are different techniques, including TEM, SEM and STEM, which vary mainly on the source of electrons used for imaging. In the next section TEM and SEM are mentioned with more detail.

2.1.2.1. Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy is based on the transmitted electrons which pass through the sample to obtain its images. It was first developed in 1931 by Max Knoll and Ernst Ruska and commercially released in 1939.

TEM microscopes have many operating modes apart from the conventional imaging. Scanning TEM (STEM), spectroscopy and diffraction modes are examples of these. Besides, the contrast can be originated from different sources, either by the atoms atomic number, crystal structure and orientation, the loss of energy by electrons that pass by, etc. That being, TEM can provide a very broad set of information according to the used protocol.

TEM provides data, mainly regarding the inner-core of the sample, as crystal structure, sample's morphology and stress state information. Due to the high vacuum and the short wavelength of electrons, TEM microscopes can reach subnanometer resolution - as low as 20 nm [169].

TEM is considered, still to this day, an essential imaging tool for nanotechnology in the biological and material fields. Its future will focus on studying the native ultrastructure of samples with higher resolution which in combination with electron tomography can provide three-dimensional imaging and supply information on specific cell structures in combination with immunofluorescence microscopy. Combination of these different techniques can provide vital information on a molecular to whole cell level.

2.1.2.2. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy, as TEM, also uses an electron beam as a source to obtain their images. The first ever SEM was developed in 1948 by Oatley [178].

SEM microscopes can reach a magnification of 300,000x and even 1,000,000X in newer models and are considered a great imaging tool for evaluation of the microstructure morphology and chemical composition of a given sample. To obtain this kind of information, SEM uses primary backscattered electrons and secondary electrons. The great advantage of this type of microscopy is the very low resolution achieved - 0.5 nm [7].

SEM has many applications in many different fields, from the study of biologic entities to the study of nanomaterials. Given its resolution, it has had a great impact on the study of subcellular structures and its understanding. In combination with energy-dispersive X-ray spectroscopy (EDS), SEM is able to provide information on the distribution of elements within a sample, with high accuracy.

2.1.2.3. Labeling tools for EM

Despite EM being one of the imaging techniques with the highest resolution achieved so far, it lacks on the number of existing reporters. As mentioned before, EM imaging relies on the level of contrast obtained, so staining protocols and immunolabeling of specific cellular structures with heavy metals are the most common EM labelling techniques.

As FM labelling techniques, in EM these can also be synthetic or genetically encoded. The most common approach is the use of antibodies [179], [180]. In 2007, Blalock et al. successfully used gold particles complexed with antibodies against MUC16 (protein present in the human cornea) for TEM and SEM visualization. The presence of gold, enhanced the electrons scattering and, consequently, improved the generated contrast by revealing clear black spots [181]. It is suggested that multilabeling is possible by using secondary antibodies that are complexed with different sized gold particles. The main issue with this technique is the antibodies' large size which unable a precise detection of the protein's location, disturbing any possible multilabeling when it comes to targeting different proteins in complexes and compromises the antibody's ability to reach tissues with higher depth [182]. Besides, the use of antibodies requires permeabilization treatments which can degrade the native structure of the cell or ultracryosectioning of sucrose-cryoprotected samples with immunostaining of each section which is time consuming [183], [184]. Finally, antibodies with high specificity can be hard to design and produce for some specific proteins of interest so using this technique is not always possible. Given their smaller size, peptide tags were studied as a possible alternative for antibodies. One of these studies was by Tabata et. al which demonstrated that by covalently binding a chemical probe to proteins via a peptide-tag, complexed with gold nanoparticles allowed the visualization of G protein-coupled receptors (GPCR) in the cell membrane in TEM [185].

Existing genetically encoded EM reporters are based on the use of horseradish peroxidase (HRP), a genetically encoded tag which catalyzes the polymerization (H_2O_2 dependent) of 3,3'-Diaminobenzidine (DAB) forming a precipitate that provides the desired contrast when treated with OsO_4 [186]. In 1983, Udin et. al successfully visualized axons on the optical tectum of frogs brains by injecting HRP and later adding DAB [187]. Others have used the same principle to observe synapses and its connections on cats with positive outcomes and even to observe the development route of protein Wingless (essential in embryos development) in *Drosophila* [188], [189], [190]. The main problem with the use of HRP is its lack of activity in the mammalian cytosol given that its constitution includes four structurally essential disulfide bonds and two Ca^{2+} binding sites which may not form in environments with low levels of Ca^{2+} [191]. Another

genetically encoded tag is mini singlet Oxygen Generator (miniSOG). This is a fluorescent flavoprotein that generates singlet oxygen ($^1\text{O}_2$) that locally catalyzes the polymerization of DAB, hence originating a precipitate that creates contrast when treated with OsO_4 [192]. However, this process is light-dependent which limits its application for thicker samples. Another approach is the use of tetracysteine with the biarsenical resofurin label (ReAsH). The tetracysteine is a peptide domain with four cysteines spatially organized to bind to molecules containing arsenic reagents [193]. The tetracysteines domains were often used as tags in association with ReAsH which presents fluorescence and can, by photoactivation, trigger the polymerization of DAB [194], [195].

To overcome such limitations, in 2012, Martell et. al showed that ascorbate peroxidase (APX), a peroxidase with no disulfide bonds or calcium binding sites, could potentially be a new genetically encoded reporter for EM [12]. They engineered a monomeric APX in order to resemble HRP and obtain similar kinetic behavior. This resulted in APEX (enhanced APX), that showed to be active in mammalian cells when stained in the mitochondrial matrix and the endoplasmic reticulum (ER) lumen, providing clear contrast to these. APEX was also able to generate contrast when fused to specific proteins as vimentin, histone 2B (-H2-b) and Cx43, providing information on their location. Besides, APEX staining is simple, given that it is resistant to fixation and staining protocols [12]. However, it requires the addition of exogenous DAB and H_2O_2 which demands for specialized procedures. Moreover, the polymerization reaction occurs in randomly directions so it can result in spatially variable contrast. Nonetheless, it can be confined to particular cellular compartments [196].

Another alternative for EM labelling is the use of metal binding-tags as ferritin, tetracysteins or methallothielineins on genetically encoded proteins to directly accumulate metals [197]. Ferritin is a protein able to store and release iron in a controlled and non-toxic manner. However due to its large size, ferritin is not considered so promising for EM applications. Tetracysteins, as mentioned before are domains composed of cysteines which have the ability to bind to metals and complexes and, therefore, present contrast in EM [198]-[200]. Methallothielineins (MT) are well-known proteins with metal binding capacity due to the rich amount of cysteine residues they possess [200]. It was discovered in 1957 by Vallee et. al by purification of a Cadmium binding protein in the renal cortex of horses [201]. MTs are physiologically important by protecting the cell from metal toxicity and oxidative stress, while regulating the level of some metals as zinc and copper. There are human-expressed MTs, as MT1, MT2, MT3 and MT4. MTs have already been successfully used as tags for EM by enhancing the generated contrast [202]-[207]. This metal binding-tags approach has shown promising results and has the potential to be the new base for genetically encoded reporters for EM however, the use of this tags require incubation with live cells and toxic metals damaging the normal status of the cells. In addition, complex post-fixation procedures follow.

However, reporters that can successfully target sub-cellular structures while being distinguishable on EM are still very few and, most importantly, they rely on the adding of exogenous compounds to the cells so the need for genetically controlled EM reporters that overcome such limitations is crucial.

2.1.3. Correlative Light-Electron Microscopy (CLEM)

More recently, a new type of microscopy has risen: Correlative Light-Electron Microscopy (CLEM). In FM, you can localize specific molecules and proteins while studying their physiological role. However, non-labelled molecules aren't visible in the microscope and there is no structural context. Besides, as mentioned before, the resolution is much lower in this type of microscopy. On the other hand, there is EM, with a much higher resolution that allows for an ultrastructure analysis on a grayscale. However, EM samples require complex protocols of preparation that aren't compatible with the preparation of FM samples. For example, OsO_4 , compounds used in EM's sample fixation and the Epoxy resins can inhibit the fluorophores photo-switching [208]. CLEM is an approach that can overcome such limitations and join these two types of microscopy. Hence, CLEM is a technique that allows to correlate the real time functional data obtained by FM with the structural data of EM from the same sample. This technique is only possible due to the most recent developments in terms of sample preparation, probes, super resolution imaging, microscopes, image overlay, data analysis, etc. [209].

There are two possible ways to perform CLEM analysis: 1) samples are analyzed in FM for visualization of their FP-tagged proteins with further fixation and processing for visualization of the cells' ultrastructure in EM. 2) samples are cut in ultrathin sections when processed for EM while still being fluorescently labeled and are then imaged with both FM and EM. Given that in standard sample preparation protocols, EM and FM are incompatible - either fluorescence is lost or ultrastructure is destroyed - CLEM requires specific protocols of preparation. In EM samples preparation OsO_4 , glutaraldehydes and epoxy resins are used for fixation and embedding, respectively. In CLEM there's the need to perform some modifications since these compounds damage the fluorophores limiting its fluorescence and create artifacts that modify the samples' ultrastructure [11], [210]-[212]. Possible alternatives are the use of acrylic resins and uranyl acetate or tannic acid as fixatives [11], [213]-[217]. However, the use of chemical fixation represents a problem given that it is not instantaneous and doesn't preserve the immediate structure of the sample. This is relevant when studying synapses and neuronal activity in which cells change from an active state to inactive state very quickly [218]. A possible solution is the use of cryo-fixation techniques - cryo-EM - where the samples are quick-freeze by high pressure freezing - samples are frozen in milli seconds while pressure increases avoiding water crystallization [219], [220] - or by putting the sample in a cold block of metal sprayed with liquid helium [221]. This allows to preserve the native ultrastructure while maintaining fluorescence and despite brightness could be decreased, it allows for long times of exposures which is beneficial in some specific applications [222].

Another concern for CLEM is the data acquisition and analysis. Since the same sample is observed with two different techniques it is necessary to match the imaged sections in both types of microscopy. Hence, to properly identify regions of interest (ROIs) and overlay these is a major problem for CLEM [209]. Several have already developed techniques to overcome this. In 1968, Price et al. used a diamond scoring system on the microscope to locate the position of the cell of interest. Essentially, it would mark the side of the glass with no cells while being recorded on LM and then mark the glass side with cells after fixation. This ensured that the marks would be preserved hence preserving the cell's position [223]. More recently, others have used a pulsed laser to mark the glass surface [224]. Another strategy is to add coordinates to the culture plates before seeding the cells by acid etching of the surface or with gridded

ibidi culture plates and coverslips [225]. This way, cells grow presenting a pattern that is then transferred to EM blocks in the resin polymerization step of preparation. Through carbon deposition on the substrate, by adding a metal grid to the sample before fixation or marking the coordinates on the substrate either manually (using a needle) or automatically (using a laser or ion beam) [226], [227]. Many of the current microscopes are coupled with software able to recognize the cells of interest [209]. For CLEM techniques where fluorescence is still preserved while being imaged in EM, correlation can be done simultaneously [228].

CLEM applications include its use to study different organelles of a cell. For example, Tanida et al. used a far-red fluorescent protein, able to maintain its fluorescence after staining with OsO₄ and Epon resin embedding, for visualization of the Golgi apparatus and the endoplasmic reticulum [208]. Another possible application is the localization and tracking of proteins. Rijnsoever et al. used the cryo-immunogold method and the growth of cells on gridded coverslips for their tracking in EM. This way they were able to monitor the kinetics and localization of LAMP-1, a lysosomal membrane protein, in live cells [229]. The study of interactions between two structures is also possible. Jun et al. observed the interaction between the virus HIV-1 and live cells using correlated cryo-electron tomography and live-cell microscopy [230].

2.1.3.1. Labeling tools for CLEM

For CLEM, the used reporters should be visible both in FM and EM. Hence, most of CLEM markers are based on the ones previously mentioned in sections “Labeling tools for LM” and “Labeling tools for EM”.

For pre-embedding CLEM methods, one possible strategy is photo-oxidation. This implies the use of a fluorophore - for FM imaging - and DAB - for EM imaging. Essentially, in the presence of a fluorophore, molecular dioxygen is converted to singlet oxygen which can oxidize DAB and, consequently, cause its polymerization which when treated with OsO₄ creates contrast [231]. This technique was used successfully with FP Lucifer yellow for the label of neurons [232]. Then, as the first genetically encoded tag system used for CLEM, tetracystein tag in combination with ReAsH, as mentioned before, was used for the trafficking of connexins [233]. More recently, miniSOG probes (also mentioned before) was used successfully for the mapping of subcellular distribution of alpha-synuclein in neurons [243], [244]. Another approach is the conjugation of antibodies with peroxidases, as the previously mentioned APEX, that obtain contrast by causing an enzymatic conversion of DAB. Particularly, APEX conjugated with GFP tagged to connexins showed positive results in CLEM [12].

Regarding post-embedding CLEM techniques, immunolabeling methods can be used by directly adding fluorescence to the embedded samples. However, the targeting of the antibodies can be disturbed by the fixation compounds as glutaraldehyde [209]. For cryo-EM, a common post-embedding approach is the Tokuyasu method optimized for CLEM [238], [264]. Here, samples are fixed with aldehydes, dehydrated using sucrose and frozen in liquid nitrogen for ultrathin cryo-sectioning. Then, sections are thawed for immunolabelling [229]. A different approach is to freeze the sample by high-pressure freezing followed by freeze substitution along with the Tokuyasu method for embedding. Usually, this technique presents higher preservation of the ultrastructure and the antibodies' epitopes [237]. Then, sections are immunolabeled with gold nanoparticles or quantum dots (QDs). Different shapes and sizes for these particles can be used to obtain multilabeling for different target proteins [238]. Another labeling

technique for post-embedding CLEM is the use of synthetic fluorophores with chemical tags, as SNAP-tag, HaloTag and CLIP-tag, since they preserve its fluorescence after EM embedding [239]. These chemical probes are self-labeling protein tags which are genetically fused to a protein of interest and covalently conjugated to a small-molecule probe (normally, a fluorescent dye) which must be functionalized with a bioorthogonal linker able to react with the protein tagged complex, as observed in Figure 13 [240]. HaloTag is derived from a *Rhodococcus* haloalkane dehalogenase (DhaA), possessing a chloroalkane linker which binds to the protein irreversibly. In addition, it shows great labeling kinetics and high stability however, it presents a larger size (33 kDa) compared to other protein tags which can interfere with the labeled proteins' function and display [241]. SNAP-tag was engineered from human O6-alkylguanine-DNA alkyltransferase (hAGT) using O6-benzylguanine as a linker [242]. Its smaller size (19.4 kDa) and faster labeling kinetics offers advantages relative to the use of Halo-tag while maintaining high stability [240]. Finally, CLIP-tag is derived from 8 mutations on SNAP-tag and uses a O₂-benzylcytosine linker [243]. This chemical probe has similar characteristics to SNAP-tag maintaining the same labeling kinetics, stability and size (19.4 kDa) [244]. This chemical probes allow for the visualization of a specific protein and its dynamics [251-254]. Immobilization and purification of proteins is also possible with these probes by using a resin with a compound that binds to the tagged proteins [249]-[252], for example Sepharose beads are used for HaloTag-labelled proteins [249]. Then, by elution with a specific protease targeting the linker, the protein bound to this resin is isolated [253]. Their use in super resolution microscopy has also been very broad [260-262]. Besides, given the different linkers they possess, they can be used simultaneously to label different proteins when incubated with different fluorescent dyes and their respective substrates [243], [256], [257].

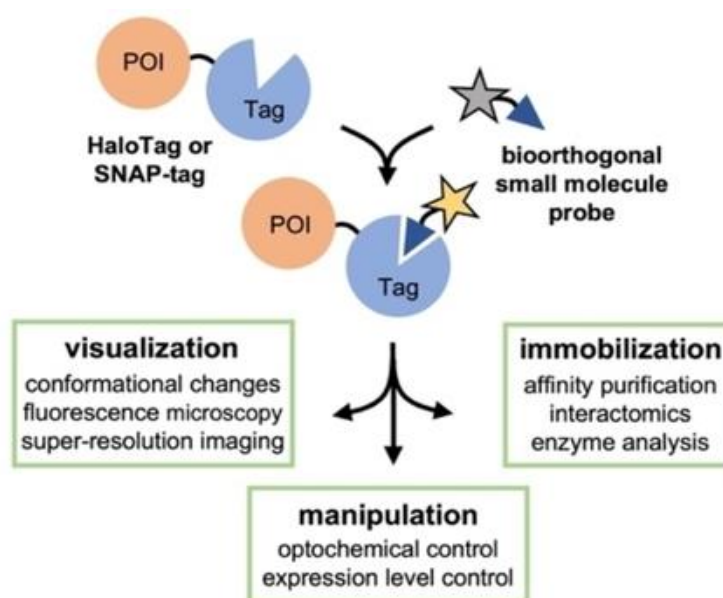


Figure 13 - Demonstration of chemical tags functioning and possible applications. Image obtained via [240]

Great improvements have been made in this field, nonetheless, there is still the need for better reporters that can efficiently preserve native ultrastructure with no artifacts while maintaining its fluorescence after EM sample preparation protocols. Encapsulins are presented below as a new potential reporter for CLEM along with its characteristics, advantages and limitations in its use.

2.2. Encapsulins

Encapsulins are existing nanocompartments in bacteria and archaea. They are proteinaceous shell proteins that auto-assemble in a ring-shaped manner spatially confining what is in their lumen. These compartments were initially discovered in 1994 by Valdés-Stauber and Scherer when they isolated a globular protein from the bacteria *Brevibacterium linens*, which had the ability to inhibit the growth of other types of bacteria [258]. Other discoveries followed, reporting the detection of similar protein compartments in *Mycobacterium tuberculosis*, *Thermotoga maritima* and *Quasibacillus thermotolerans* containing proteolytic enzymes [259]-[261]. In 2017, more than 900 different encapsulin systems were reported between fifteen Bacteria and two Archaea *phyla* and despite their genome being highly conserved among most bacteria, encapsulins present themselves with different sizes, structures and functions [262].

Encapsulins' structure is based on icosahedral complexes (12 vertices, 20 faces and 30 edges) formed by the self-assembly of its shell proteins. They are usually classified according to their triangulation number (T), a virology term to define the number of structural units per each face of the icosahedron and can be calculated by dividing the number of subunits by 60 [263]. So, each encapsulin will self-assemble in different icosahedrons and present different Ts. For example, encapsulins from *Thermotoga maritima* are composed of 60 protomers, T=1 while those from *Myxococcus xanthus* consist of 180 protomers which corresponds to T=3. Encapsulins from *Quasibacillus thermotolerans* have 240 protomers and, consequently, T=4.

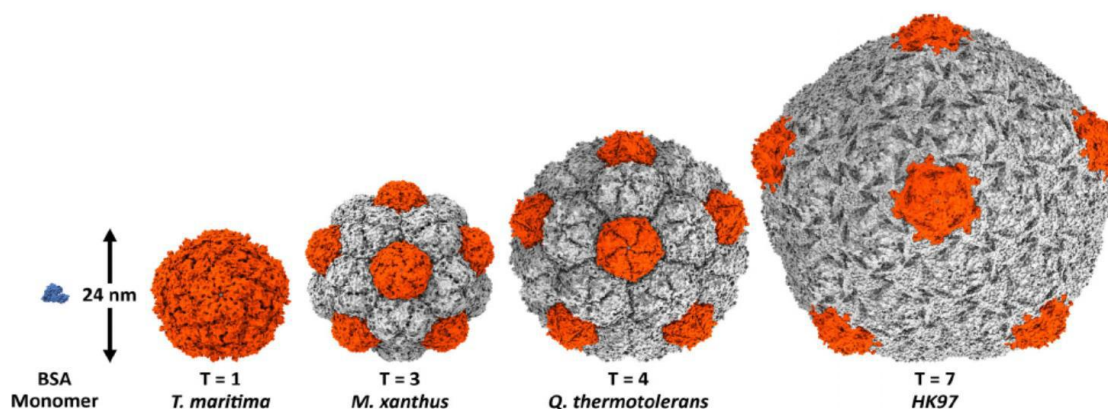


Figure 14 - Structure of Tm, Mx, Qt and HK97 nanocompartments. Image obtained via [269]

For most encapsulins it is considered that its shell proteins have a HK97-like fold [264]. Hong Kong 97 (HK97) is a bacteriophage with a triangulation number of 7 (T=7) and its fold is conserved in many capsid proteins of other bacteriophages [265]. The structure of the four mentioned nanocompartments is observed in Figure 14 with the structure of each monomer on Figure 15. That being, in homology to viral capsid proteins, the encapsulins monomers consist of three parts: P (peripheral domain), A (axial domain) and E (elongation loop) [266]. The P domain forms the main body of the structure and is where the N-terminal is located. The A-domain is responsible for the compact structure of the encapsulin and where the C-terminal is found. Besides, in this domain a pore is formed between the other protomers. The E domain corresponds to a protein loop.

Encapsulins are involved in many physiological processes of bacteria. Their function depends on the cargo proteins that are within the nanocompartment, in the C-terminal [267]. Several cargo proteins have been identified and studied so far. Dye decolorizing-type peroxidases (Dyp), haemerythrins (Haem), ferritin-like proteins (Flp) and, more recently, an iron-mineralizing encapsulin associated Firmicute (IMEF) [261], [268], [269]. These cargo proteins have a short peptide sequence on the C-terminal, called cargo loading peptide (CLP), which is responsible for the transport of the cargo to the interior of the encapsulin before full assembly by enhancing the affinity between the protomers via hydrophobic interactions [266], [267], [269], [270]. Encapsulins able to compartmentalize Dyp and Haem enzymes give bacteria higher resistance to the detrimental action of peroxides and nitrates [262]. Another possible function is the storage and collection of iron by encapsulins that encapsulate the IMEF cargo protein. Finally, encapsulins integrating FLPs are used for iron storage and regulation of their basal levels while protecting bacteria from ROS [262].

Specifically addressing *Thermotoga maritima* (Tm), forms a spherical protein (31 kDa) shell with an external diameter of around 24 nm and an internal diameter of 22 nm [266]. It has 12 pentameric capsomers and a pore size of approximately of 4 Å [271], [275]. Despite the homology between encapsulins and HK97, for Tm encapsulins this homology is less significant given that only the A and E domains correspond to the ones in HK97. The E domain for Tm is shorter which can explain why these form tighter interactions and, consequently, a T=1 capsid [266]. This encapsulin also has the ability to load iron, capturing almost 400 iron atoms per nanocompartment [272].

As for *Myxococcus xanthus* (Mx), it consists of a shell protein (EncA-32.5 kDa) and three internal proteins (EncB-17 kDa, EncC-13 kDa and EncD-11 kDa). It has 12 pentameric and 20 hexameric capsomers in icosahedral vertices and faces, respectively, forming a shell of around 32 nm of external diameter and 26 nm of internal diameter. The internal proteins attach to the inner surface of the compartment, being that EncB and EncC have ferritin-like domains that attract 10-fold more iron atoms than ferritin [273].

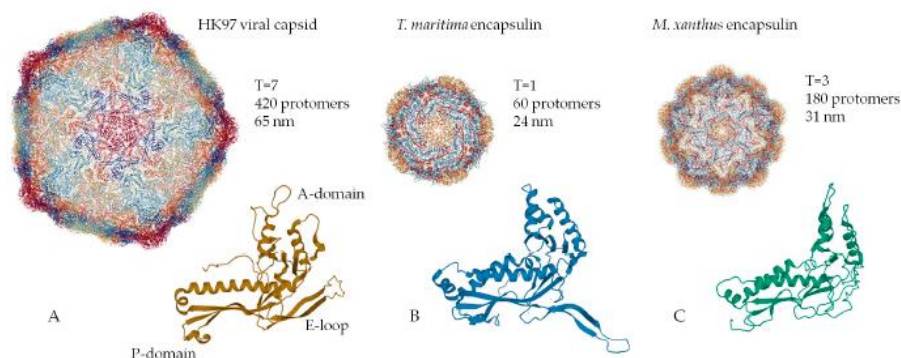


Figure 15 - Demonstration of homology in the structure of HK97, Tm and Mx nanocompartments. Image obtained via [268]

Finally, *Quasibacillus thermotolerans* (Qt) is the biggest encapsulin yet discovered [261]. It consists of 12 pentameric and 30 hexameric capsomers in icosahedral vertices and faces, respectively. After assembly it forms a nanocompartment with 42 nm of diameter. It is composed of four capsid proteins: EncA, EncB, EncC and EncD, with a molecular weight (MW) of 32.2 kDa, and presents an extra domain compared to T=3 and T=1 encapsulins - G loop. In addition, this encapsulin presents many pores, all negatively charged and around 7.2 Å, which

attract iron ions, hence making this encapsulin one of with the highest iron loading capacity (23.000 iron atoms per encapsulin) [261].

Given the great modularity that encapsulins can provide along with their different structures and functions, it is clear that they can be used for multiple applications. Moon et al. engineered Tm encapsulins to present the SP94 peptide, a hepatocellular carcinoma cell binding peptide, on its surface and later loaded the lumen of the encapsulin with Aldoxorubicin, an anticancer prodrug. Results showed that the targeting was successful and presented similar killing efficiency compared to the use of free drugs. However, with the advantage of not being cytotoxic to the cells opposed to the established platforms for drug delivery [274].

Encapsulins have also been studied extensively as nanoreactors for biotechnology applications. Their inner diameter allows for the spatial confinement of enzymatic reactions and deposition of its products [269]. Lau et al. successfully assembled a synthetic Mx T=3 system in the eukaryotic yeast *Saccharomyces cerevisiae* [275]. They proved that the encapsulins could protect unstable enzymes from proteolytic degradation and enhance the complementation of split proteins confined in the nanocompartment. Besides when using a tetrameric pyruvate decarboxylase yeast enzyme (Aro10p) as a substitution for the native cargo protein, it resulted in the confinement of the catabolic enzymatic reaction, avoiding metabolic interference and toxicity from occurring metabolites [275]. Using the same Mx system, Sigmund et al. also proved that encapsulins protect its cargo from degradation. The encapsulation of mEos4b, a photoactivable FP, fused to a FKBP12-derived destabilizing on its N-terminal, signaling for its degradation by proteosomes, successfully shielded the cargo in the lumen [276].

Another potential application of encapsulins is in the field of biomaterials. Kunzle et al. used these nanocompartments to encapsulate gold nanoparticles (AuNPs) by adding CLP to the surface of the AuNPs which allowed the assembly of the encapsulin protomers. Thus, generating inorganic-organic nanostructures [277]. Giessen and Silver also showed that by genetically modifying the Tm shell - deletion of the Flp protein and fusing a silver precipitating peptide (AG4) to the N-terminus of the encapsulin - led to the precipitation of silver ions and consequent formation of silver nanoparticles (AgNPs) with higher antimicrobial activity than silver nitrate and citrate-capped AgNPs [278].

Besides, encapsulins have already proved to be great tools for imaging techniques as MRI, enhancing the contrast via iron biomineralization due to its ability to load iron [276]. This iron loading ability has also proved advantageous in TEM imaging. On another study, Sigmund et al. observed higher contrast in TEM by means of ferroxidase activity from the ferritin domains of the Qt encapsulin [279]. In addition, by co-expressing Mx and Qt encapsulins in mammalian cells it was possible to conclude that these can be differentiated using TEM [279]. Its use in FM has also been studied. Putri et al. successfully engineered encapsulins to express photo-switchable fluorophores - spiropyran without interfering in the assembly of the nanocompartment. By using ultraviolet (UV) light and visible light it was possible to observe the switch on and off of spiropyran using super resolution microscopy [280].

Particularly for this Master Thesis, encapsulins will be studied as a potential genetically encoded reporter for EM, FM and CLEM. In this case, encapsulins present a set of promising advantages.

- Encapsulins are composed by a single shell protein, implying that no proteolytic process is required for its formation. More importantly, its structure is readily visible in EM without the need for modifications or exogenous compounds. They assemble according to different structures and sizes making

them differentiable between each other which is important for multiplexing studies [273]. In addition, the existence of ferritin-like domains allow for the deposition and storage of iron inside the encapsulin with a 10-fold increase on the amount of iron atoms that bind to these domains in comparison with ferritin [282], [290]. This can be used to enhance the contrast generated by the encapsulins in EM.

- Another point in favor is the extension of the encapsulins termini to both the inner and outer surface of the shell, which allows for the functionalization of the encapsulins surface or lumen with multiple moieties, for example ligands for labeling of specific proteins [274]. Furthermore, it's possible to include non-native proteins inside the encapsulins via an encapsulation signal, to protect them from proteolytic degradation or to keep resulting metabolites entrapped from the cytosolic environment, for example, for entrapping a GECl. [282]. Finally, encapsulins are biorthogonal, meaning that they can be successfully expressed in mammalian cells, they can be genetically engineered to respond to a specific requirement or application and genetically encoded which means that its expression can be controlled by the means of different promoters and genetic engineering tactics [276], [279], [283].

- Its assembly is regulated by different encapsulation tags, defined by a CLP on its C-terminus [261]. The compartments are pH resistant and present high stability with different temperatures [281]. The existence of a pore on the inside of the encapsulin, around 5 Å, allows for the passage of small molecules in and out of the encapsulin [281]. These provide flexibility for encapsulins to be used in a broad range of applications and environments.

Given these characteristics, encapsulins present themselves as a very promising genetic marker for FM, EM and CLEM. In this Thesis, encapsulins were engineered to possess different targeting moieties, GECl, metal-binding domains, FPs, among other characteristics for applications on different imaging tools.

Methods

3.1. Cell culture

Low passage number HEK293T cells (ECACC: 12022001, obtained via Sigma-Aldrich) were cultured in advanced DMEM with 10 % FBS, 1% GlutaMax and penicillin-streptomycin at 100 µg/ml at 37 °C and 5% CO₂. Cells were split two times a week using 5 mL of Accutase Cell Detachment Solution by Sigma-Aldrich for 5 min in a 37 °C incubator for detachment and centrifugation for 5 min at 400 rpm. 500 µL of the resulting cell suspension was added to 15 mL of medium in a T75 flask. For transfection, cells were previously washed with PBS and counted using a Neubauer-Improved chamber to seed cells at the recommended density, according to the well-plate to seed. HEK293T cells were transfected, after 24 h, using 100 µL of OptiMEM and X-tremeGENE HP (Roche) transfection reagent according to the protocol of the manufacturer (3 µL reagent per µg of DNA). All actions were performed under the cell culture hood.

3.2. Molecular Biology

All DNA constructs were custom-synthesized by IDT DNA Technologies or obtained by assembly of multiple fragments. In the latter, template plasmids were digested using enzyme restrictions - EcoRI, EcoRI-HF, NotI, NotI-HF and EcoNI, accordingly - by 1 h incubation at 37 °C with the CutSmart® Buffer by NEB. Then, incubation for 20 min at 65°C for heat inactivation or gel electrophoresis of 1% agarose gels during 30 min at 100 V followed by gel extraction of the correct band using the Monarch® DNA Gel Extraction Kit by New England BioLabs (NEB).

After digestion, ligation was performed via HiFi assembly, Gibson Assembly or traditional ligation cloning, according to NEB standard protocols. PCR-based mutagenesis methods were also employed, using the Q5® High-Fidelity 2X Master Mix by NEB or the Platinum™ SuperFi II PCR Master Mix by ThermoFischer Scientific along with the respective primers and followed by a 5 min incubation with the KLD mix (kinase, ligase and DnPI enzymes) at room temperature (RT). Table 1 shows the conditions of the performed PCRs. The resulting amplicon was cloned into pcDNA 3.1 (+) Zeocin for mammalian expression or pGEMHE for future translation into mRNA. The length of the PCR product was evaluated by gel electrophoresis of 1% agarose gels in 30 mL of 1X TAE buffer during 30 min at 100 V. Observation of the gels were made under an UV illuminator. All obtained DNA and RNA products were purified using the Monarch® PCR and DNA Cleanup Kit by NEB and the Monarch® RNA Cleanup Kit by NEB, respectively.

All plasmids were transformed into *Escherichia coli* in agar plates with carbenicillin or kanamycin antibiotics, accordingly, and incubated overnight in a hot room with a controlled temperature of 37°C. Colony PCRs were performed to evaluate the length of the plasmid transformed into *E. coli*, using the DreamTaq Green PCR Master Mix (2x) by ThermoFischer Scientific. For each plasmid, 3 clones were incubated for either miniprep or maxiprep. The

obtained plasmids were isolated and prepared using Monarch® Plasmid Miniprep Kits and Monarch® Plasmid Maxiprep Kits acquired from NEB, respectively. All plasmids were sequenced by Azenta®, using their universal primers.

RNA constructs were obtained using the respective DNA plasmids as a template. Translation was done using the HiScribe™ T7 ARCA mRNA Kit (with tailing) by NEB.

All resulting plasmids used Qt encapsulins and are displayed in Annex I of the Supplementary Data, along with the used cloning method and description.

Table 1 - Thermocycler conditions for PCR-based mutagenesis

Step	Temperature	Time	N° of cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	5 sec	30
Annealing	60°C	10 sec	
Extension	72°C	20 sec per 1 kb	
Final extension	72°C	5 min	1
	4°C	Hold	---

3.3. Gel Electrophoresis

To separate proteins according to their size either clear native (CN-PAGE) or sds page (SDS-PAGE) gels were performed. CN-page separates acidic water-soluble and membrane proteins in an acrylamide gradient gel according to the protein charge and size. SDS-PAGE uses polyacrylamide gels to separate proteins based on its size. In this case, proteins are denaturated by adding SDS (sodium dodecyl sulfate) detergent, giving them a uniform negative charge making their separation dependent on the proteins molecular weight (MW).

Previous to the running of the gels, cells were washed 3 times with 200 µL of PBS, after removal of the medium under the hood. Lysis was achieved by adding 250 µL of mammalian protein extraction reagent (MPER) plus protein inhibitor (PI) and shaking at room temperature for 10 minutes. The lysate was then centrifuged for 10 min at 13400 rpm, at 4 °C. The amount of protein on the supernatant was measured using the NanoDrop Spectrophotometer via OD 280 nm.

In CN-PAGE mode, cell lysates containing 100-500 ng of nanocompartments were loaded along with 4X NativePage buffer onto pre-cast NativePAGE™ Novex Bis-Tris Gel System (Invitrogen) to 12% gels. To the first well of each gel was loaded with a NativePage Unstained Protein Ladder from ThermoFisher. Cathode buffer containing 0.05% of anionic detergent sodium deoxycholate in 1X NativePage buffer, dilution of 20X NativePAGE™ Running Buffer by ThermoFischer in deionized water, was added to the outer chamber while Anode buffer constituted by 1X NativePage buffer was added to the inner chamber. All gels ran at 150 V for 2 h at room temperature. In the case of separating assemblies with fluorescent proteins, the chamber was shielded from light to avoid bleaching. After the running was completed, gels were washed 3 times with water. To detect fluorescently-labeled protein assemblies, CN-PAGE gels were illuminated on a standard UV table. To stain for the total protein content in the cell lysates, Coomassie staining was performed on the CN-PAGE gels by adding InstantBlue® Coomassie Protein Stain by Abcam until fully covering the gel and leaving under gentle agitation for 15 min. After decantation of the Coomassie stain, water was added to wash the gels during

5 min on agitation for 3 times. All resulting gels were documented using a standard cell phone camera.

In SDS-PAGE mode, the used electrophoresis buffer was 1X Tris-Glycine-SDS by BioRad. 50 μ L of 2X Laemlli Sample Buffer by EcoTech BioTech was added to the cell lysates containing 100-500 ng of the total protein content. Then the samples were boiled at 95 °C for 5 minutes, loaded onto Mini-PROTEAN TGX PreCast Gels (BioRad) and ran at 150 V for 1 h at room temperature. SDS-PAGE gels were analyzed after Coomassie staining. The used ladder for reference was the PageRuler Plus Prestained Protein Ladder by ThermoFischer. As CN-PAGE, resulting gels were documented using a standard cell phone camera.

3.4. Microscopy

3.4.1. Fluorescent microscopy

Widefield fluorescence microscopy was performed on an EVOS fluorescence microscopy system equipped with filter cubes to image DAPI, GFP, and RFP. The GFP filter cube was used to image eUnaG, mNG, EGFP and the RFP filter to image mCherry, sfCherry and mScarlet fluorescence.

A Leica SP5 (Leica Microsystems) equipped with 405, 488, 561, and 633 nm laser lines along with the 63 X with oil objective were used for confocal microscopy. According to the specific fluorophores used in each experiment, the spectrum filters were adjusted. Images were taken according to the x,y and z axis with a resolution of 1024 x 1024 and 100 Hz of frequency. In calcium experiments, cells were imaged according to the dimensions x,y and t with a 512 x 512 resolution and 400 Hz of frequency to minimize the time between acquirement of frames.

For super resolution microscopy, external services were employed from ZEISS company. Images were taken using an Elyra 7, a structured illumination light super-resolution system. Two lasers were employed: 488nm (green) and 561nm (yellow).

In all experiments, cells were visualized 24 h after being transfected.

3.4.2. TEM

TEM images were acquired on a Libra120 TEM (Carl Zeiss GmbH, Germany), equipped with a CCD camera (TRÖNDLE Restlichtverstärkersysteme, Germany) using ImageSP software (SYSPROG, Belarus). All samples were pre-irradiated with 120 kV beam voltage, 200 μ rad illumination angle without insertion of apertures. Image acquisition took place with the activated BIO-AIS condenser aperture system and 60 μ m objective aperture under the same beam conditions as pre-irradiation, except with an illumination angle of 100 μ rad.

For TEM sample preparation, the Adapted Briggman Protocol was used. The first step is fixation followed by staining. Cells were lysated and centrifuged at 1000 g for 5 min at room temperature (RT). Fixation was performed using a 2.5% Glutaraldehyde/0,1M sodium cacodylate solution with pH between 6.7 and 7.0 for 20min on ice. Post-fixation followed with 10 mL OsO₄, 10 mL of a 0.3 M Cytoskeletal Buffer solution (CB) and 0.3 g of Potassium hexacyanoferrate, for 20 min on ice. After removal of OsO₄, cells were rinsed 2 times for 5 min with 2 mL of 0.1 M sodium cacodylate buffer and 2 times for 5 min with MilliQ-water. For staining, 2 mL of 1% tannic acid (10 min at RT) was used along with 2 mL of 1% uranyl acetate (30 min at RT) and 3% lead aspartate (15 min at RT), each followed by 5 washes with MilliQ-

water during 5 min. The next step is dehydration and Epon embedding. Starting with by incubating cells with 2 mL of 75% ethanol (EtOH) for 10 min, followed by 1 mL of 90% EtOH for 10 min. Then, incubation with 2 mL of 100% EtOH for 30 min and replacing with fresh 100% EtOH for another 30 min. All these should be performed on ice. Upon removal of EtOH, there is an incubation with 5 mL of 100% propylene oxide for 5 min at RT and replacement with a new 100% propylene oxide for another 5 min incubation at RT. After, cells are incubated in a solution with 50% Epon with 50% propylene oxide for 30 min at RT. Finally, two incubations in 100% Epon for 30 min and overnight at RT, respectively. Finally, on day 2, the sample is rinsed in new Epon resin and baked for 72 h at 60 °C.

In experiments with Calcium, a Calcium Ionophore (10 μ M) or Histamine (100 μ M) was added after centrifugation and before fixation, in the referred timepoints.

3.5.Data Analysis

EVOS and confocal images were analyzed using the FIJI-ImageJ v2.3.0/1.53q software [284] with brightness and contrast being adjusted accordingly.

TEM data was analyzed using the Fiji software. For all tested conditions, 20 particles were manually selected using the ROI manager tool and plot lines of gray values over x-distance were traced.

For experiments involving Calcium, the used software was Calcium Imaging Analyzer v2.1 - CALIMA [285] - which automatically selects the regions of interest and measures the signal intensity levels over time according to the defined parameters.

Results and Discussion

4.1.Engineering Encapsulins to Obtain Different Contrast Patterns

Encapsulins were genetically engineered to have different patterns of contrast - “barcodes” by adding MT3 domains to both the N- and C-terminal of Qt encapsulins. To fulfil such purpose, different plasmids were cloned: pcDNA_Zeo_MT3-QtEnc-FLAG - a Qt encapsulin with a MT3 domain on the inside of the encapsulin - pcDNA_Zeo_MT3-QtEnc-eUnaG - similar to the latter with a surface-exposed eUnaG and pcDNA_Zeo_MT3-QtEnc-MT3-eUnaG - the same but with an extra MT3 domain on the C-terminal of the Qt encapsulin. All three plasmids are presented in Figure 16.

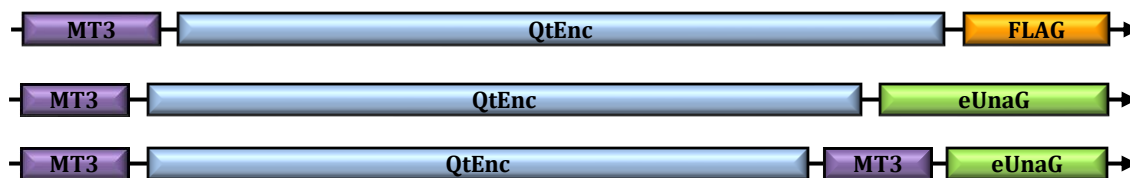


Figure 16 - Representation of pcDNA_Zeo_MT3-QtEnc-FLAG (above), pcDNA_Zeo_MT3-QtEnc-eUnaG (middle) and pcDNA_Zeo_MT3-QtEnc-MT3-eUnaG (below).

The obtained TEM data shows that, in fact, different levels of contrast can be obtained according to the number and localization of MT3 domains. As seen in Figure 17, encapsulins with two extra MT3 domains present themselves darker and with enhanced contrast compared to the encapsulins with only one MT3 domain. A quantitative analysis on the resulting TEM data was performed to confirm what is stated before. Traces of the calculated mean of the gray values of encapsulins are presented according to the x-distance, as demonstrated by the white arrow in Figure 17. These graphs are presented in Figure 18. Both traces have a similar pattern, with lower gray values between 0 to 10 nm and 35 to 45 nm and higher gray values between 10 nm and 35 nm, corresponding to the encapsulins shell and lumen, respectively. Contrary to what was seen in TEM, the measured gray values for the outer ring are very similar between both conditions (around 70 and 90), meaning that the level of contrast does not significantly differ. Nonetheless, gray values comprised in the 10 to 35 nm interval are significantly lower for pcDNA_Zeo_MT3-QtEnc-MT3-eUnaG meaning that these encapsulins lumen is darker compared to the condition with one MT3 domain only. Given the results, quantitative analysis did not show the level of difference that was expected for the presented pattern of brightness however, by TEM visualization is clear that two different classes of encapsulins are presented. To make such results clearer it is suggested that the number of particles under analysis is enhanced to 100 particles for each condition, for example, to take more robust conclusions. In addition, different approaches for particles analysis with higher complexity and fidelity should be taken into account.

Using pcDNA_Zeo_MT3-QtEnc-eUnaG, it was tested whether the expression of eUnaG on the surface would have any effect on the assembly of encapsulins or in the emission of fluorescence by eUnaG itself. The results are presented in Annex II. The fluorescence data shows a clear cytosolic pattern - expected encapsulins display - while the CN page gel with Coomassie stain shows clear bands representing the Qt encapsulins. Given this, encapsulins can successfully express FPs on its surface leaving the lumen free for encapsulation of other POIs, for example. In addition, these encapsulins are visible in both FM and TEM and can be used for pre-embedding CLEM.

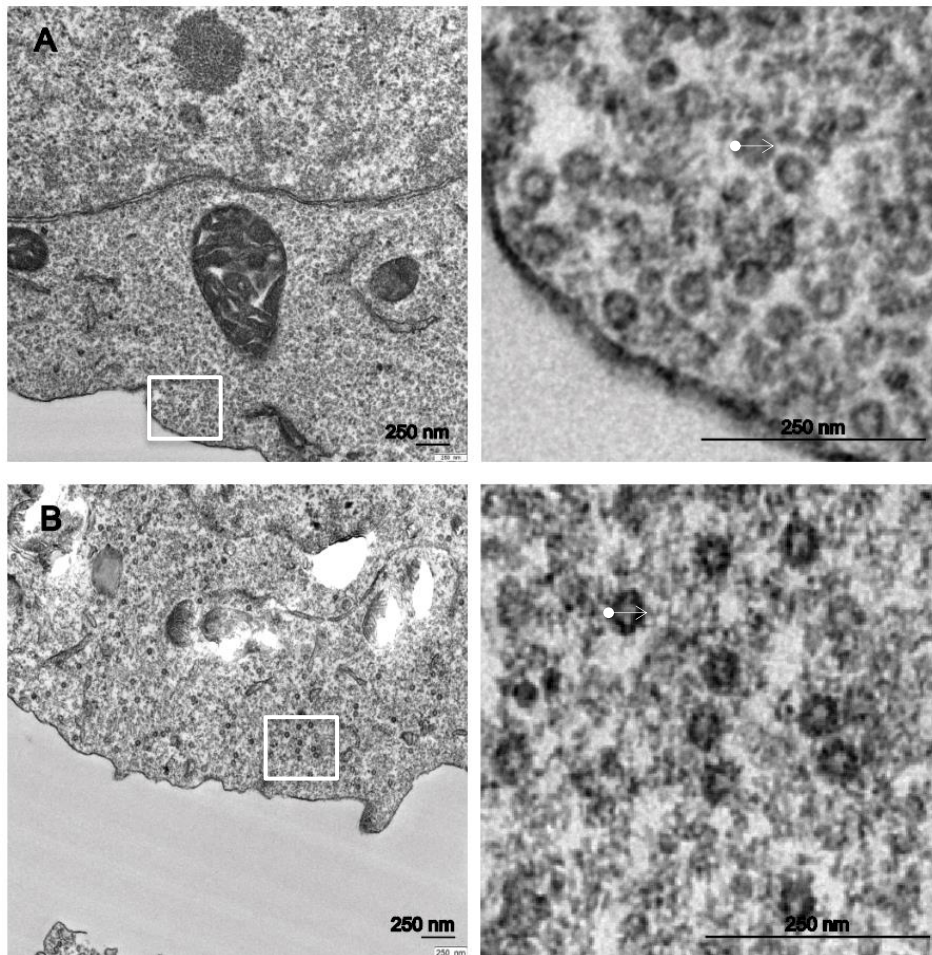


Figure 17 - Comparing different patterns of contrast by expressing encapsulins with a different number of metal binding tags. Images are presented on a 250 nm scale bar. A) TEM micrograph expressing HEK293T cells expressing pcDNA_Zeo_MT3-QtEnc-FLAG. B) TEM micrograph of HEK293T cells expressing pcDNA_Zeo_MT3-QtEnc-MT3-eUnaG.

In short, a new class of encapsulins were attained by adding MT3 domains to the outer shell of the encapsulin, which resulted in a different pattern of contrast visible in TEM. These different classes were distinguishable in EM either at the naked eye or using analysis tools. For further experiments, extra or mutated MT3 domains can be added on both ends of the Qt encapsulin to investigate its impact on the obtained contrast. Besides, encapsulins proved that they can be visible in FM by expressing FPs on its surface without any disruption of its assembly.

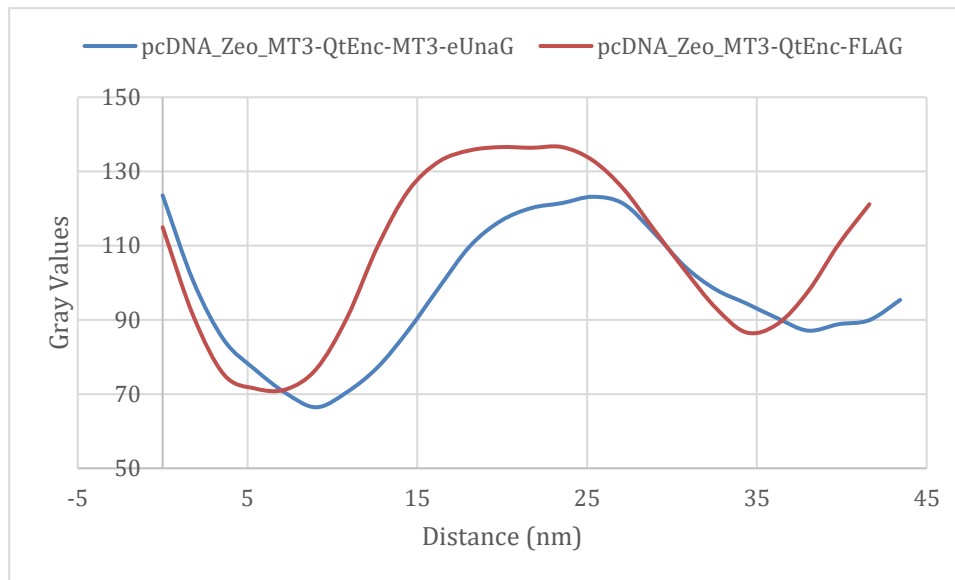


Figure 18 - Trace representing the mean of all gray values vs. the x-distance in nm according to the white arrows showed in Figure 17. For each condition, 20 particles were manually selected and gray values were automatically measured by FIJI. Red line represents the pcDNA_Zeo_MT3-QtEnc-FLAG. Blue line represents the pcDNA_Zeo_MT3-QtEnc-MT3-eUnaG.

4.2.Using Encapsulins to Target Cellular Structures

4.2.1.Expressing an intrabody on the outer surface of encapsulins

Another goal was to show that encapsulins could target specific cellular structures when genetically engineered to have an intrabody on its surface. For that, two constructs were designed: pcDNA_Zeo_MT3-QtEnc-eUnaG and pcDNA_Zeo_MT3-QtEnc-antimCherryIB(LAM-4), as shown below in Figure 19. The fused eUnaG would allow to follow the pattern of expression of the encapsulins on the cells in FM while having the MT3 domain for enhanced contrast in EM. LAM-4 is an intrabody against the RFP mCherry, which in this case was fused to a membrane signal - pcDNA_Zeo-memSig-mCherry - meaning that mCherry would be located at the cellular membrane. If successful, the intrabody would bind to mCherry and, consequently, target the cellular membrane.

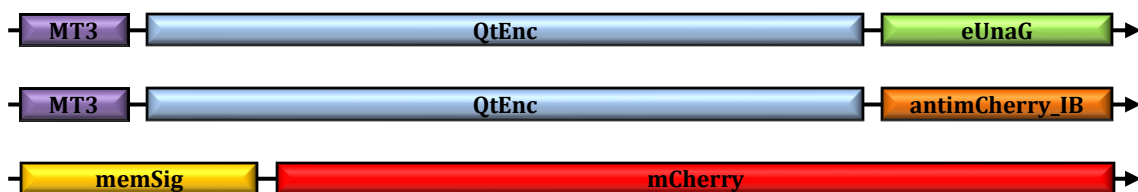


Figure 19 - Representation of pcDNA_Zeo_MT3-QtEnc-eUnaG (above), pcDNA_Zeo_MT3-QtEnc-antimCherryIB (middle) and pcDNA_Zeo_memSig-mCherry

In this case, HEK293T cells were co-transfected with these plasmids in different ratios - 50:50, 80:20 and 95:5, with 20% of the total DNA representing the membrane bound mCherry. These ratios were tested to minimize the amount of used intrabody while maintaining the desired targeting efficiency. The excess of intrabodies in the surface of the encapsulins can

cause sterically constraints in the binding to the target and possibly, on a smaller scale, be toxic to cells. On the other hand, the lack of intrabodies can limit the obtained targeting.

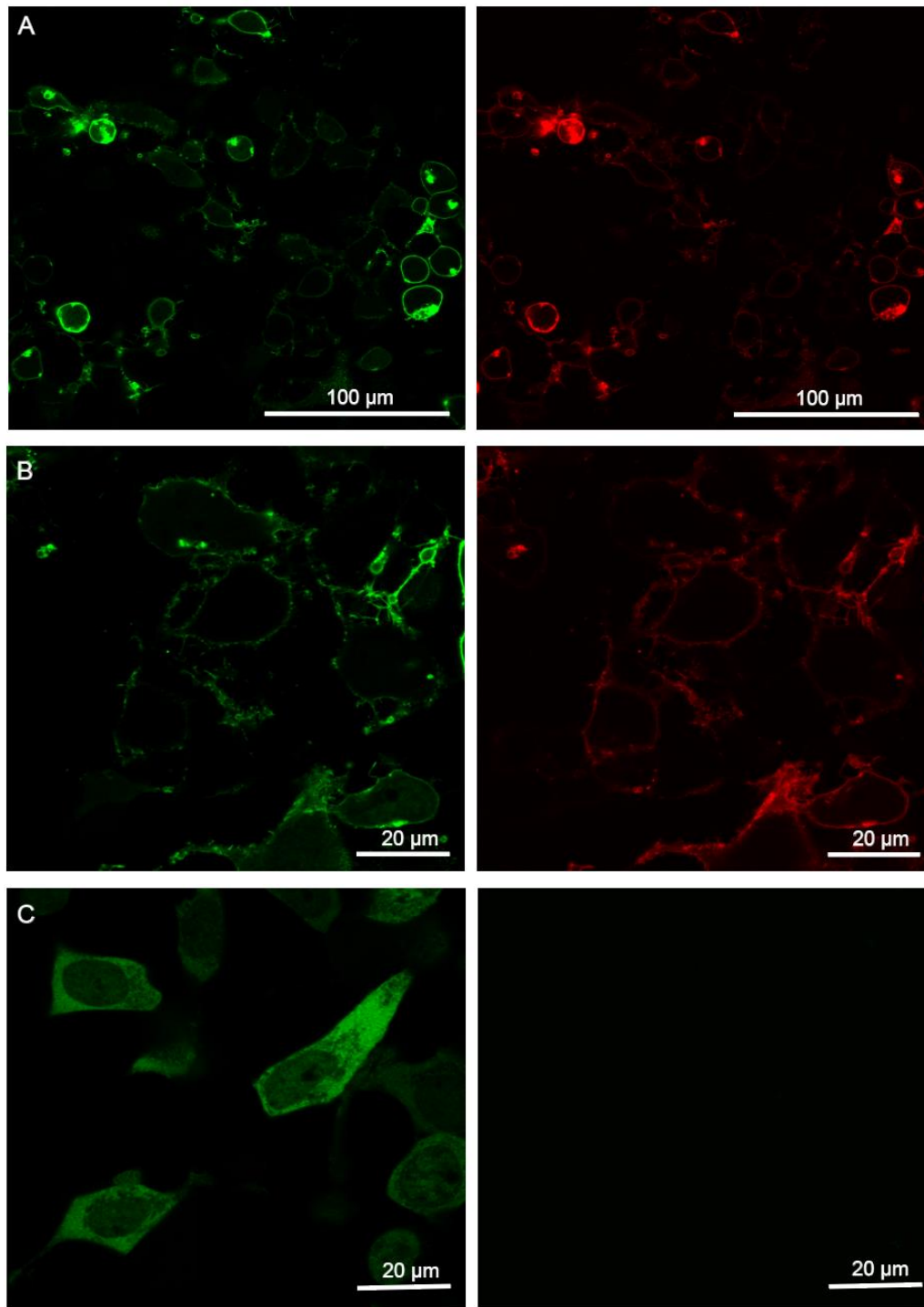


Figure 20 - Targeting of encapsulins to the cellular membrane by expressing an intrabody against mCherry on the surface of Qt encapsulins. Images were taken using a Leica SP5 confocal microscopy system. At the left eUnaG signal and at the right mCherry signal. A) HEK293T cells co-expressing pcDNA_Zeo_MT3-QtEnc-eUnaG and pcDNA_Zeo_MT3-QtEnc-antimCherry(LAM-4) at a 80:20 ratio, with pcDNA_Zeo_memSig-mCherry-FLAG (20% of total DNA). Image presented in a 100 µm scale bar. B) Same condition as A presented in a 20 µm scale bar. C) HEK293T cells expressing pcDNA_Zeo_MT3-QtEnc-eUnaG and pcDNA_Zeo_MT3-QtEnc-antimCherry(LAM-4) at a 80:20 ratio. Negative control.

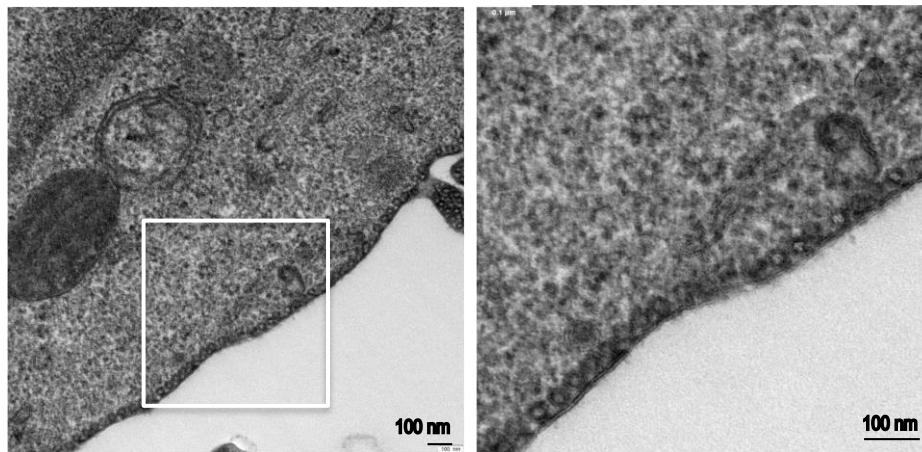
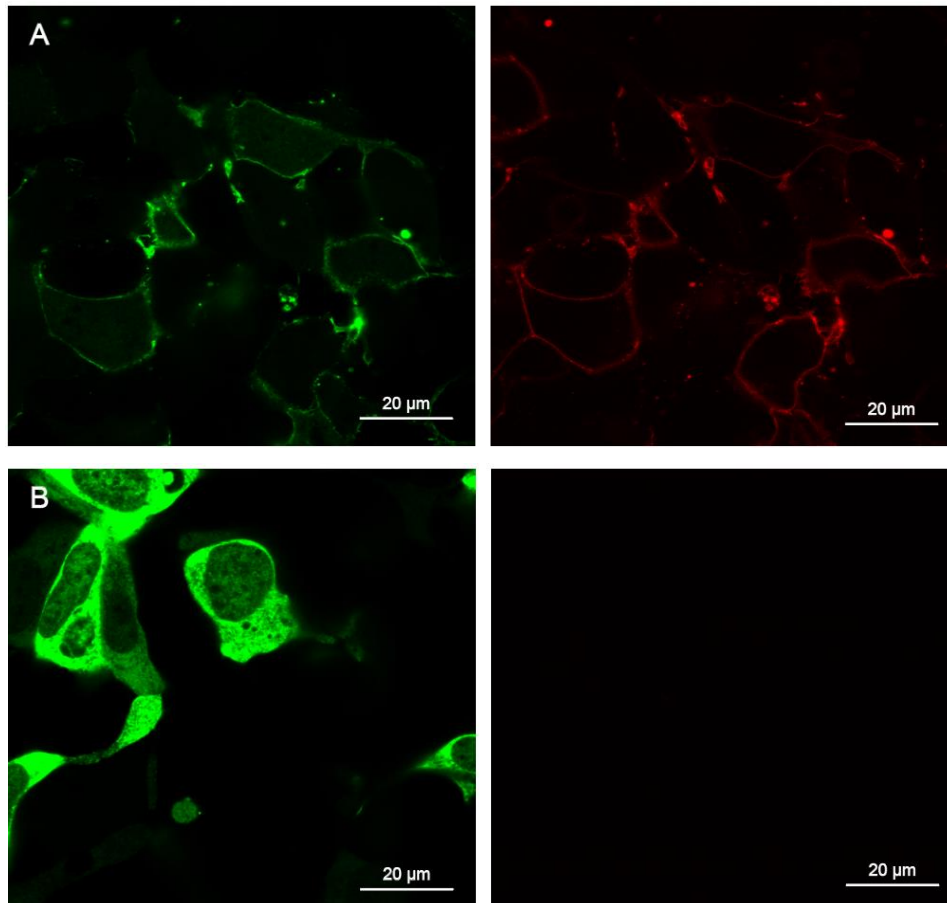


Figure 21 - Targeting of encapsulins to the cellular membrane by expressing an intrabody against mCherry on the surface of Qt encapsulins. Images were taken using a Lyra 7 TEM system. TEM micrograph of HEK293T cells co-expressing pcDNA_Zeo_MT3-QtEnc-eUnaG and pcDNA_Zeo_MT3-QtEnc-antimCherry(LAM-4) at a 80:20 ratio, with pcDNA_Zeo_memSig-mCherry-FLAG (20% of total DNA). On the right is a zoom-in of the white square. Images are presented in a 100 nm scale bar.

Results showed that there is a clear co-localization of the encapsulins in the membrane for the 80:20 ratio, as seen in Figure 20-A,B. For other ratios the results were similar, although the targeting is less clear for the ratio 95:5 where it is visible a cytosolic pattern for eUnaG, in Annex III. As a negative control, the 80:20 ratio condition was tested without the addition of the membrane bound mCherry. As shown in Figure 20-C, the encapsulins present no fluorescence and green fluorescence is expressed across the cytosol proving that the targeting is solely due to the binding of the mCherry to the intrabody located in the encapsulins.



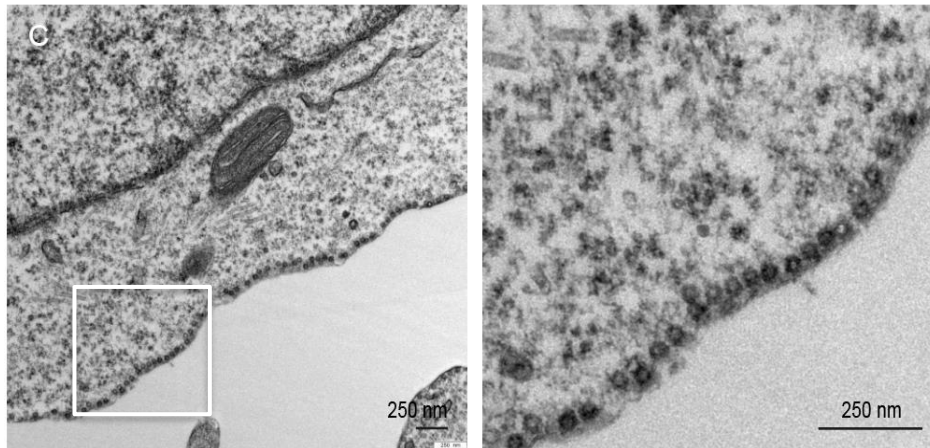


Figure 22 - Targeting of encapsulins to the cellular membrane by expressing an intrabody against mCherry on the surface of Qt encapsulins. eUnaG signal on the left and mCherry signal of the right. A) CM images of HEK293T cells co-expressing pcDNA_Zeo_MT3-QtEnc-MT3-eUnaG and pcDNA_Zeo_MT3-QtEnc-antimCherry(LAM-4) at a 80:20 ratio, with pcDNA_Zeo_memSig-mCherry-FLAG (20% of total DNA). B) CM images of HEK293T cells co-expressing pcDNA_Zeo_MT3-QtEnc-MT3-eUnaG and pcDNA_Zeo_MT3-QtEnc-antimCherry(LAM-4) at a 80:20 ratio. Negative control. Images presented in a 20 μ m scale bar. C) TEM micrograph of the same condition as A. On the right is a zoom-in of the white square. Images are presented in a 250 nm scale bar.

The sample of cells observed in confocal microscopy was then prepared for TEM visualization. As shown in Figure 21, the previous results are confirmed. The encapsulins target the membrane-bound mCherry showing a darker area (higher contrast) across all membrane.

The same experiment was designed with the plasmid pcDNA_Zeo_MT3-QtEnc-MT3-eUnaG to understand if the extra MT3 domain on the C-terminal of the encapsulins would not interfere with the intrabody and mCherry binding, maintaining the same results. Given the previous results, the 80:20 condition was used due to a better balance between the amount of intrabody and the achieved targeting efficiency. As before, there was a clear co-localization of the encapsulins with the cellular membrane, both in CM and TEM, as seen on Figure 22.

These results prove that encapsulins can have targeting moieties on its outer surface without disrupting their assembly and serve as markers for specific proteins or organelles in mammalian cells. Despite successful targeting with all tested ratios, the 80:20 condition should be used for further experiments as explained before. In addition, CLEM was achieved as the observation of the same sample was performed with both CM and TEM. Future experiments on this matter should focus on the targeting of other organelles and structures with higher biological relevance for clinical applications.

4.2.2. By expressing split fluorescent proteins on the surface of encapsulins.

Encapsulins targeting was also tested by using the split fluorescence mechanism allowing to study if they would interfere with the complementation process of the FP in question. In this case, the FP sfCherry2 was used by splitting strands 1 to 10 from strand 11 - mentioned above. The cloned plasmids were: pcDNA_Zeo_MT3-QtEnc-sfCherry2_1_10 - strands 1 to 10 fused to the C-terminal of the Qt encapsulin - and pcDNA_sfCherry2_11-eGFP-CAAX - strand 11 of sfCherry2 on the N-terminus of eGFP which shows green fluorescence and CAAX a ligand which binds to phospholipids, hence targets membranes. In this case, only a 50:50 ratio of the two plasmids was tested. These constructs are presented in Figure 23.



Figure 243 - Representation of pcDNA_Zeo_MT3-QtEnc-sfCherry2_1-10 (above) and sfCherry2_11-eGFP-CAAX (below).

As shown in Figure 24, there is green and red fluorescence detected, from eGFP and sfCherry2 complementation, respectively. The green signal is more intense along the cellular membrane, as expected. Red fluorescence from sfCherry2, despite having a weaker signal, it is also present at the membrane in co-localization with the green fluorescence.

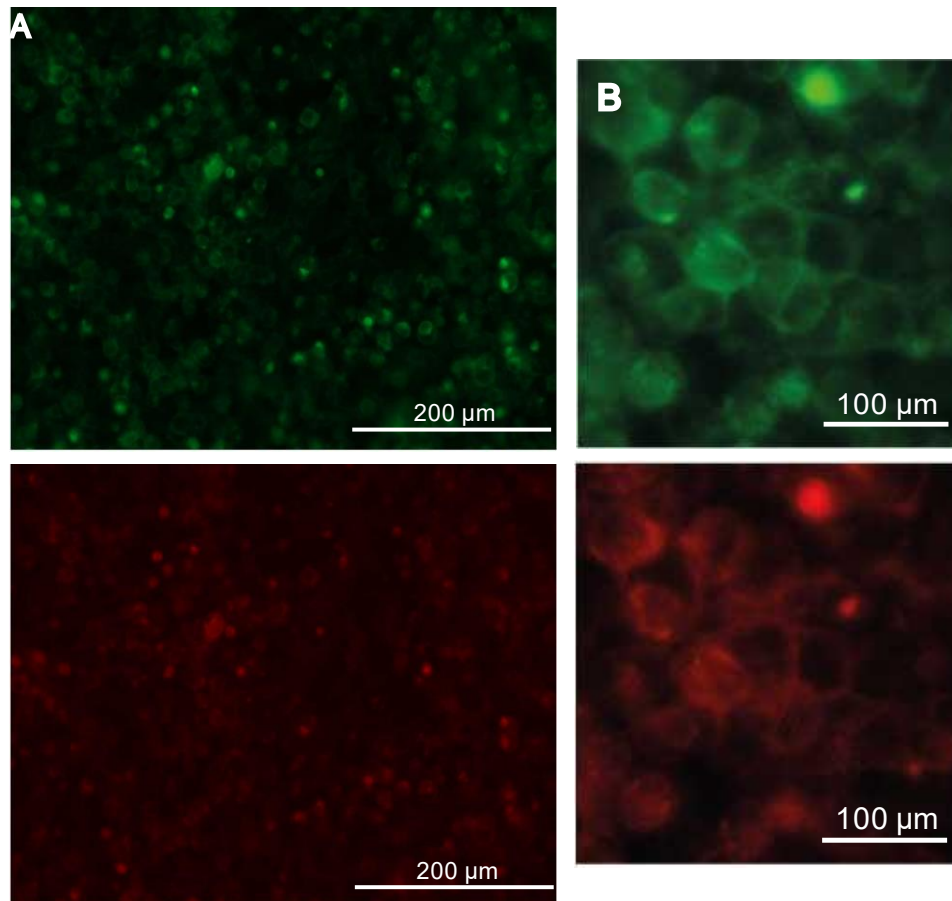


Figure 234 - Targeting of encapsulins to the cellular membrane by complementation of strand 1-10 and 11 of sfCherry2 - split fluorescence. EVOS images of HEK293T cells co-expressing pcDNA_Zeo_MT3-QtEnc-sfCherry2_1-10 and sfCherry2_11-eGFP-CAAX in a 50:50 ratio. eGFP signal (above) and sfCherry2 signal (below). A) Images are presented in a 200 μm and B) in a 100 μm scale.

Following the split fluorescence mechanism of sfCherry2, targeting was achieved successfully. As expected, red fluorescence followed the pattern established by green fluorescence since complementation demands that strand 1 to 10 reaches the membrane to successfully complement to strand 11. Given the results, it can be assumed that encapsulins do not interfere with this complementation allowing for the restoration of the native structure of sfCherry2 resulting in emission of fluorescence. The same experimental setup should be repeated in CM, as well as TEM for better resolution and more robust results. Future experiments should focus on the use of other FPs as mNG and possibly, applying this technique with CRISPR-Cas9 for the creation of one stable cell line. This would allow the targeting of other proteins by knock-in of strand 11 of a FP in genes of interest without the need for co-

transfection of different plasmids. One possible advantage of this method is that it does not require the use of intrabodies, as the previous experiment, since these can be potentially cytotoxic.

4.3. Encapsulins as Sensors for Calcium Levels

4.3.1. Fluorescence enhancement induced by Calcium

Encapsulins were also tested to serve as potential markers for changes in the cells environment, specifically to be sensible to Ca^{2+} levels modifications by changing its conformation (for EM) and by changing brightness of attached fluorophores (for FM). For this, a construct of pcDNA_Zeo_Twitch2b-QtSig was developed. Twitch2b serving as the calcium sensor incorporated with a Qt encapsulin signal so they would target and enter the encapsulin lumen. This plasmid was co-transfected with pcDNA_Zeo_QtEnc-FLAG, in a 90:10 ratio, into HEK293T cells and later analyzed under confocal microscopy. The mentioned plasmids are shown in Figure 25. While on the confocal, the cells medium was removed, and after 30 seconds,



Figure 25 - Representation of pcDNA_Zeo_Twitch2B-QtSig (above) and pcDNA_Zeo_QtEnc-FLAG (below).

replaced by a 2uM solution containing DMEM and a calcium ionophore (CI). When exposing the samples to UV light the expected result would be that the detected fluorescence is enhanced by the added Ca^{2+} . Furthermore, a CN-page gel for these conditions (two wells for each) was performed to also evaluate the effects of calcium stimulation. Specifically, in the first well of each condition, a Ca^{2+} solution was added and for the second well of each condition, an EDTA solution (Ca^{2+} chelator) was added right before starting the running of the gel. Later, the gel was evaluated under an UV illuminator and Coomassie stained to check if Twitch2b would not disrupt the assembly of encapsulins by entering into its lumen.

The results showed that Twitch2b successfully enters the encapsulins, not disrupting their assembly, as it is possible to see a clear band above 1.3 MDa (MW marker limit) representing the Qt encapsulins, see Figure 26-A. The calculated MW of the Qt encapsulins and Twitch2b assembly is around 7 MDa. However, the lower weight bands, representing Twitch2b are much brighter, implying that the amount of Twitch2b that actually entered the encapsulins was low. Regarding brightness enhancement, by the naked eye it is possible to see that the bands where calcium was added are brighter than the ones with EDTA, as expected. Since, the difference of signal intensity was less clear for the upper bands, these were analyzed for quantification of its brightness. Using Fiji, the intensity of each band was measured by defining a threshold of intensity values that isolated both bands from the background, see Figure 26-B. On Figure 26-C,D, the obtained trace and the resulting area measurements, which equals the intensity signal, are shown. As observed, intensity of the band corresponding to Ca^{2+} stimulation was 40% higher than the one with EDTA stimulation.

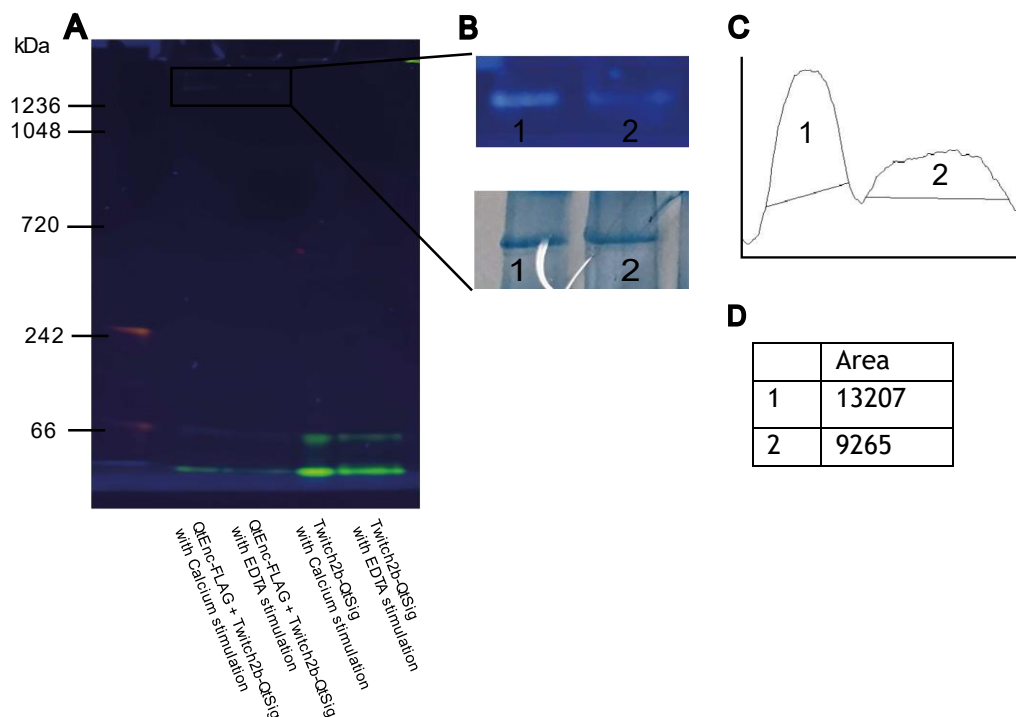


Figure 26 - CN page gel of Twitch2b with Qt encapsulins and Twitch2b only, respectively. Each condition was loaded with a Calcium solution or EDTA. **A)** CN page gel observed under an UV illuminator. Square is around the bands that represent the Qt encapsulins. **B)** A zoom-in of the square in **A** (above) zoom-in of the square of the gel after Commassie Staining. Each band was labelled 1 and 2 to distinguish Calcium and EDTA conditions, respectively. These images were treated in Fiji for brightness and contrast enhancement. **C)** Trace of pixel intensity over x-distance. Traces for Area calculation were defined manually. **D)** Table shows measured Area values for each band. Analysis performed on Fiji using the Gel Analyzer Tool.

In Figure 27 and 28, it is shown the averaged intensity of signal of all detected ROIs by CALIMA over time, in minutes. The positive control (Figure 27), with only Twitch2b being expressed, had a very fast response to the Ca^{2+} with only 20 seconds separating the timepoint of adding calcium and Twitch2b reacting to it. Regarding the second condition (Figure 28), where Twitch2b is co-expressed with Qt encapsulins, there's also a continuous enhancement of brightness after adding the CI, however it took around 3 minutes and 10 seconds to show significant differences and reach its peak. This, most likely, means that only after about 3 minutes, calcium enters the encapsulins. One possible explanation lies on the kinetics of the reaction, since the CI must enter the encapsulin to only then react with Twitch2b.

In Figure 29-A,B, there is one single cell at two different timepoints: when the CI is added to the cells ($t=0$) and when the CI reacts with Twitch2b ($t=20\text{sec}$ and $t=3\text{min } 10\text{sec}$ for Twitch2b alone and Twitch fused to the Qt encapsulin, respectively). Here, it is clear that brightness is enhanced with the adding of the CI. On the other hand, as mentioned above, it should be expected that the Ca^{2+} binding would lead to the contraction of Twitch2b. In this experimental setup, Twitch2b is fused to the encapsulin on its lumen. Hence, it should be expected that the contraction of Twitch2b would also contract the nanocompartment, resulting in a different representation of the encapsulin, with a thicker outer ring and smaller inner diameter. To confirm this theory, samples with the same conditions described above were imaged on TEM with and without Ca^{2+} stimulation. Contrary to what was expected, the encapsulins showed no significant differences in its diameter size when exposed to a $2\text{ }\mu\text{M}$ Ca^{2+} solution, see Figure 29-C.

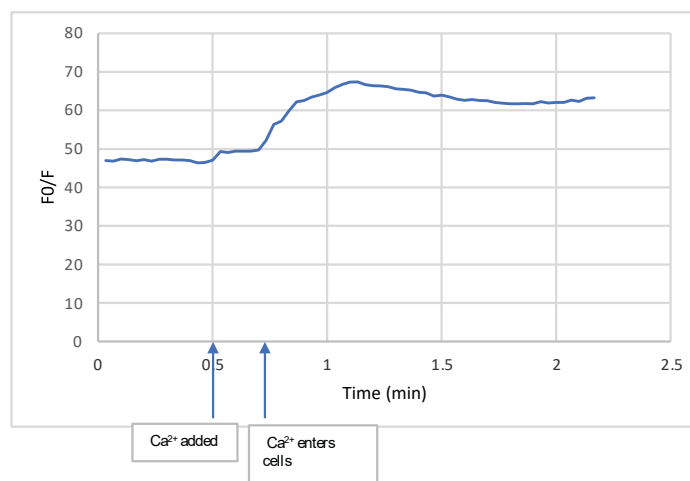


Figure 28 - Brightness enhancement of Twitch2b by reaction with a 2 μ M Cl solution. Expression of pcDNA_Zeo_Twitch2b-QtSig. Signal intensity is presented time-dependently, in minutes.

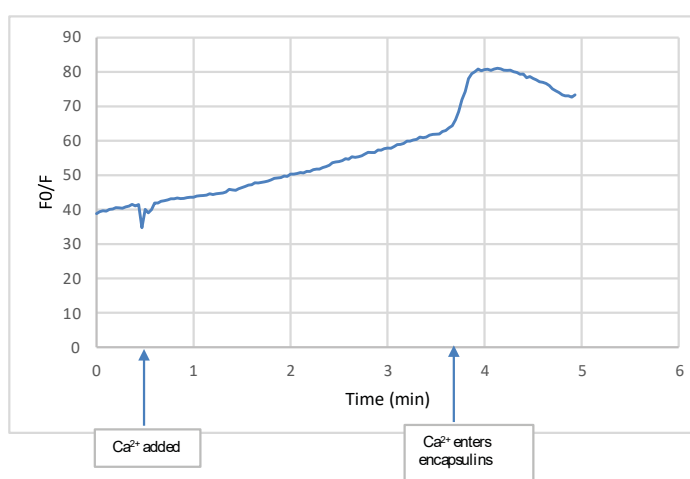


Figure 27 - Brightness enhancement of Twitch2b by reaction with a 2 μ M Cl solution when locally restricted to the lumen of Qt encapsulins. Co-expression of pcDNA_Zeo_Twitch2b-QtSig and pcDNA_Zeo_QtEnc-FLAG. Signal intensity is presented time-dependently, in minutes.

In short, encapsulins were able to sense the calcium levels within a cell by successfully entrapping the FRET-based sensor Twitch2b in its lumen. This encapsulation does not disrupt the normal behavior and function of Twitch2b and its enhancement of brightness is clear by either visualization on confocal microscopy or analysis on CALIMA software. On the other hand, TEM data wasn't clear on the structural impact of Twitch2b over the established nanocompartment. Unfortunately, follow-up experiments with Twitch2b have not shown the same favorable results as the ones presented. Possible explanations rely on the fact that the Twitch2b response is not significant due to their dynamic range being too small for visualization on either TEM and CM. One alternative would be to use a known GCaMP as GCaMP6, entrapping them into encapsulins and studying their response to Ca^{2+} . Despite the conformational change in GCaMP6 being less relevant than the one of Twitch2b, their dynamic range is higher which can show improvements compared to the obtained results. Finally, it is suggested that the extension of the period of exposure of TEM samples to Ca^{2+} before fixation is enhanced (to 3 minutes and 10 sec as it was the peak of brightness) or that the concentration of the used Ca^{2+} solution to be higher.

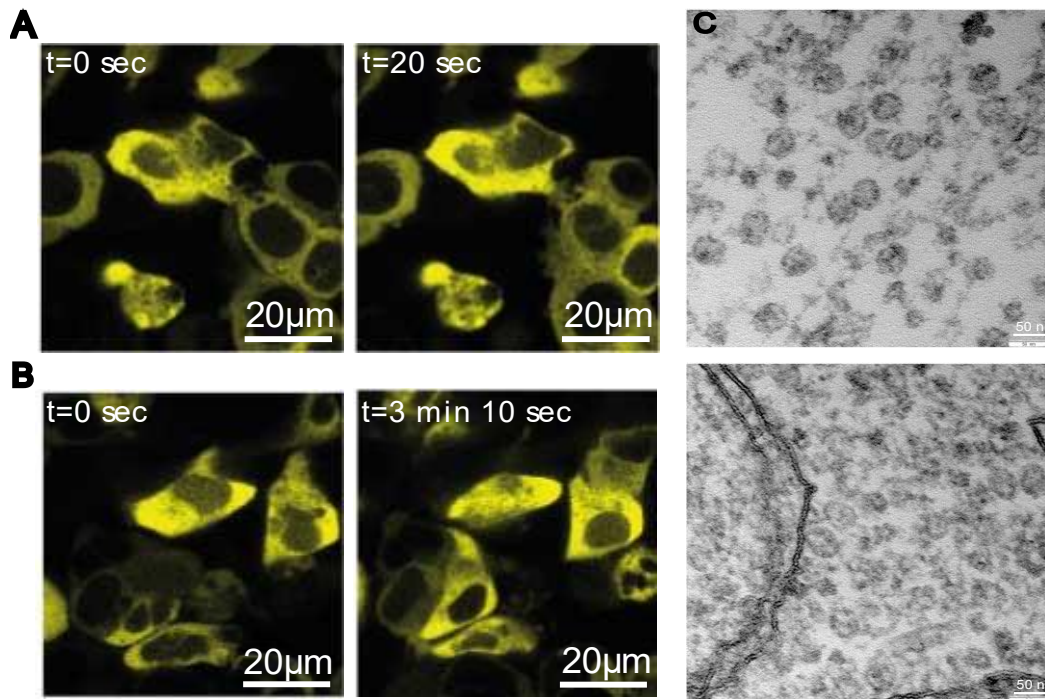


Figure 29 - Brightness Enhancement using Twitch2b by reaction with a 2μM Cl solution on a single-cell level. A) Twitch2b only. B) Twitch2b fused to the N-terminal of Qt encapsulins. Both imaged at two different timepoints: before Ca²⁺ stimulation and after Ca²⁺ stimulation (at the peak of brightness). C) TEM images of the latter condition with no Ca²⁺ stimulation (above) and with Ca²⁺ stimulation (below).

4.3.2. Encapsulins aggregation induced by Calcium.

Another calcium experiment was based on the CaM-RS20 complex ability to bind to Ca²⁺ and HpFtn, a ferritin from the bacteria *Helicobacter pylori* which loads and binds to iron. That being, the RS20 peptide was fused to the C-terminal of two different plasmids creating: pcDNA_Zeo_MT3-QtEnc-eUnaG-RS20 and pcDNA_Zeo_MT3-QtEnc-MT3-eUnaG-RS20. As for CaM and HpFtn, the plasmid pcDNA_Zeo_CaM-HpFtn was cloned. These plasmids are shown in Figure 30.

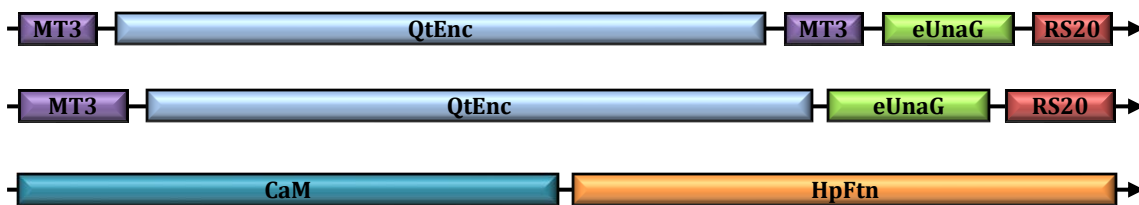


Figure 30 - Representation of pcDNA_Zeo_MT3-QtEnc-MT3-eUnaG-RS20 (above), pcDNA_Zeo_MT3-QtEnc-eUnaG-RS20 (middle) and pcDNA_Zeo_CaM-HpFtn (below).

Similar to the previous experiment, HEK293T cells were co-transfected with these plasmids and evaluated under confocal microscopy while replacing its current growth medium with a 100 μM histamine solution with DMEM. The addition of Ca²⁺ should result in the binding of RS20 to CaM and consequent conformational change which would allow for the fused HpFtn to bind to others HpFtns and form clusters. In this case, histamine was used as the Ca²⁺ solution since it presents transient expression patterns and would allow to study the formation and disintegration of the formed clusters, sequentially. To attend such purpose, cells were imaged

in real-time over three different timepoints: right before histamine was added (t=0 sec) and 30 seconds (t=30 sec) and 3 min (t=3 min) after histamine addition.

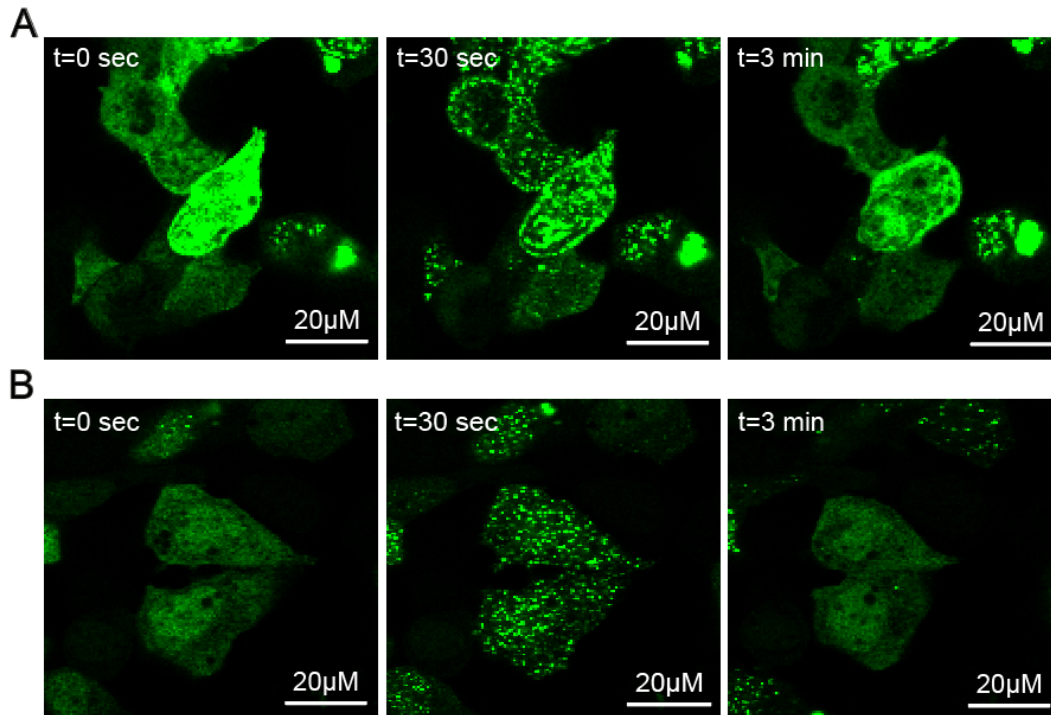


Figure 31 - Calcium-induced aggregation of encapsulins. Images obtained with a Leica SP5 system and presented in a 20 µm scale bar. A) HEK293T cells co-expressing pcDNA_Zeo_Mt3-QtEnc-eUnaG-RS20 and pcDNA_Zeo_CaM-HpFtn. B) HEK293T cells co-expressing pcDNA_Zeo_MT3-QtEnc-MT3-eUnaG-RS20 and pcDNA_Zeo_CaM-HpFtn. Both conditions were captured in three different timepoints: before calcium stimulation and 30 sec and 3 min after calcium stimulation.

As expected, the addition of Ca^{2+} highly triggers the clustering of the encapsulins for both conditions tested (Figure 31). In addition, after 3 minutes of histamine stimulation the clusters are almost fully disintegrated and the encapsulins go back to a cytosolic pattern. TEM data of these conditions is not available due to poor EM sample preparation which limited the quality of the obtained images and, consequently, the robustness of any conclusions to be taken.

Again, encapsulins showed to be able to serve as sensors for Ca^{2+} levels. In this case, encapsulins suffer a structural change when stimulated with histamine by binding to other encapsulins and form clusters. These clusters are visible on CM which proves their potential as genetically encoded reporters to evaluate the physiological state of a cell. Given the transient profile of histamine stimulation, the formed clusters are disintegrated over time, which can be advantageous in further experiments regarding the study of the switching on and off effect occurring in neurons, for example. The same experiment should be repeated for TEM analysis and confirmation of the results obtained in CM.

4.4. Encapsulins as labelling tools for Super Resolution Microscopy.

Other type of microscopy tested for encapsulins was super resolution microscopy. In this case, the same experimental setup as the one for targeting experiments were tested: pcDNA_Zeo_MT3-QtEnc-eUnaG and pcDNA_Zeo_MT3-QtEnc-antimCherry(LAM-4) in a 80:20 ratio with pcDNA_Zeo-memSig-mCherry as 20% of the total DNA.

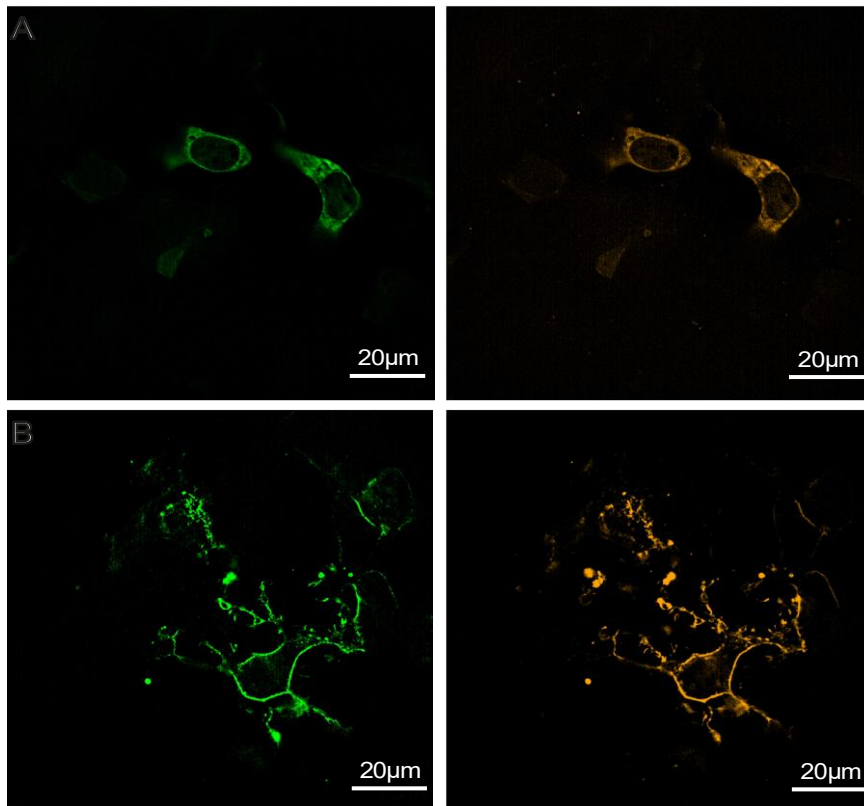


Figure 32 - Super Resolution Microscopy data. On the left eUnaG signal and on the right mCherry signal. A) negative control - pcDNA_Zeo_MT3-eUnaG with pcDNA_Zeo-MT3-QtEnc-antimCherry in a 80:20 ratio. B) pcDNA_Zeo_MT3-eUnaG with pcDNA_Zeo-MT3-QtEnc-antimCherry in a 80:20 ratio with memSig-mCherry (20% total DNA).

The acquired results, presented in Figure 32, confirm what was observed before in CM with the same conditions. As expected, these super resolution images show the targeting succeeded with much more detail than before. Serving the purpose of this Thesis, the results prove that encapsulins can be used as a genetic reporter in multiple imaging tools.

4.5. Encapsulins as Genetically Encoded Reporters for CLEM using Chemical tags.

Encapsulins were tested as genetically encoded reporters for post-embedding CLEM by fusing SNAP-tag or CLIP-tag on the C-terminal of Qt encapsulins. Both plasmids are presented in Figure 33. As mentioned above, these tags preserve its fluorescence during EM sample

preparation so its successful fusion to encapsulins would allow to use them on both FM and EM using post-embedding sample preparation methods.



Figure 33 -Representation of pcDNA_Zeo_MT3-QtEnc-CLIPtag (above) and pcDNA_Zeo_MT3-QtEnc-SNAPtag (below).

Initially, these encapsulins were tested with CN-page gels to prove its assembly. While for CLIP-labeled encapsulins there were no visible bands to show that, SNAP-labeled encapsulins showed a band which would most likely indicate the assembly of encapsulins. One possible explanation for this difference in the resulting gels is due to accumulation of the used chemical dye which unable the running of the proteins in the gel for CLIP-tag encapsulins. Unfortunately, due to technical problems this biochemical data is not available to present.

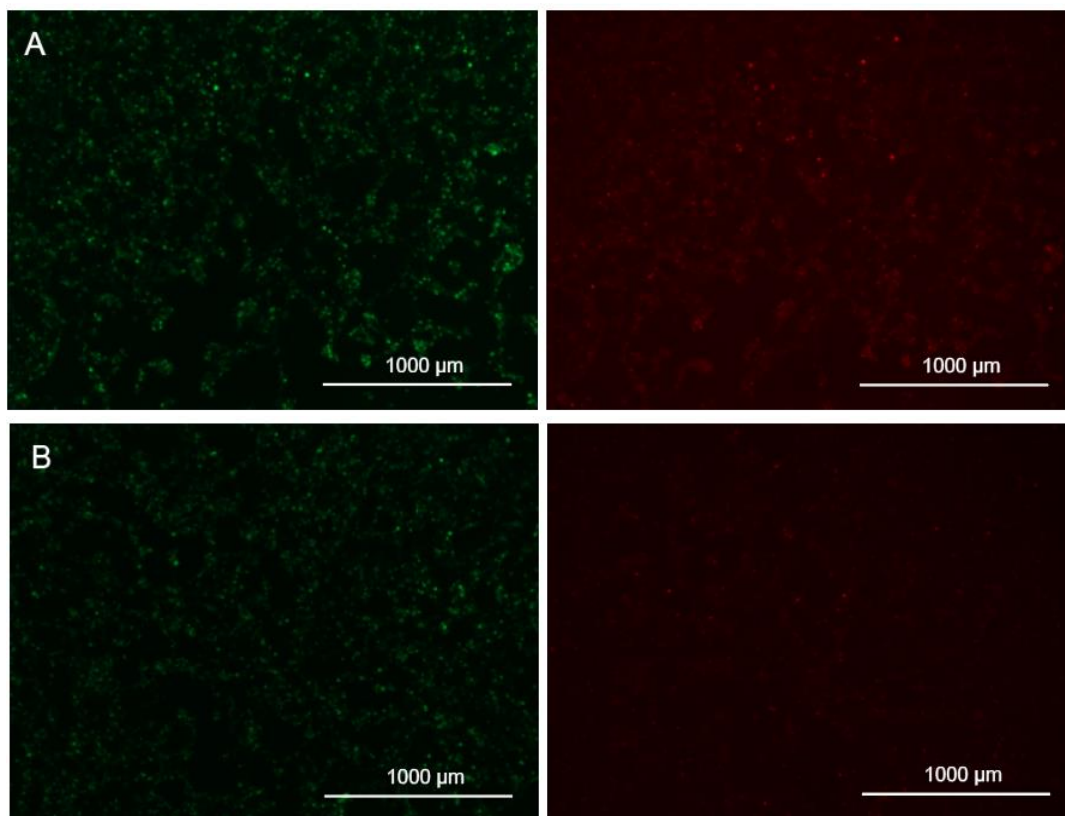


Figure 34 - Encapsulins with fused chemical tags on its surface. Image acquisition performed with EVOS microscope. eGFP signal on the left and CLIP-TMR signal on the right. A) HEK293T cells expressing pcDNA_Zeo_MT3-QtEnc-CLIPtag and pcDNA_Zeo_eGFP-CAAX in a 75:25 ratio. B) HEK293T cells expressing pcDNA_Zeo_eGFP-CAAX.

Nonetheless, fluorescence and TEM data was obtained for C-terminal CLIP-tag and C-terminal SNAP-tag encapsulins, respectively. In Figure 34, EVOS fluorescence data is presented regarding encapsulins with surface-exposed CLIP-tag. HEK293T cells were co-transfected with pcDNA_Zeo_MT3-QtEnc-CLIPtag and pcDNA_Zeo_eGFP-CAAX. In this case, eGFP was used as a control for fluorescence and transfection efficiency. As a substrate for CLIP-tag, CLIP-TMR was chosen, which emits red fluorescence at 580nm. Contrary to what was expected, there is red fluorescence detected in the negative control condition, meaning that there is some non-specific uptake of the dye. Nonetheless, the red signal is relatively stronger in the CLIP

condition, indicating that CLIP-tag is functional and is responsive to the used chemical dye. TEM results are not shown given that the lack of proof for assembly disregarded its preparation for TEM imaging. Regarding SNAP-labeled encapsulins, following its biochemical results, they were prepared for TEM imaging, as shown in Figure 35. These TEM results show the encapsulins assembled presenting the expected round shape. Due to lack of time, no FM data was obtained.

In summary, the results did not match the initial expectations. SNAP-tag did assemble but no fluorescence data is available. On the other hand, CLIP-tag had no proof of assembly, so these samples were not prepared for TEM. In addition, fluorescence results were not as expected having a non-specific dye uptake. More experiments should be performed in order to be able to take more conclusions on the possibility of using encapsulins for post-embedding CLEM, particularly for SNAP-tag which showed positive TEM results.

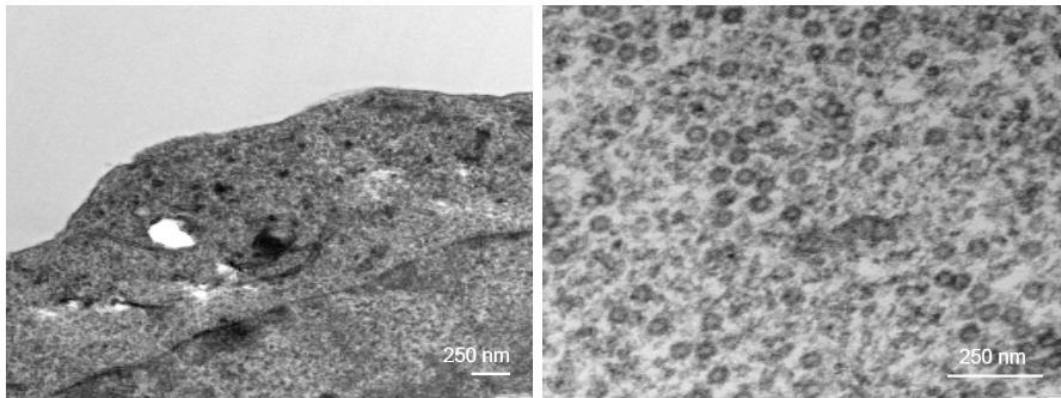


Figure 35 - Encapsulins with fused chemical tags on its surface. TEM micrograph of HEK293T cells expressing pcDNA_Zeo_MT3-QtEnc-SNAPtag. Images are presented in a 250 nm scale bar.

4.5.Expression of encapsulins after RNA transfection.

Lastly, as a final note, encapsulins were expressed in cells via mRNA transfection opposed to all previous experiments - DNA transfection. In this case, plasmids pGEMHE_MT3-QtEnc-eUnaG and pGEMHE_MT3-eUnaG-QtEnc-antimCherry(LAM-4) were translated to RNA and then expressed in HEK293T cells.

As seen in Figure 36, green fluorescence is detected in both setups however transfection presented a low yield overall. Particularly, expression of pGEMHE_MT3-eUnaG-QtEnc-antimCherry(LAM-4) is scarce probably due to mRNA instability. On the other hand, pGEMHE_MT3-QtEnc-eUnaG shows good levels of expression indicating good perspectives on its use in further experiments.

These results shows that it is possible to obtain expression of encapsulins via RNA transfection, despite its low efficiency. Nonetheless, it is promising for further experiments where the transfection is advantageous due to showing its results faster (mRNA does not need to reach the nucleus for expression) and ability to be expressed in cells where transfection of DNA is impossible. On possible application relies on encapsulins being transfected as RNA and DNA, respectively, to be used in experiments where a sequential expression is beneficial, for example to study the intermixing of different classes of encapsulins.

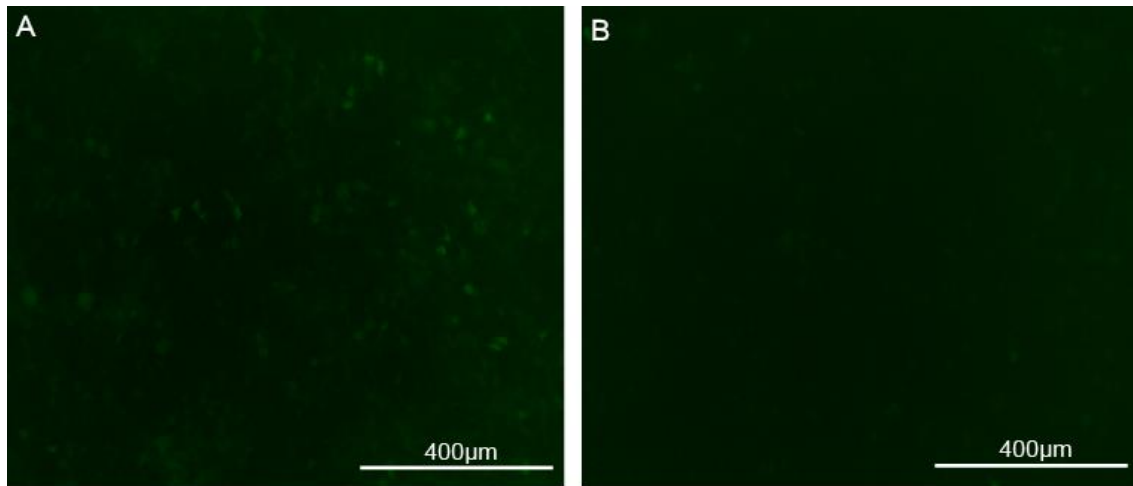


Figure 36 - Expression of Qt encapsulins on HEK293T cells via RNA transfection. A) pGEMHE_MT3-QtEnc-eUnaG. B) pGEMHE_MT3-eUnaG-QtEnc-antimCherry(LAM-4).

Conclusions and Future Work

Labelling tools are crucial for the understanding of all biological processes occurring in a cell. They allow to track a protein and understand its interactions with the surrounding environment, providing information on its functions and role in physiological events. Current reporters, despite greatly developed for FM, for EM are scarce and with very limited applications due to the creation of artifacts during EM sample preparation or their dependency on the addition of exogenous compounds.

The ultimate goal of this dissertation was to develop a genetically encoded reporter visible in both FM and EM without disrupting the normal behavior of a cell to provide the necessary functional and structural data, using encapsulins. These reporters, “EMcapsulins”, were tested for their targeting and sensing capacities, tested to obtain different patterns of contrast, among others.

The first performed experiment consisted of comparing two Qt encapsulins with a different number of MT3 domains, on the inner and/or outer surface of the nanocompartment. In addition, it was tested if eUnaG being exposed on the surface of the encapsulin would affect its assembly or distribution in the cells. The results showed different contrast patterns visible in TEM creating a new class of encapsulins and eUnaG surface exposure did not compromise the normal behavior of encapsulins allowing for FM and EM imaging. Furthermore, the addition of these extra MT3 domains do not interfere with the assembly of encapsulins as they formed the recognized round shape.

Two different methods were tested to prove encapsulins could label a specific cell structure, particularly, the cellular membrane. On one of the methods, encapsulins were engineered to expose an intrabody, against mCherry, on the outer surface while mCherry was expressed on the cellular membrane. Encapsulins successfully bound to mCherry and targeted the membrane. This was clearly observed in CM by co-localization of eUnaG and mCherry fluorescence signals and in TEM by a darker contrast along the membrane. The second method was based on split FPs. While encapsulins expressed strands 1 to 10 of sfCherry2, strand 11 was fused to a FP and a lipid-binding ligand, CAAX. Fluorescence would only be achieved in the case of re-complementation of sfCherry2, meaning that the encapsulins would need to target the membrane. Targeting was successful as, again, there was a clear co-localization of green (eGFP) and red (sfCherry2) fluorescence.

Encapsulins were also tested on their ability to detect changes in the cell environment, particularly, calcium levels. For that, a FRET-based Ca^{2+} sensor was used - Twitch2b. Twitch2b responds to calcium by enhancing its fluorescence and by contracting its structure. Twitch2b was designed to have an encapsulation signal, which when co-expressed with Qt encapsulins would result in its entrapment on the lumen of the encapsulin. Encapsulins successfully entrapped Twitch2b and kept its response to calcium with fluorescence being enhanced, as confirmed in CM. On the other hand, the conformational change of Twitch2b, expected to also cause the contraction of the encapsulin had no confirmation results when observed under TEM with calcium stimulation. Another tested methodology was the detection of Ca^{2+} levels by using

the CaM-RS20 complex mechanism. The RS20 peptide was exposed in the outer surface of Qt encapsulins while CaM was fused to a HpFtn. The addition of Ca^{2+} led to the binding of RS20 and CaM, leading to a conformation change that allowed for HpFtn to bind to other Ftms and form clusters. Unfortunately, TEM data was compromised due to problems during the EM sample preparation. Nonetheless, these clusters were visible in CM.

Encapsulins can also be used as labeling tools in SRM. The same experimental setup used for targeting studies was used for visualization in SRM. The results showed a clear targeting on the membrane with sub-diffraction resolution.

Regarding the use of chemical tags fused to encapsulins, unfortunately the results were not as expected. Due to lack of time and inconclusive results, no post-embedding CLEM data was attained given that only one of the components were tested for each of the tested conditions.

Finally, encapsulins also proved that they can be expressed by directly transfecting RNA instead of DNA as, despite the weak signal, green fluorescence (eUnaG) was detected on EVOS.

In summary, the work developed in this dissertation fulfilled the established goals to a high extent. Encapsulins showed different patterns of contrast on TEM and can efficiently present fluorescence by exposing FPs on its surface. This opens the possibility for multiplexing on both FM and EM. These nanocompartments can be used to label specific cellular structures as the cellular membrane and can detect changes on calcium levels within a cell in a way that it is visible in both FM and EM by enhancing its fluorescence or forming clusters, respectively. Furthermore, encapsulins can be engineered to label structures in SRM and be visible in CLEM (pre-embedding methods). On the downside, there is a clear lack of robustness in the Twitch2b experiments as repetitions of the same conditions did not show the same outcome. Even so, the acquired positive results show great potential and with continuous improvements can lead to a reliable genetically encoded reporter for Ca^{2+} sensing in FM, EM and CLEM. Another limitation is focused on the SNAP and CLIP-tag experiments. The existing results have failed to show the potential use of encapsulins in post-embedding CLEM. There was no acquisition of FM and EM images, in simultaneous, for none of the constructs, which was vital to conclude on possible correlations on this data.

Regarding the future, as mentioned before, the work developed in this dissertation is part of the “EMcapsulins” project, which aims to develop a new genetically encoded reporter for FM, EM and CLEM using encapsulins. Following the goals of this project, future experiments should focus on the use of the proposed reporters in animals experiments *in vivo*.

For such purpose, the performed calcium sensing studies should be further developed for detection on both FM and EM with the use of different GECIs for study of synapses behavior and interactions along with the study of neurons activity with higher fidelity and efficiency. Moreover, studies that can combine the targeting and sensing abilities tested in this dissertation would be extremely beneficial to define the profile of Ca^{2+} levels in specific receptors of neurons which can indicate possible disfunctions in these. In addition, experiments where RNA encoding for encapsulins could be directly injected in neurons could be used for the better understanding of its mechanisms without the need for DNA transfection. Furthermore, the development of stop codon readthrough motifs that can translate the same information as different plasmids to the cells can be helpful to establish stable cell lines avoiding co-transfection methods. Ideally, these reporters will be able to investigate neurological systems by its observation in multiple imaging tools for diagnosis and disease or potential therapies progression.

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Supplementary data

Annex I

Table 2 - Description of all plasmids with the corresponded cloning methods

Plasmid	Cloning Method	Definition
pcDNA_Zeo_MT3-QtEnc-eUnaG	PCR-based mutagenesis	Encapsulin with surface exposed eUnaG.
pcDNA_Zeo_MT3-QtEnc-MT3-eUnaG	PCR-based mutagenesis	Encapsulin with an extra MT3 domain on the C-terminal.
pcDNA_Zeo_MT3-QtEnc-antimCherryIB	PCR-based mutagenesis	Encapsulin exposing a mCherry intrabody on the surface.
pcDNA_Zeo_memSig-mCherry	---	mCherry with a membrane targeting signal.
pcDNA_Zeo_Twitch2b-QtSig	Traditional cloning ligation	Twitch2b with an encapsulation signal.
pcDNA_Zeo_QtEnc-FLAG	---	Encapsulin with a FLAG domain
pcDNA_Zeo_MT3-QtEnc-eUnaG-RS20	PCR-based mutagenesis	Encapsulin with surface-exposed RS20 for CaM binding.
pcDNA_Zeo_MT3-QtEnc-MT3-UnaG-RS20	PCR-based mutagenesis	Encapsulin with surface-exposed RS20 for CaM binding.
pcDNA_Zeo_CaM-HpFtn	Gibson assembly	CaM bound to a HpFtn.
pcDNA_Zeo_MT3-QtEnc-sfCherry2_1-10	Gibson assembly	Split sfCherry2 expressed on the surface of an encapsulin.
sfCherry11_eGFP-CAASX	PCR-based mutagenesis	Split sfCherry2 tagged by eGFP and a lipid-binding ligand.
pcDNA_Zeo_MT3-QtEnc-CLIPtag	PCR-based mutagenesis	Surface-exposed CLIPtag for CLEM.
pcDNA_Zeo_MT3-QtEnc-SNAPtag	PCR-based mutagenesis	Surface-exposed SNAPtag for CLEM.
pGEMHE_MT3-QtEnc-eUnaG	PCR-based mutagenesis	eUnaG exposed on the surface for RNA translation.
pGEMHE_MT3-QtEnc-antimCherry	PCR-based mutagenesis	Encapsulin exposing a mCherry intrabody on the surface for RNA translation.

Annex II

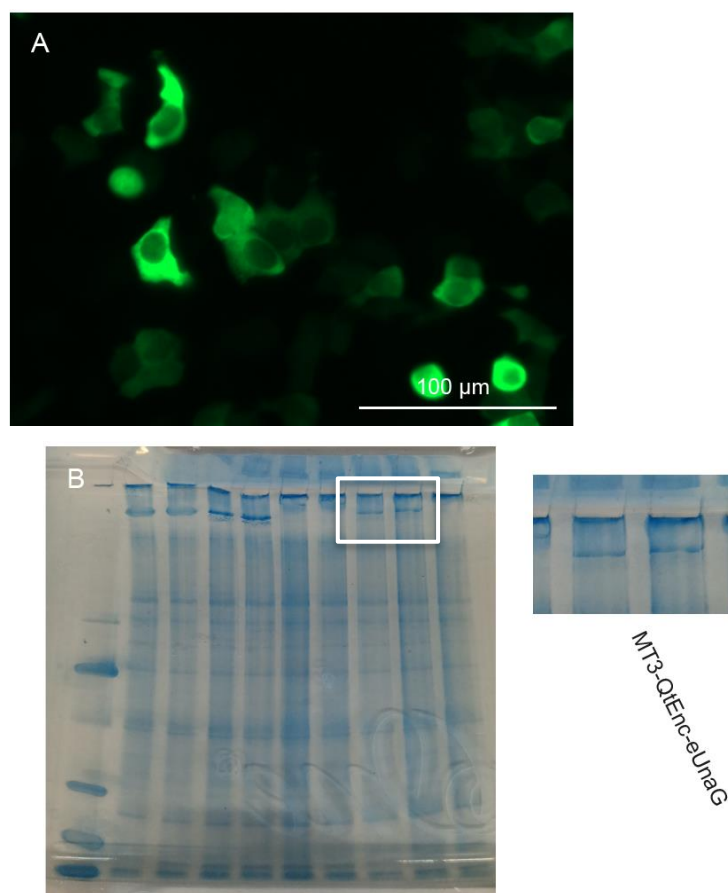


Figure A.1 - Additional results on encapsulins exposing eUnaG on the surface. A) EVOS imaging of HEK293T cells expressing pcDNA_Zeo_MT3-QtEnc-eUnaG. Image is presented in a 100 nm scale bar. B) CN-PAGE gel with Coomassie staining of pcDNA_Zeo_MT3-QtEnc-eUnaG.

Annex III

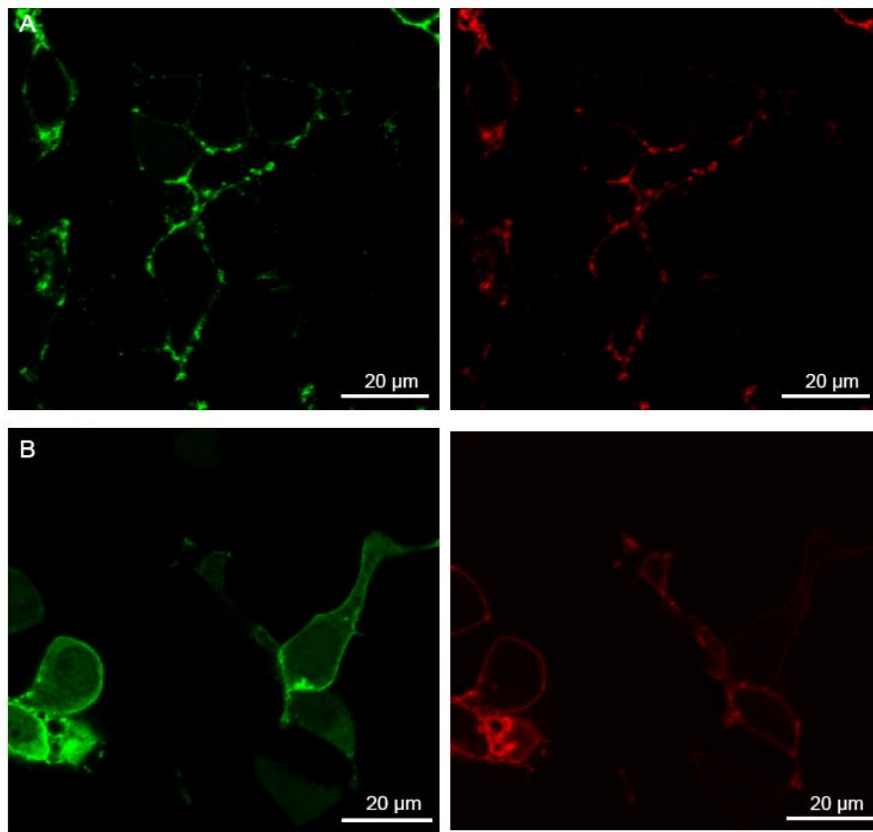


Figure A.2 - Additional results on the targeting of encapsulins to the cellular membrane by expressing an intrabody against mCherry on the surface of Qt encapsulins. eUnaG signal on the left and mCherry signal of the right. Images presented in a 20 μm scale bar. A) CM images of HEK293T cells co-expressing pcDNA_Zeo_MT3-QtEnc-eUnaG and pcDNA_Zeo_MT3-QtEnc-antimCherry(LAM-4) at a 50:50 ratio, with pcDNA_Zeo_memSig-mCherry-FLAG (20% of total DNA). B) CM images of HEK293T cells co-expressing pcDNA_Zeo_MT3-QtEnc-eUnaG and pcDNA_Zeo_MT3-QtEnc-antimCherry(LAM-4) at a 95:5 ratio.

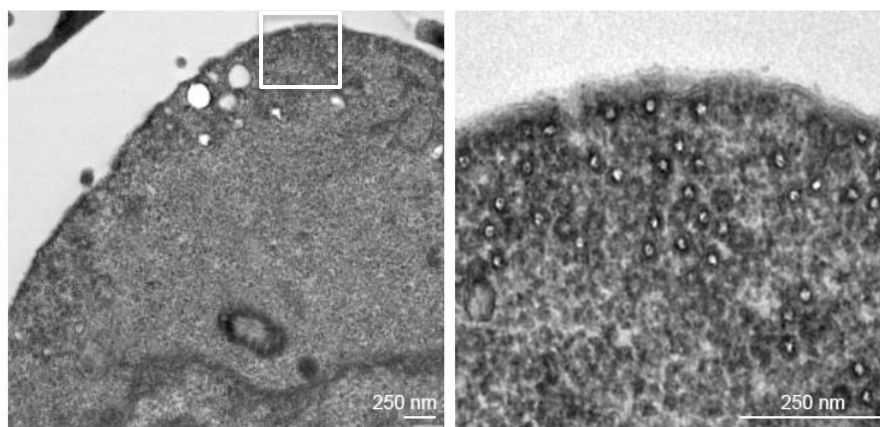


Figure A.3 - Additional results on the targeting of encapsulins to the cellular membrane by expressing an intrabody against mCherry on the surface of Qt encapsulins. On the right is a close-up of the white square. Images presented in a 250 nm scale bar. TEM micrograph of HEK293T cells co-expressing pcDNA_Zeo_MT3-QtEnc-eUnaG and pcDNA_Zeo_MT3-QtEnc-antimCherry(LAM-4) at a 95:5 ratio, with pcDNA_Zeo_memSig-mCherry-FLAG (20% of total DNA).