

## Magnetic manipulation of intracellular signals Elie Balloul

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# Magnetic manipulation of intracellular signals

# Manipulation de signaux intracellulaires à l'aide de nanoparticules magnétiques

## Manuscrit de thèse d'Elie BALLOUL

Soutenance le 13 septembre 2019

Dirigé par Mathieu Coppey and Maxime Dahan

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#### Abstract

Cells are sensing their environment using receptor that transmit the information to the intracellular side where pathways of signaling molecules are processing the information to decide of their behavior. In our project we propose a new way to hijack these internal signals. We propose to use magnetic nanoparticles to target intracellular engineered protein and to move them in the cytoplasm. This magneto-molecular actuation on cells is part of the Magneuron H2020 project which intend on the long run to provide tools to control implanted cells in the context of regenerative medicine targeting the Parkinson disease. In this pathology neurons connecting two regions of the brains are lacking and a proper cure would need a way to implant cell in one of this location the substancia nigra, and to control the neurons to make them grow toward the striatum.

Magnetic manipulation of intracellular proteins offer interesting characteristic on paper. First magnetic fields can go through tissue without limitation and have no interaction with biological structure. Second actuating intracellularly allow the highest specificity and will limit potential adverse effects on other cells. Thus using magnetic nanoparticles (MNPs) we targeted Guanine exchange factors (GEF) proteins which are known to be the activator of the Rho small GTPases. These RhoGTPases are playing many roles, and specially they can control cell migration and cell polarization. The later is very important during the axonal growth of neurons, the first one is easy to visualize in cell cultures. We produced sets of experiments showing that we could generate in single cell experiments protrusions of a few micrometers that are related to the activation of the RhoGTPases. As we also observed different limitation to our technique we studied the environment in which our particles are, and what could be the causes of these limitations. The environment of the particles is the cytoplasm, we explored how different particles interact with this environment and how it affect their diffusion. We also explored spatially if this environment is homogeneous. We showed that small particles (less than 70nm) diffuse can diffuse freely in the cell when they are properly passivated.

Concerning the magnetic manipulation we present some preliminary data that start to explain why the technique is limited to activate intracellular signals.

Keywords: RhoGTPases, diffusion, cytoplasm, magnetic manipulation

**Résumé**: Les cellules captent des informations sur leur environnement en utilisant des récepteurs qui transmettent l'information au milieu intracellulaire. L'information est traitée par des protéines de signalisation qui in fine produiront un comportement cellulaire adapté. Dans ce travail nous proposons une méthode pour pirater ces signaux intracellulaires. Nous proposons d'utiliser des nanoparticules magnétiques pour cibler et déplacer des protéines intracellulaires. Ce contrôle magnetico-moléculaire fait parti du projet H2020 Magneuron qui prévoit le développement d'outils pour contrôler des cellules implanté chez des patients dans le contexte de la médecine régérative pour la maladie de Parkinson. Dans cette maladie des neurones dopaminergiques qui connectent deux régions du cerveau sont manquant, et un soin curatif nécessite d'implanter des neurones dans la substancia nigra et de contrôler la croissance de leur axone en direction du striatum. Les manipulations magnétiques intracellulaires ont plusieurs avantages sur le papier. Tout d'abord les champs magnétiques traversent les tissues biologiques sans difficultés et avec peu d'intéractions. Deuxèmement en agissant de manière intracellulaire il est possible d'agir de manière spécifique sur certaines cellules et de limiter les effets indésirables sur d'autres tissus. En utilisant des nanoparticules magnétiques nous avons ciblé les facteurs d'échange de guanines (GEF) qui sont les activateurs des petites Rho GTPases. Ces rhoGTPases ont différents rôles, entre autres le contrôle de la migration cellulaire et de la polarisation. Cette dernière est importante pour la formation des axones, tandis que la première est facilement observable dans les cultures cellulaires. Nous avons donc produit des expérimentation sur des cellules unique en culture, et nous avons pu contrôler la formation de protrusion de plusieurs micromètres en réaction à nos manipulation. Nous avons toutefois observé un certain nombres de limitations à notre technique et nous avons donc cherché à en comprendre les causes.

Premièrement nous avons exploré l'environnement des particules, le cytoplasme. En utilisant différent type de particules nous avons dans la continuité de précédent travaux montré que les interactions non spécifiques jouent un rôle prépondérant dans le mode de diffusion de ces particules. Nous avons aussi montré que s'agissant de particules ayant peu d'intéractions, la diffusion de ces particules est homogène dans tout les cytoplasme. Concernant les manipulations de nanoparticules magnétiques nous présentons un ensemble de données préliminaires pour tenter d'expliquer les limitations de notre technique. **Mots clefs**: RhoGTPases, diffusion, cytoplasme, manipulation magnétiques

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# Acronyms

 $\alpha GFPnb$ : Anti GFP nanobody

CB : Carboxybetain

DHPH : Dbl-homology Pleckstrin-homology protein domains

DLVO : Made by Boris Derjaguin, Lev Landau, Evert Verwey and Theodoor Overbeek.

It is about the stabilisation of particles in solution.

ECFP : Enhanced Cyan Fluorescent Protein

EGFP : Enhanced Green Fluorescent Protein

ER : Endoplasmic reticulum

FCS : fluorescence correlation spectroscopy

FKBP : FK506 binding protein

FPLC : Fast protein liquid chromatography

FRAP : Fluorescent recovery after photobleaching

FRB : FKBP12-Rapamycin Binding domain

fMNPs : Ferritin based magnetic nanoparticles

GAP : GTPases activating protein

GEF : Guanine exchange factor

GFP : Green Fluorescent Protein

iLID : improved Light induced dimer, subdomain of the LOV2 protein

iPSCs : Induced pluripotent stem cells

QD : Quantum Dots

MNPs : Magnetic nanoparticles

MSD : Mean squared displacement

OD600 : optical density at 600nm, used to monitor bacterias growth. At OD600=1 cell are about 8.10 cells per ml

PC : Phosphocolin

- PEG : Polyethylene glycol
- pMOXA : poly(methyloxazoline)
- SiMNP : Silica Core Shell Magnetic nanoparticles
- ${\rm SB}\,$  : Sulfobetain
- SPT : single particle tracking
- SspB : Stringent starvation protein B, partner protein of iLID in optogenetics
- ROI : Region of interest
- TRPV : transient receptor potential vanilloid

# Publications made during this work

Debayle, M., **Balloul, E.**, Dembele, F., Xu, X., Hanafi, M., Ribot, F., Monzel, C., Coppey, M., Fragola, A., Dahan, M., Pons, T., Lequeux, N., 2019. Zwitterionic polymer ligands: An ideal surface coating to totally suppress protein-nanoparticle corona formation? Biomaterials 219, 119357. https://doi.org/10.1016/j.biomaterials.2019. 119357

Etoc, F., Balloul, E., Vicario, C., Normanno, D., Liße, D., Sittner, A., Piehler, J., Dahan, M., Coppey, M., 2018. Non-specific interactions govern cytosolic diffusion of nanosized objects in mammalian cells. Nature Materials 17, 740–746. https://doi.org/10.1038/s41563-018-0120-7

Toraille, L., Aïzel, K., **Balloul, E.**, Vicario, C., Monzel, C., Coppey, M., Secret, E., Siaugue, J.-M., Sampaio, J., Rohart, S., Vernier, N., Bonnemay, L., Debuisschert, T., Rondin, L., Roch, J.-F., Dahan, M., 2018. Optical Magnetometry of Single Biocompatible Micromagnets for Quantitative Magnetogenetic and Magnetomechanical Assays. Nano Letters. https://doi.org/10.1021/acs.nanolett.8b03222

# Chapter 1

# Introduction

### 1.1 Generalities

Cells have to perceive cues of various nature (physical, chemical) surrounding them to interact with their environment. In the context of an organism it means sensing chemotactic cues, differentiation cues, survival cues, having inter-cellular communication, or detecting foreign elements. Many events occur during the embryonic development with cell shuffling around, differentiating and organizing themselves into various tissues. For all these events, the involved cells need to integrate and process many sources of information. Once developed, an organism still have many cells traveling around.

These cells continue to communicate with their neighbors and further away located cells to maintain the homeostatis of the body. Understanding the intracellular mechanisms would allow to get the full picture of the organism, from the tissue scale down to the molecular mechanisms. Mechanisms that rely on biochemical reaction that transmit and process the signal across cascade of reaction are called signaling pathways. That processed information give rise to cellular behaviors. But these intracellular transmission of signals are not straightforward but are rather composed of many paths that cross regulate themselves. Knowing how a multicellular system organizes and maintains itself would obviously help the development of new strategies for regenerative medicine.

#### 1.1.1 Magneuron project

My PhD project has grown in the context of the Magneuron OPEN-FET H2020 program. Its purpose is to develop new strategies to be able to restore lost brain connection in the Parkinson Disease. If the etiology of this disease seems to be multifactorial (Poewe et al. 2017), the disease is always related to the accumulation of  $\alpha$ -synucleine in the brain. The non mutated form of this protein is normally soluble but in the Parkinson disease a conformational change will make it agregate and accumulate. These accumulation named "Lewy bodies" leads to cell death of neurons at diverse location in the brain depending of the stage of development of the disease (Braak et al. 2003). One of these locations, the *substancia nigra* located in the *basal ganglia* is affected by the loss of dopaminergic neurons. These specific neurons have their cell body in the *substancia nigra* and their



Figure 1.1: The nigrostriatal pathway link the *substancia nigra* to the striatum. The neurons responsible for this connection are lost during the Parkinson disease. From Wikimedia Commons (Patrick J. Lynch et al. 2015)

axonal projection reaches the *striatum*. When the cell death reaches 50% to 60% (Gibb 1991) the motor symptoms specific of the disease are developing.

The gold standard treatment for the disease is the use of L-DOPA (Poewe et al. 2017) but it is not a perfect treatment as it can trigger drug induced diskynesia (movement disorders) but also because the drug loses effect in advanced stages of the disease. For this reason, as for many degenerative disease the best treatment would be the restoration of the damaged tissues in its original state.

This strategy is becoming more and more plausible with the recent advancement in induced stem cells (iPSC) (Takahashi et al. 2006; Shi et al. 2017) being developed as well as progress in controlled cell differentiation to specific neuron types. Still rebuilding a connection between two separate areas of the brain would require a way of controlling cells after implantation in the patient to allow the cell to direct its axonal growth.

The Magneuron project proposes to develop magnetic tool to keep control of implanted cells in the brain of patient to guide the growth of their axons. The project is divided in



Figure 1.2: Magneuron project : the strategy

Magneuron schematics showing the different steps of the therapeutic strategy to be developed. After biopsy cell would be undifferentiated to iPSCs, multiplied and differentiated to dopaminergic precursor neurons. After loading with magnetic nanoparticles the cells would be transplanted in the patient's brain where they would be guided with an external magnetic field using spaceMode (deplacement of particles) for axonal growth and polarization. tempMode (Force activation of membrane receptors) is developed by partners to induce the differentiation of precursors into dopaminergic neurons. (Schematic based upon the Magneuron project, drawing of the patient is from Sir William Richard Gowers 1886, User Beao from Wikimedia)

six work package for scientific development from WP2 to WP7. WP2 for the engineering of magnetic nanoparticles (MNPs) headed by the PHOENIX laboratory, WP3 for the biofunctionalization and internalization of the MNPs headed by the Osnabruck University. Our WP is the forth, it consists in the development of the manipulation tools and techniques for the spacemode manipulation (Intracellular manipulation of signalling activities; Figure 1.3). The WP5 and WP7 are directed by the Keele University and intend to use the tempmode magnetic actuation (Stimulation of receptor with a defined frequency; Figure 1.3) for cell differentiation and apply manipulation of neuronal cell in tissue culture respectively. Finally WP6 is applying the spacemode for triggering fiber outgrowth and is headed by the Bochum university.

The final assembly of the different works should result in the following strategy. First



Spacemode

Figure 1.3: Magneuron project : Tempmode and spacemode

spacemode on the left: is used to control axonal growth by controlling intracellular mechannisms with magnetic nanoparticles; tempmode on the right: tempmode is used to stimulate membrane receptor to differentiate membrane neurons. (From the Magneuron project)

some cells are recovered from a biopsy of the patient. Second, the cells are reprogrammed in iPSCs cells and further differentiated to neuronal precursor and then to dopaminergic neurons using the tempmode. The tempmode rely on mechanical activation of membrane receptors by magnetic particles. Then, these cells are loaded with MNPs and are implanted in the substancia nigra of the brain. From there using the MNPs to control intracellular signals (spacemode), we trigger the axonal outgrowth and guide the axon elongation toward the striatum allowing the reconstruction of the link between these two regions of the brain. Our charge was the development of a magnetic toolbox to control intracellular signals that permit a directed growth of the axons of the newly implanted cells. Axonal growth is mainly occurring during the organism development and is guided by several cues. Studies over classical model organisms have highlighted the role of the extra cellular matrix (ECM)(Barros et al. 2011) as well as chemo-attractants(Stoeckli 2018). Reports are showing diversity of cues effect depending of the cellular type, showing either chemo-attractant effect, or trophic effect for example. Moreover graft of precursors in adults don't allow the recovery of interconnection between separated brain areas which suggest that many cues are not available anymore in the adult brain. One need to provide the information to the cell to allow the axonal growth. To do so without affecting surrounding cells it seems really interesting to be able to control the process from the inside of the cell. In the cell, axonal growth and polarization is under the control of members of the Ras superfamily of small GTPases (Hall et al. 2010). This superfamily is subdivided in many subfamilies: the Ras, Rho, Ran, Rab, Arf and Sar and G $\alpha$  (Colicelli 2004). Of which mainly the Ras and Rho families are involved in axonal growth.

From the actual knowledge the Ras family play a role at the initiation and the growth of the axon, and the Rho family is in charge of the polarization and the specification of the axon (Hall et al. 2010).

We decided to focus our attention on the Rho family because it is involved in axonal formation and shares with the Ras family its mechanisms of activity. In addition, the Rho family is involved in the process of polarization which is important for other cellular processes as cell division and cell migration (Jaffe et al. 2005). The later process is happening in many cell type for normal(ie:white blood cells) and anomalous(invading cancer cells) behaviors. Moreover can be observed easily at the single cell level in a petri dish (Figure 1.4). Therefore, it is a good candidate to establish our technique and study intracellular signal processing.

The defined strategy that will be discussed further in this chapter consist in manipulating spatially and temporally signaling proteins. To do so we engineered fusion proteins that can combine with internalized magnetic nanoparticles (MNPs). These complexes protein-MNPs can then be manipulated using magnetic fields.

Previous work has shown the possibility of controlling Rho small GTPases activity using



Figure 1.4: Schematic of a migrating cell

A migrating cell need to remodelate its structure to move. The cortical network of actin play an important role but the organization and the localization of organelles are also correlated to cell polarization. (From Mathieu Coppey Unpublished)

engineered proteins based of the subdomain of their activators (Inoue et al. 2005). Across the coming chapter we will first discuss the detail of the Rho GTPases signaling pathways and present the previous strategies that have been used to study and manipulate them. Finally we will detail the project and introduce the framework of our approach with the different challenges to we had to face.

#### 1.1.2 RhoGTPases during cell migration

#### 1.1.2.1 The Rho family

The Rho small GTPases family (RhoGTPases) (Table 1.1) is constitued by 21 members (Boureux et al. 2007) in humans of which importance for cell migration may vary depending on the cell type (Ridley 2015). But we can highlight the major importance of three proteins of this family, RhoA, Rac1 and Cdc42. They are described as being keyplayers for the actin remodeling at the front edge of a cell protrusion but also to play a role for the polarization of the cell that will allow directionnality in the cell migration. They are also among the most well studied protein of the family as they are the better conserved between species.

They all promote actin remodeling but with different modalities. Rac1 and Cdc42 are actively promoting actin branching via ARP2/3 complex whereas RhoA favor actin nucleation and elongation via mDia but also acto-myosin contraction. The general spatial

Q 1 (		Subfamily	RhoGTPases
Subfamily	RhoGTPases	Bho	BhoA
Cdc42	Cdc42	10110	BhoB
	RhoJ		
	BhoO		RhoC
Dee	De e1	RhoBTB	RhoBTB1
Rac	Raci		RhoBTB2
	Rac1bb	BhoDF	BhoD
	Rac2		DhoE
	Rac3		Rnor
	BhoG	RhoH	RhoH
		Rnd	Rnd1
RhoUV	RhoU		Bnd2
	RhoV		Dnd2
			ппаэ

#### Table 1.1: Rho subfamilies

This table recapitulates all the 21 members of the RhoGTPases family, and their division in subfamilies. Based on Boureux et al. 2007

description of each GTPase is to associate Rac1 and Cdc42 with the front of the migrat-

ing cell whereas RhoA is linked to rear of the cell (Figure 1.5).



#### Figure 1.5: Rho GTPases localization during migration

General localisation of the RhoA at the rear and Cdc42, Rac1 at the front. Even though reality is more subtle this give a general picture of the asymptric distribution of Rho GTPases. (From Mathieu Coppey Unpublished)

But reality is more complex, RhoA is also present in the protrusion but at a different location than Rac1 and Cdc42. Whereas RhoA is located at the tip of the protrusion, Cdc42 and Rac1 are located just a few  $\mu$ m behind (Machacek et al. 2009). Their regulation is complex as they can be activated by different activators and themselves actuates on different targets. Rac1 and Cdc42 are also crossregulating eachother (Burridge et al. 2004; Beco et al. 2018; Nobes et al. 1999).

The regulation of the activity of the small GTPases is similar inside the family. It rely on a conformational change to an active form upon binding to GTP and the conversion of GTP to GDP to go back to its inactive form. Both events are catalysed by external protein as they are relatively slow otherwise (Boureux et al. 2007). Guanine exchange factors (GEF) catalyse the exchange of the GDP by GTP and are thus considered as activator of the Rho small GTPases. Guanine activating protein (GAP) is the inhibitor and favor the hydrolysis of the GTP to GDP to go back to the inactive form (Figure 1.6).



#### Figure 1.6: Rho small GTPases regulation

Regulation of the activity of the Rho family small GTPases. GEFs are catalyzing the exchange of GDP to GTP whereas GAPs catalyse the hydrolisis of the GTP to GDP. Under its GTP bound form the GTPase can bind effector proteins.

Many GEFs and GAPs have been described for each RhoGTPase some beeing more or less specific. For example, Rac1 can be activated by Tiam1,  $\beta$ -PIX, and Dock180 (Ridley 2015). But  $\beta$ -PIX has also been shown to be a GEF for Cdc42 and only Tiam1 is rather specific of Rac1. On its side Intersectin1 is considered to be more specific of Cdc42.

Once activated, RhoGTPases are able to form complexes of proteins that among the different effect can recruit the ARP2/3 complex which allows actin branching. This allows the nucleation of new actin filament that can push the membrane forward. Cdc42 acts through the WASP complex and Rac1 interacts with the WAVE complex. RhoA on its side is more responsible of retraction linked to recruitment of the ROCK protein which activates Myosin motors, but also play a role in actin nucleation.

Our work has focused on Cdc42 and Rac1 activity using GEFs mostly specific for each of

them. But we also tested more upstream activity regulators like PI3K.

#### 1.1.2.2 Intersectin: A GEF for Cdc42

Intersectin1 (ITSN1) (O'Bryan 2010) is a multi target protein. It regulates Cdc42 through it's Dbs homology (DH) domain(Hussain et al. 2001). The other domains have



#### Figure 1.7: Itsn1 effectors

Itsn1 interactions with other proteins. If we consider only actin cytoskeleton remodeling activity, it has been shown to interact with Cdc42 through the DH domain, N-WASP and SOS through the SH3 domain and WIP through the EH domain (From interactions described in Herrero-Garcia et al. 2017)



#### Figure 1.8: Itsn1 protein

Itsn1 domains scematic. The SH3 domains play a role in the autoinhibition of the GEF. The PH domain is necessary for the membrane localization. (Inspired by Herrero-Garcia et al. 2017)

been shown to allow interaction with other proteins but also to regulate the activity of the DH domain. The absence of the Pleckstrin homology (PH) domain is reducing the catalyzing activity because of its potential action on the conformation of the DH domain but also because the PH domain is expected to favor the membrane localization of the GEF by interacting with the lipids of the membrane (Whitehead et al. 1999; Hussain et al. 2001). Moreover, intersectin could inhibit itself through its SH3 (Zamanian et al. 2003) domain by interacting with the DH domain. This was proved by the fact that morphological changes appearing through the over expression of DH domain of ITSN1



#### Figure 1.9: Tiam1 protein

Tiam1 exhibits a DH-PH domain for the regulation of Rac1 but also different regulatory domains as PHn-CC-Ex domain wich seems important both for regulation and localization.

that can be reversed by the expression of the SH3 domain (Kintscher et al. 2010). To lift this autoinhibition, it is suggested the necessary participation of other proteins of which Numb could be a candidate (Nishimura et al. 2006).

#### 1.1.2.3 Tiam1: A GEF for Rac1

Tiam1 is as ITSN1 a GEF but specific of Rac1. It's a multidomain protein that is describe to be mostly specific to Rac1 but that can also regulate other proteins through other interactions. It carries two PH domains one in C-terminal downstrean of DH domain which serves the same purpose as the one of ITSN1 and a PH domain in N-terminal which seems to auto inhibit Tiam1 (Mertens et al. 2003). This inhibition could be lifted by interaction with different proteins like CD44, Par3 or ephrin. (Xu et al. 2017)

But the PHn domain together with the CC-Ex subpart has also a positive role for the localization of Tiam1 (Stam et al. 1997). It presents a high affinity for phophorylated inositides which favor the localization at the membrane to activate Rac1.

#### 1.1.2.4 GEF regulation

There exists many more GEFs. They are divided in two families, the DH-PH family and the DOCK family. Their regulation is based on many post translational modification as phosphorylation, acetylation, ubiquitynilation. The member of the DH-PH subfamily are also regulated by phosphoinositisides lipids (PIP) as PIP serve the GEFs to relocate at the membrane.(Mertens et al. 2003)(Hodge et al. 2016)

This last regulation is linked to a higher affinity of PH domains for more or less phosphorylated PIP. A process which is regulated by PI3K. Making this last one an upstream regulator of the GEFs.

#### 1.1.2.5 PI3K: Phosphoinositol regulation of GEF activity

As said earlier the PH domain of GEFs has an affinity for different kind of lipids. A subset of GEFs has a higher affinity for Phospho inositol(PI) 3,4,5 Phosphate (PIP3) and/or PI 4,5 Phosphate (PIP2). The phosphorylation of PI are regulated among other enzyme by the Phopho inositol 3 kinase (PI3K) of class 1 (Fruman et al. 2017). It acts through its Ish2 domain. The effect will vary among the different GEF, for some they seem to be just an anchor point like ITSN1, for others they increase the GEF activity like for Tiam1 and P-rex (Campa et al. 2015).

#### 1.1.3 Intracellular pathways

From this descriptive point of view about molecular signals, we highlight that there is many different step of signal transmission which are modulated by many regulatory step. For decades attempts have been made for understanding the mechanisms of regulation of this cascade of proteins. The work is still ongoing and an effort is now done to understand how the information is processed through these pathways to output the correct choice for the cell.

These efforts to get a better understanding of the intracellular pathways were done using various perturbation techniques which are discussed in the following section.

### **1.2** Perturbation of intracellular pathways

The exploration of the intracellular mechanisms relies on many techniques. They all offer benefits and have allowed to explore different aspect of biological systems. Some have the capability of probing a cell population whereas other are suitable of single cell experiments. Here we review different approches that brought valuable information on intracellular pathways and we will conclude presenting our approach for this project.

#### 1.2.1 Manipulation of cell functions: Multicellular approach;

#### **1.2.1.1** Functional genomics

Around the 80's, the discovery of restriction enzymes and the development of Poly Chain reaction (PCR) mark the start of gene editing. Deletion, point mutation, gene fusion permitted gain or loss of function in protein and opened the path to study intracellular mechanism at the protein level. Not only, these techniques allowed to highlight the role of a protein in a specific function but also the role of its subdomain in executing and regulating its functions. The continuous improvement of gene editing tools but also of high throughput techniques in gene sequencing produced a large part of the description of protein family through the constitution of large proteomic databases that allow us to relate proteins in between them and to have an overview of their main functions.

Concerning RhoGTPases, the role of gene editing tools were valuable. It allowed to link together the different small Rho GTPases in families sharing functions (Figure 1.1) through gene conservation studies. It also pointed out the similarities between their activators, the GEFs, and their inhibitors, the GAPs.

Through mutations of the GEF, the conserved role of their domains have been shown. For example, the common role of the DH-PH for the interaction with the GTPases. It also highlighted the role of specific amino acids for the specificity of interaction both for their effector targets as well as for their lipids affinity through their PH domain. It also revealed the importance of the other subdomains of the proteins for their regulation or alternative mode of action (Mertens et al. 2003; Stam et al. 1997)

Finally, mutations have allowed to understand the role of this proteins through constitutively active (Q61L) form or inactive forms (T17N) for Rac1 (Revach et al. 2016) and Cdc42. The study of these mutants also helped to understand anomalous behavior of cancer cells that often upregulate these protein activity.

The development of gene editing opened the door to bioengineering of many sort of protein that allow scientists to get control on intracellular protein activity. These different techniques to control intracellular proteins are discussed below. We will start discussing chemically induce dimerizer as they were the proof of concept that showed intracellular signal manipulations, then optogenetic and finally magnetically controlled signals using engineered proteins.

#### 1.2.1.2 Drug induced dimerization

Different kind of drugs can be used to control cellular activity. One kind of drugs can interact with proteins to inhibit or enhance their activity, these are not discussed here. The kind we discuss is made of couples of chemical/proteins that have been used to create artificial functions in cells. Functions which are triggered by exposition to the corresponding chemical.

The main advantage of engineered systems is that in theory they have a low risk of interaction with preexisting signaling mechanisms of the cell. Such system appeared in the early 90s with the development of doxycycline dependent gene expression. First, with the tTa protein that promote gene expression in absence of doxycycline. Then with rTTA which induces gene expression in presence of the doxycycline molecule. Both were based on the fusion of the TetR protein from *Erischerischia Coli* with the activating domain V16 of the *herpes simplex* virus(Gossen et al. 1995; M. Gossen et al. 1992). It offered the ability to control gene expression in a reliable manner to study the role of genes in specific mechanisms. The downside of this system is that it needs a delay before the desired gene is expressed. It also requires some time before the protein gets degraded. Together these issues limit the possibility to study the effect of fast on-off switch of a protein activity.



Figure 1.10: Chemical inducement of dimerization.

At about the same time, the discovery of the FRB fragment that bind the FKBPrapamycine couple offered a tool to control the dimerization of proteins (J. Chen et al. 1995). In practice, two proteins can be engineered one carrying the FRB fragment and the other fused to FKBP.

These two proteins can then dimerize upon addition of rapamycine with a high affinity (Kd in the nM range). This solved the problem of having a fast activation but it result that the activation if not easily reversible. Still using this strategy Inoue et al. 2005 showed that it was possible to use the DH-PH domain of a GEF to manipulate Rho GTPase activity. Tiam1 DH-PH domains were fused to FKBP and a second protein was a fusion of FRB and Lyn11. The later is a peptide targeting the plasma membrane. On addition of Rapamycine analogues they could increase the formation of lamellipodia in the cell expressing the previous constructs. The coexpression of a reporter PAK1-YFP confirmed that this effect was mediated through an increase of activity of Rac1.

#### **1.2.2** Single cell manipulations

Even if the aforementioned techniques were efficient to explore intracellular mechanisms they suffer from from the possibility to generate fast (in minute time scale) on-off stimulation of intracellular signaling proteins. Genetic modification produce a permanent effect once it is applied, thus no live interaction can be produced. Chemically induced mechanisms allow to turn on an engineered mechanisms but the reversal of these events can take as long as the time necessary to degrade the affected proteins.

To study the dynamics of intracellular event, it is required to have access to a tool that can be modulated spatially and temporally in minute timescales. Here, we start to present first the optogenetic tools that represent the gold standard for intracellular protein manipulations. On the following, we will then move to the strategies implying magnetic manipulation and explain the chosen strategy for this work.

#### 1.2.2.1 Light based signaling manipulation

There is a wide range of tools based on light that allow to experiment on single cell either to test their physical parameters, like optical tweezers or to modulate protein activity. We focus here on the manipulation of proteins using photosensitive proteins. This category of tools are grouped in a family named optogenetics. All of them are based on light sensitive proteins discovered in different species that have been successfully used to manipulate intracellular mechanisms using light. Two groups can be defined. The first is based on opsin proteins which are sensitive to light. The channel rhodopsin protein family identified in some bacterias and plants is a ionic channel that allows the transfer of cations or anions (depending on the channel type) through the membrane (Nagel et al. 2003). This is a useful tool to study all phenomenon based on electric polarization of the plasma membrane of the cell. Other opsin proteins regulating G protein (also know as guanine binding proteins) activity, have also been identified in many species. Endogenously these last are responsible for light detection in mammalian (Karunarathne et al. 2015).

The second group allows the manipulation of protein thanks to couple of dimerizing proteins that have been identified over the last decades. Starting with PIF3/PhyB (Ni et al. 1999; Shimizu-Sato et al. 2002) discovered in *Arabidopsis* is photoactivated with red light and disactivated with far-red light. As precise control of the dimerization requires the use of two wavelengths making it complicated to use. Moreover, PhyB also requires a chromophore which is not available endogenously in mammalian cells. Blue light sensitive proteins are represented by the CRY2/CICBN couple (Kennedy et al. 2010) and the iLID/SspB couple (Guntas et al. 2015). All these proteins offer a wide panel of tools with different properties. For example just variants of iLID/SspB offer different affinity, from nM to  $\mu$ M range in the dark to hundred of nM and  $\mu$ M range respectively in the light (Zimmerman et al. 2016).

Also, for these two couples of proteins, the dissociation of the dimers once put back in the dark is in the range of minutes (Spiltoir et al. 2019). Their binding half life range from hundred of seconds for iLID/SspB to 5 minutes for CRY2/CIBN.

Optogenetic is of interest to this work because they were used to control the activity of Rho GTPases in the continuation of the previously cited work using FRB/FKBP of Inoue et al. 2005 and more broadly to control proteins related to migration signaling. Using the PIF3/PhyB system, Levskaya et al. 2009 showed the possibility to use optogenetics to induce Rac1 activity at the membrane using the DH-PH domain of Tiam1 recruited at the membrane. The monitoring of Rac1 activity was done using the PAK1-GBD fused to a fluorescent protein. This construct could report the presence of GTP



#### Figure 1.11: Optogenetic schematic

General schematic of recruitment of dimerizing strategy using blue light to control the subcellular localization of proteins.

bound Rac1 (activated form). A similar experiment was done with Itsn1 DH-PH domain but with a reporter based on N-WASP who showed the activation of Cdc42.

Later with CRY2/CIBN, Idevall-Hagren et al. 2012 proved the possibility to regulate PIP phosphorylation at the membrane. The regulation of the phospholipids being implicated in the regulation of the localization of the GEFs.

Finally optogenetics have been used extensively in our lab to study the Rho GTPases activity. First Valon et al. 2015 used the CIBN/CRY2 system in order to characterize the system for manipulating GEFs DH-PH domains. And then to study the activity of the Rho GTPases. Remorino et al. 2017 showed the increase of formation of nanocluster of Rac1 where it is activated. Finally Beco et al. 2018 studied the relation between Cdc42 and Rac1. Showing that a feedback exist in both direction between them but also that each protein support a different information for the cell. The Rac1 activity would be mostly responsible for the orientation of the migration and thus favouring the polarization. Meanwhile Cdc42 would control the speed of the migration.

In conclusion optogenetics has been shown to be reliable tool for manipulating GEFs to control Rho GTPases activity. The main limitation of optogenetic remains its complexity when one wants to operate in a tissue due to the low penetration of blue light in deep tissue because of light scattering and absorption by the tissue itself. Second even if blue light sensitive proteins have a peak of absorption at 450nm they are sensitive to most wavelengths less that 520nm which requires to avoid the use of any fluorescent probe with excitation/emission wavelength less than 520nm. It is then restricting the number of fluorophores available to monitor cell activity during an experiment.

For these reasons the development of a magnetic tool could be of interest both for application in vivo but also for in vitro experiments. In the present work, optogenetics will serve for comparison and will help to understand how our tool can be more effective.

#### 1.2.2.2 Magnetic control

Finally, magnetic manipulation is going to be discussed. It is the chosen strategy for this work. But magnetic manipulation have been developed thoroughly during the last decades using many different strategies. For the sake of the completeness of this journey about manipulation tools we will first introduce the different strategies that have been used and conclude with ours.

As for light based tools the first developed tools were used to explore the physical properties of biological elements. Magnetic tweezer are using big particles mostly in the micrometer range to generate forces from pico (Gosse et al. 2002) to nano (Kollmannsberger et al. 2007) newtons. In Strick et al. 1996 they were used to test the elasticity of supercoiled DNA for example.

But coming back to signal manipulation magnetic manipulation was applied in different ways for many application. As reviewed by Monzel et al. 2017, we can define three forms of actuation of signals, the magneto-mechanical actuation, the magneto-thermal actuation, and finally the magneto-molecular actuation.

Magneto-mechanical (Figure 1.12) actuation is made by the binding of a magnetic particles to a membrane receptor that will be activated by stretching. The stretching is done by a pulling or torque force exerted by the particles upon application of a magnetic field. An application of this strategy has been applied to study the stretching sensitivity of the Notch receptor. Gordon et al. 2015 showed that a switch occurs between 3.5 and 5.4pN to activate the Notch receptor. The resulting stretching is assumed to open access to a cleavable sequence to a metaloprotease allowing a peptide to be released in the cell to transduce signaling.

Magneto-thermal actuation (Figure 1.12) rely on particles heated by radiofrequency. The local increase of heat is then activating heat sensitive receptors that activate intracellular signals.



Figure 1.12: **Magneto-mechanical** actuation is done via pulling on single (like Notch) or multidomain receptors (like TRPV4). **Magneto-dimerization** is a subclass of magneto-molecular manipulation. The dimerization is done by particle particle interactions.(EGFR) **Magneto-thermal actuation** Thermal actuation on heat sensitive receptor TRPV1, radiofrequencies are used to heat particles who open the ion channel.

Magneto-thermal activation are mainly mediated through TRPV receptor family. The family of TRPV receptor are made of calcium channel that is sensitive to temperature or stretching. TRPV1 is activated above  $42^{\circ}C$  (Monzel et al. 2017) while other receptors like TRPV3 or TRPV4 are respectively sensitive to temperature  $\geq 38^{\circ}C$  and  $35^{\circ}C$ which make them difficult to use for mammalian cell cultured at  $37^{\circ}C$ .

With different strategy people have been able to locally increase the local temperature either through direct binding to the targeted receptor, either through generic binding to the plasma membrane of the cell (Figure 1.12). Heat is generated using radio wave at high frequency for nanoparticles (from hundred of kHz to MHz). Particles when excited with radiofrequencies can generate heat through Neel relaxation or Brownian relaxation. The preferred dissipation mode and the adapted frequency depend on the parameter of the particle. Three characteristic are important core composition, anisotropy, and size of the magnetic core. Small particles usually require higher frequencies and depend more on the Neel relaxation whereas bigger particles may heat at more lower frequency and the heat dissipation mechanism depends more on the Brownian relaxation. This strategy, heating a TRPV channel using a magnetic particle is very interesting as it can control the depolarization of a cell through an increase influx of calcium. It is useful to control neuronal activity in tissue for example.

In Huang et al. 2010, they proved the ability to locally heat the membrane targeted by magnetic particles. They measured a resulting calcium influx in the cell produced by the opening of the TRPV1 channels. They also showed the possibility to control muscle contraction in a *C. Elegans* worm. In R. Chen et al. 2015 they showed similar results but in mice neurons. They showed that they could induce neuron stimulation monitored by an increase of c-fos expression in brain cells.

Finally magneto-molecular manipulation is the technique that propose to manipulate spatially and temporally the localization of protein at subcellular level.

In this kind of manipulation one can use magnetic particles to move spatially an active protein. It can be either a receptor clusterized at the membrane upon exposure to magnetic field (magneto-dimerization Figure 1.12). Or either a displacement of freely diffusing protein in the cytoplasm to create controlled concentration of protein to generate signaling activity. For the first case, we can cite the work of Bharde et al. 2013 in which they use magnetic particles targeting EGFR receptors to induce its clustering. Its activation monitored by the increase phosphorylation of the EGFR receptors.

The second case is the manipulation of free protein. The concept is to manipulate them to create locally active concentration of these proteins (Figure 1.13). This is the technique that I worked on during this PhD. The strategy is based on a magnetic nanoparticles (MNPs) functionnalized with functional protein. This complex protein-MNPs can then be manipulated to generate localized concentration of the protein to induce biochemical reactions.

First *in vitro* work was done by Hoffmann et al. 2013 to generate pattern of microtubules using MNPs coated with Ran GTPase who stabilized microtubules formation. In further work, Bonnemay et al. 2013 biased the localization of microtubules asters formation in *Xenopus* egg extract droplets. The explanation was that MNPs biofunctionalized with the Ran GTPase were able to stabilize microtubule nucleation. By doing so, they pushed away the aster of the microtubules from the center of the droplets.

In our team work to manipulate GTPase activity was done in single cell experiments. First, using large magnetic particles (F. Etoc et al. 2013) of 500nm they could induce the formation of actin comets around the particles. Using Cdc42Q61L, the constitutively active Cdc42 to biofunctionlized in vivo the particles they showed the co-localization of a NWASP fluorescent reporter of the activity of Cdc42. Also with the Tiam1-DH-PH domain similar to the one used in optogenetic experiment they could induce the formation of actin comets around the particles.

Second, they showed that using smaller particles below a 70nm hydrodynamic diameter threshold(Fred Etoc, Vicario, et al. 2015), they could generate gradient of particles and thus of proteins inside a cell. In the same concept, (Liße, Monzel, et al. 2017) but using semi-synthetic particles based of the fusion of ferritin protein to mEGFP loaded with an iron core they could manipulate signaling proteins or target organelles with these magnetic particles. Finally Vicario 2016 showed that with MNPs it was also possible to induce localized protein activity by monitoring the colocalization of a downstream reporter. It was shown for both DH-PH domain of Tiam1 and Itsn1 activating respectively Rac1 and Cdc42, that manipulation with MNPs of various size could induce the targeted protein activity. All this previous work were promising for the development of the sometimes



Figure 1.13: Magneto-molecular actuation General schematic of manipulation of intracellular signaling proteins.

called magnetogenetics to become another tool for intracellular signal manipulation.

As for optogenetics, an efficient tools to manipulate intracellular proteins could help to study how signals are processed by the cells, how the different proteins operate and coordinate together to produce the desired behaviors. We can even think of combination between optogenetics and magnetic manipulation to increase the number of input we can give to the cell. Finally magnetic manipulation if translated to clinical application could be of interest to keep control of implanted cells as we suggest it in the Magneuron project. But manipulation of proteins inside the cell requires the tools to have a specific design in term of passivation, magnetic properties and targeting. With this work, we have tried to improve and understand the parameter that are needed. The continuation of the previously described work was also the source of the present project.

A final note is necessary. The reader may have noticed than three rather recent works which are Stanley et al. 2016; Qin et al. 2016; Wheeler et al. 2016 on magnetic manipulation of intracellular signals have not been discussed. They were challenged by Meister 2016 upon the physical reality of their claims and to my knowledge a better explanation to their result has not been brought yet which led me to avoid presenting these results. But this discussion raise the point of assessing which are requirements for such technique
and to ensure that it fits with the basic law of physics.

# 1.3 Developping manipulation of intracellular magnetic objects

In order to get an efficient tool for manipulating intracellular proteins, one wants it to avoid unspecific interaction so that it only affect the target. Also our magnetic nanoparticles (MNPs) have to be able to move freely in the cytoplasm to allow the manipulation with low forces. The cytoplasm is crowdy in terms of proteins, but also in terms of structures (organelles, cytoskeleton). For these reasons the cytoplasm was often described as a poro-elastic medium (Moeendarbary et al. 2013; Hou et al. 1990; Luby-Phelps et al. 1986). When a particles is below a certain threshold it sees a viscous fluid in which it can diffuse as a brownian object going through the pores of the meshwork of structures. If it is bigger, the particles can't go further retained by the different structures. But the cytoplasm is also a crowdy environment which is full of proteins with which a particle could interact unspecifically. In conclusion a particle must have a specific size and a proper passivation. Also, to control that these requirements are achieved it is useful to measure the diffusing properties of particles. This is the topic of the next section. Then we continue with a discussion on the magnetization of the particles and the forces we can generate. This will led to a final discussion on how to generate the field to manipulate MNPs and how to target them on specific proteins.

### 1.3.0.1 Diffusion of particles in the cytoplasm

The nanoparticles experience brownian diffusion when put in a solution because of the thermal agitation. From physic equation the square speed of a particles should be:

$$\langle v^2 \rangle = \frac{K_B T}{m} \tag{1.1}$$

In practice this movement is random, thus the particles don't go anywhere in mean. For this reason the diffusion is rather analyzed as a surface explored over time.

This surface is of course not explored entirely but is full of holes. The "explored surface"

has been defined as the Mean squared displacement(MSD).

$$\langle x^2 \rangle = MSD = 2dD \triangle t$$
 (1.2)

Where d is the number of dimension (d=2 in 2D, d=3 in 3D), D is the diffusion coefficient. The MSD is proportional to the time( $\Delta t$ ) elapsed during the exploration when the movement of the particle is Brownian.

The diffusion coefficient is defined by the Stokes-Einstein equation:

$$D = \frac{K_B T}{6\pi\eta R_H} \tag{1.3}$$

This makes the D coefficient dependent on temperature (T) and on the viscosity ( $\eta$ ) of the medium it diffuses into. It is also dependent on the hydrodynamic radius of the particles  $(R_H)$ . D is usually expressed in  $\mu m^2 . s^{-1}$  and if one calculates the MSD of a trajectory it is basically the slope of the increase of the MSD over time.

This description is valid as long as the diffusion is purely brownian. In the cytoplasm the obstacles and the non-specific interactions can modify the behavior of the particles. Anomalous behaviors were studied by Saxton (Saxton 2007). To characterize the behavior of the particle a simple way is to add a coefficient of anomalous diffusion denominated  $\alpha$ . MSD is then no longer proportional to time. It follows that:

$$MSD \propto t^{\alpha}$$
 (1.4)

With  $\alpha = 1$  being equivalent to the brownian diffusion situation.  $\alpha > 1$  mean that the particle is superdiffusive. this can be the situation where the movement is biased in a direction (movement on a molecular motor for example).  $\alpha < 1$  on the countrary means that the particle is subdiffusive meaning that the particles is exploring less than expected either because of unexpected interactions either because of confinment which limits the expansion of the explored surface (Figure 1.14).

We can use this description to assess if a particles is having anomalous behavior or a Brownian behavior. A good particle candidate should have as few non specific interaction as possible and a size small enough to diffuse in the mesh of the cytoplasm.



Figure 1.14:  $\alpha$  influence on MSD

 $\alpha$  equal to one means that the MSD is proportionnal to time. In anormal behavior this is not the case. If less than one the particle will explore less than expected (yellow curve), it is the case when a particle have unspecific interactions. If it is above one the particle is probably having a biased movement in one direction, it is the case if a particle is attach to a molecular motor.

This later parameter is depending on the physical nature of the cytoplasm which has been considered as a poroelastic medium as previously discussed.

### 1.3.0.2 Limitation to diffusion in the cytoplasm

As said, the cytoplasm is described as a poro-elastic medium. This can be imagined as the organelles and cytoskeleton are dividing the cytoplasm in many chambers linked together by pores with a defined size. This description has also been confirmed by recent work in our team (Fred Etoc, Balloul, et al. 2018).

Because of the different structures in the cytoplasm it appears that particles above a certain threshold cannot diffuse freely in the cytoplasm. Because of this to move large magnetic particles in the cytoplasm requires high forces to force their way inside the mesh. In F. Etoc et al. 2013 they show that weakening of the intracellular mesh improve the particle mobility inside the cells and it was shown that particles small enough to go through the pores diffuse in a brownian fashion (Fred Etoc, Balloul, et al. 2018).

The size of the pores of the meshwork seems to vary depending of the cell but seems to stay in a close range around 50nm. In Luby-Phelps et al. 1986 and Kalwarczyk et al. 2011 estimate the size of the pores to 30nm in 3T3 cells, whereas it is estimated in between 50 to 75nm in Hela CCL2 (Fred Etoc, Balloul, et al. 2018) and up to 80nm in Hela (Kalwarczyk et al. 2011).



Figure 1.15: Limits to diffusion in the cytoplasm From Fred Etoc, Balloul, et al. 2018 Limitation to diffusion depending on particles size

The second parameter that can limit the diffusion of a particle is its interaction with the surrounding elements. In one case the particles can interact with a mobile protein that will increase its hydrodynamic diameter, it is referred as the corona of the particle. The hard corona is use to described proteins adsorbed at the surface of the particle definitively. The soft corona is referring to proteins that can be desorbed if the particles are put back in a free protein medium. In both case this will have an impact on the diffusion coefficient of the particles. In the other case the particle can interact transiently or permanently with an immobile object which will decrease the apparent diffusion coefficient in the first case or immobilize definitively the particle in the second case making it sometimes impossible to move with the magnetic forces we can generate.

Thus the criteria for having freely diffusing particles would be to use particles around 50nm or less in Hela cells and maybe even smaller if one wants to apply the technique to other cell type with smaller pores. The second point is that the particles need to avoid unspecific interaction, this will be discussed in colloid stability and passivation subsection after discussing the tools used to monitor the diffusion in a cell.

### 1.3.0.3 Measuring diffusion in intracellular space

There is different approaches to measure diffusion. They allow to characterize the behavior of particles using indirect or direct means. Most commonly used techniques are fluorescent recovery after photobleaching (FRAP) (Daddysman et al. 2013), fluorescent correlation spectroscopy (FCS) (Schwille et al. 1999), single particle tracking (SPT) (Li et al. 2015), and more recently photo converted intensity profile expansion (Gura Sadovsky et al. 2017). Except SPT all rely on inferring physical parameters from the datas. They are useful to describe the properties of the population of particles. They also have to take in account fluorophore parameters such as its photostability or as if it is prone to photoblinking. SPT on its side allows to analyze for each individual trajectory. And from the trajectory it can highlight one or another behavior. Of course SPT have it's own limitation as the need of using fast acquisition systems and stable fluorescent emitters to get long enough trajectories. In our context as we suggest of working with large objects(compared to other proteins) with limited diffusivity ( 1 to 10  $\mu m^2.s^{-1}$  ) and that carry multiple fluorescent molecules per particles, it is an interesting tool.

In this work we will present the contribution to assess the role of non-specific interaction in the behavior of nanoparticles and we will use SPT to qualify the magnetic particles we use.

### **1.3.1** Particles stability

To be manipulated the particles have to stay stable in the cell meaning that they need to stay separate and free to move. First we will discuss the colloidal stability in itself presenting the DLVO theory which concern the stability in between them, then we will discuss about the passivation of the particles to avoid unspecific interaction with the biological environment.

### 1.3.1.1 Colloidal stability

In a stable colloid suspension, the particles have to stay dispersed without precipitating. The risk of precipitation is intimately linked to the chance that the particles interact with each others. To avoid interaction the idea is to equilibrate the Van de Waals (attractive) forces with an electric (repulsive) force. For this reason the particles need to be



Figure 1.16: **DLVO theory** 

Following DLVO theory the stability of a colloid rely an the balance between Van der Waals (attraction) forces and Coulombic forces (repulsion). Adapted from Gregor Trefalt et al. 2014

charged in order that the electric repulsion stay higher than attraction force.

As many cellular elements are charged negatively such as plasma membrane lipids the electrical potential named  $\zeta - potential$  has to be negative. In Fred Etoc, Vicario, et al. 2015 a  $\zeta - potential$  of -20mV was sufficient to get the suspension of particle stable in the cell.

### 1.3.1.2 Passivation

Charge on its own is not sufficient. Silica coated particle tend to adsorbe protein on their surface (formation of corona of proteins) and the ferritin based nanoparticles as any proteins may interact with other cytoplasmic proteins. The classic strategy is to use a polymer which will have antifouling properties because of its charge and/or conformation and steric effects. The most classical polymer used in that context is the poly-ethylene glycole (PEG).

PEG is used in two conformation. Either using short chains with a high density grafting PEG can form brushes close to each other protecting the desired surface. Either using long chain of PEG that will fold itself, in this second form less density of molecule of PEG is needed as one will cover a larger surface. PEG has many practical and formal advantages. First, it used for a long time has been well studied for its interaction in biological systems. Second, even though all effect are not clear its effect of passivation has been described in many applications. Finally in terms integration in the Magneuron project its a polymer that is already used in medical application lifting already a barrier for potential translational applications.

But the PEG is now beeing challenged by zwitterionic polymer. Indeed PEG can be affected by hydrolysis in biological systems which can reduce the viability of a particle on the long term and so alternatives are being developed. Zwitterionic polymers would be more stable over long time and according to Estephan et al. 2011 they would disfavor protein adsorption because their alternate charges limit the number of counter ions that would beavailable for release in exchange for interaction with a charged protein. This explanation relies on a possible explanation that adsorption is driven by ions release of ions as shown in Henzler et al. 2010. Since the development is ongoing and successfull particle have been developed using zwitterionic polymers that display interesting stability over time (Tasso et al. 2015). It is an interesting alternative but at the time of this work no MNPs with this type of coating was available.

We discussed the processes by which one can make a particle stable inside the cytoplasm, and how we can evaluate its stability. Once a particle achieve such criteria we need to be able to manipulate it, which is the topic of the next section.

### **1.3.2** Magnetic forces on nanoscale magnetic objects

In order to move particles less than 50nm in diameter in the cytoplasm, the required forces are much lower than the one to activate a membrane receptor or to move bigger particles that need to force their way in the mesh of the cytoskeleton. In our case, aN to fN forces are sufficient as we rather need to biase diffusion. Vicario 2016 based on some work of Leroyer et al. 2010 considered that this can be described as diffusion with drift. Based on this work we will first discuss the forces that apply on a particle of less than ten nanometers and the resulting effect on the localization of the particles.

Type, core size, composition	$M_s$ in emu/g	Reference
Synthetic, 9.6nm, 15.1% $Fe_3O_4$ 84.9% $Fe_2O_3$	66	(Frison et al. $2013$ )
Synthetic, 20nm, 69.7% $Fe_3O_4$ 30.3% $Fe_2O_3$	72.2	(Frison et al. $2013$ )
Synthetic, 30nm, 100% $Fe_2O_3$	74	(Cao et al. 2016)
Ferritin, 7nm, $100\% Fe_2O_3 \ 0\% \ Co$	75	(Fantechi et al. 2014)
Ferritin, 7nm, 95% $Fe_2O_3$ 5% $Co$	96	(Fantechi et al. 2014)
Ferritin, 7nm, 90% $Fe_2O_3$ 10% $Co$	51	(Fantechi et al. 2014)

Table 1.2: Magnetization of Ferritin cages This table present different result of magnetization measured with SQUID at  $300^{\circ}K$ 

#### **1.3.2.1** Magnetic particles characteristic and Magnetic field

The force that is applied on a magnetic dipole is dependent on the gradient of the magnetic field B gives eq 1.5

$$F = m\nabla B \tag{1.5}$$

As we discussed earlier we use particles of nanometric size. They are made of two different material classicalily used for ferrofluids. It is either magnetite  $Fe_3O_4$  or Maghemite  $Fe_2O_3$ . These materials can be doped either with Zinc or Cobalt to modify their magnetic properties (Moise et al. 2017; J. M. Byrne et al. 2013; James M. Byrne et al. 2014). Pure magnetite or maghemite have close magnetization at saturation The table 1.2 presents the magnetization at saturation of different materials previously published showing similar magnetization at saturation for magnetite and maghemite. It also shows that dopping with Cobalt (Co) is a also allow better magnetic properties (Fantechi et al. 2014) whereas it is normally associated with Zinc (Zn) doping(J. M. Byrne et al. 2013).

Concerning particles manipulation in biological system it is worth it to have in mind the discussion of Meister 2016. We propose a short discussion on the range of forces we could apply in our case. As an example we use a theoritical ferritin containing 5000 Iron atoms with a magnetization at saturation of 70 emu/g (in the range of reported values for magnetite). We first need to establish the  $M_s$  of a single ferritin.

$$M_s = M_{sbk} \times M_c \tag{1.6a}$$

$$M_c = N \times M_W / N_A \tag{1.6b}$$

$$M_s = 70 \times 5000 \times 56/6.022 \cdot 10^{-23} \tag{1.6c}$$

$$M_s = 3.26 \cdot 10^{-17} emu = 3.26 \cdot 10^{-20} J/T \tag{1.6d}$$

From Bulk SQUID measurement we can measure the mean value of the magnetization of the particle normalized per gram of Iron.  $M_c$  calculation 1.6b calculate the mass of Iron of a core of a ferritin particle.  $M_{sbk}$  is the magnetization at saturation at 310 °K of a ferrofluid of ferritin particles in emu/g.

We then consider that we will use magnetic field with a gradient of  $10^3$  to  $10^4 T.m^{-1}$  (typical gradients obtained with our tools) which gives from 1.5.

$$F = 3.26 \cdot 10^{-20} * 10^3 \tag{1.7a}$$

$$F = 3.3 \times 10^{-17} N \tag{1.7b}$$

$$F = 33aN \text{ if } B = 10^{3}T/m \text{ or } F = 0.33fN \text{ if } B = 10^{4}T/m$$
(1.7c)

From the extrapolated value of the magnetization of a particle and based on the range of gradient in T/m we can estimate the forces on one particle. Which gives a force of 33aN at  $10^{3}T/m$  and up to 0.33 fN at  $10^{4}T/m$  respectively (calculations 1.7). These forces even if they are not sufficient to activate mechanical receptor are sufficient to move particles inside the cytoplasm considering empirical and theoretical results. (Fred Etoc, Vicario, et al. 2015) Such magnetic field gradient is produced by small soft ferromagnetic materials that are shaped according to requirements. Two strategies have been used to reach the required gradient. Either the crafting of iron magnetic tips which have an ending of 10 to 30 microns resulting in gradient of  $10^{3}T/m$  to  $10^{4}T/m$ (Vicario 2016). This crafting beeing more or less reproducible depending on the methods. The most easy is to pull an iron string under high temperature until it elongates and breaks. Some also use etching of wire of permalloy. The latter technique is more reproducible but need some expertise and development to be done in house as no company are easily providing such service. The main disadvantage is to be limited in the number of cells that can be manipulated

during each experiments because the type has to be approched of each cell one by one. Either as proposed by Kunze et al. 2015 using of microfabricated array of micromagnets made of permalloy. It is made of nickel-iron alloy that possess high magnetic permeability and low magnetic coercitvity providing a soft material that can be turned on on demand using an external magnetic field. This technique offers reproducible gradient and in theory offers the possibility to scale up experiments by manipulating particles in many cells at a time.

In both cases these materials play the role of concentrating the magnetic field line of an external magnetic field to generate higher gradients.

It is thus necessary to generate an external field both for magnetizing the particles but also to magnetize the tip or the micromagnet. Because of the complexity to build and cool an electromagnetic device that could reach a high field intensity we relied on the now cheap and easy to find Neodyme Iron Bore (NdFeB) permanent magnets. NdFeB alloy has a high magnetic remanence (1.3T) and a high magnetic coercivity (750KA/m ) allowing us to have a reliable high intensity magnetic field.

We continue the present discussion by describing the kind of gradient we can produce.

#### 1.3.2.2 Manipulation of proteins inside the cytoplasm

The purpose of manipulating particles inside the cells is it's potential application of generating asymptrical distribution of an element with predictible modalities. Considering the previous section we can now explain what would be expected from such setup. As discussed from the beginning of this part the resulting distribution of the particle could be described as diffusion with drift. Following the work in the PhD of Vicario 2016 the concentration of the particles could be described as follows when at steady state:

$$C(x,\infty) = C_0 \frac{h}{l} \frac{e^{-x/l}}{1 - e^{-h/l}}$$
(1.8)

considering  $C_0$  the concentration in the cell, h the size of the compartment (the cell diameter for example), x in the interval 0 < x < h and l being a distance corresponding to:

$$l = K_B T / F \tag{1.9}$$

so in our case with a force of 33 aN

$$l = 130\mu m \tag{1.10}$$

or with a force of 0.33fN

$$l = 13\mu m \tag{1.11}$$

With gradient of  $10^3$  to  $10^4$ T/m we can create gradients that spread over tenth of microns to hundreds of microns at 37 °C which is adapted to produce asymptrical distribution of particles in a mammalian cell (about  $40\mu$ m) (Figure 1.17). This shows the feasibility and the potential versatility of manipulation that we could produce.



Figure 1.17: **Different gradient depending on forces** For a  $40\mu$ m cell we can generate different gradient that will be very localized or will extend over the full cell.

Finally, to coordinate the movement of cytoplasmic proteins we need to target them, that is the last requirement for our technic.

### **1.3.3** Specific targeting of proteins

The targeting to the desired protein is an important step. Many strategies for specific binding have been developed over time. One can choose between different tools with different properties. Here we shortly discuss between three of them, the biotin-strepatavidin binding, the Halotag system and the nanobody targeting of mEGFP.

Biotin-strepatavidin is a very standard and well established targeting system. It has a

very high affinity in the femtomolar range (DeChancie et al. 2007). But the unbinding is thus considered as very unlikely. The streptavidin is quadrivalent which may result in particles clustering in the case streptavidin is expressed on the protein of interest, because one protein could bind different particles. Also if one wants to use biotynation of intracellular proteins in mammalian cells it requires the co-expression of an enzyme for biotinylate specific peptide site.

These issues are getting fixed as monovalent streptavidin with high affinity has been developed (Howarth et al. 2006) and a peptide with an affinity for streptavidin is also now available (Fogen et al. 2015).

Then comes the halotag systems (Los et al. 2008). It is made of a ligand the Halotag Linker (HTL) and a protein the Halotag Protein (HTP). The protein and the ligand are binding with a very high specificity but a slower rate than streptavidin biotin. But as the binding is covalent after few minutes the entire the reaction between ligand and protein can be complete.

Finally, the progress in the development of single chain antibodies has led to the development of nanobodies based on the antibodies heavy chains of Camelide species (Kubala et al. 2010). It result in a small peptide (114 amino acids), easy to integrate in a fusion protein and to express it inside a cell. The availability of nanobody targeting fluorescent proteins like mEGFP with high affinity (named here  $\alpha GFPnb$ ) allowed the development of intracellular application in combination with classically used fluorescent marker. The strategy has been used in Liße, Monzel, et al. 2017 with success to manipulate GEF in cells.

These three examples are showing the availability of many set of tools to achieve a good coupling of particles to a protein to manipulate.

### 1.3.4 Final words

Having discussed all the different aspect to build a magnetic toolbox for intracellular signal manipulation we can dive into the presentation of the work done during this PhD. I could rely on all previous development made in the team done previously and on collaborations with other team members.

The general strategy already presented in Figure 1.13 consist in the assembly of the dis-

cussed techniques to achieve magnetic nanoparticles passivated to avoid unspecific interaction with intracellular elements but that carries a specific targeting systems toward an engineered signalling protein. The displacement of the particle-protein couple allow assymetric distribution of protein activity to control the cell behavior.

We tried to push a step further the manipulation of intracellular signal to achieve a reproducible tool for parallelized manipulation of intracellular signals. Our goal was to untangle intracellular signal processing mechanisms and to achieve cellular control for the Magneuron project.

I also participated to the continuation of the work to explore cellular cytoplasm to get a better picture of how our particles evolve in this complex environment.

Finally we tried to understand what are the limitations of this work and how eventually we could overcome them.

# Chapter 2

# Material and methods

# 2.1 Cellular biology

# 2.1.1 Cell lines

### 2.1.1.1 Hela CCL2 (Hela)

The Hela CCL2 cell line is a common cell line used in many experimental approaches that have been derived from a human cervix carcinoma (Scherer et al. 1953). They were purchased from ATCC. It as been used with success for optogenetic experiments on cellular migration. It is also a cell line which is known for its robustness which is an obvious advantage for a developing technology because it reduces the risk of cell death and let us focus on technical development. These cells were cultured in a DMEM medium.

#### 2.1.1.2 Retinoid Pigmental Epithelium hTERT (RPE1)

Retinoid pigmental epithelium hTERT cells are a human immortalized cell line derived from the RPE-360 cell line (Bodnar 1998). They were purchased from ATCC. Compared to cell line derived from cancer cells they suffer much less from genomic defects. For these reasons they appear as good step forward when reaching the biological aspects of the project development. They are a good intermediate between Hela cells and Primary cell lines which are much more complicated to maintain. These cells were cultured in a DMEM:F12 medium.

### 2.1.1.3 human Mesenchymal Stem Cells (hMSC)

This primary human cell line was cultured with a dedicated kit (MesenCult MSC Basal Medium 05401, MesenCult MSC Stimulatory Supplement 05402, Stemcell Technologies). These cells were provided to us by Maïté COPPEY-MOISAN (Jacque Monod Institut). As primary cells they underwent less passages and so are much closer to endogeneous cells. Passage were kept as low as possible. Experiments were done with cells from P7 to P20 maximum.

### 2.1.1.4 SH-SY5Y cells

These cells were derived from human neuroblastoma cell cultures (Biedler et al. 1973). They have been described as precursor for generating dopaminergic like neurons. They can thus be used as a cell model for studying Parkinson disease (Xicoy et al. 2017). They were cultured in DMEM:F12(1:1) medium. We used them to assess the effectiveness of our technique on a cell type closer to neuronal cells for the benefit of collaborators experimenting on SOS/Ras signaling molecules.

### 2.1.2 Medium

### 2.1.2.1 DMEM medium

When referring to DMEM medium it means cell culture medium made of DMEM (31966 ThermoFischer Scientific) with 10% FBS (10270106 Life technologie). This medium is only buffered with bicarbonate. It has to be used in a  $CO_2$  controlled environment set at 5% of  $CO_2$ .

### 2.1.2.2 DMEM/F12 (1:1) medium

When referring to DMEM:F12 medium it means cell culture medium made of DMEM:F12 (1:1) (D6434 Sigma Merck) with 10% FBS (10270106 Life technologie) and supplemented with glutamax (35050061 ThermoFischer Scientific). This medium is buffered both with HEPES and Bicarbonate allowing to use it in non  $CO_2$  controlled environment.

### 2.1.3 Transient transfection

In order to integrate our engineered DNA sequences and to make the cells express the bioengineered proteins we used classical synthetic lipofectant techniques. We used Viafect (E4981 Promega) according to manufacturer recommendations and we adapted to achieve the desired transfection rates and level of expression. Unless specified otherwise cells were transfected the day before the experiment. We used transfection ratio of 1:3 for Hela cells or 1:4 for RPE1 cells meaning that for  $1\mu g$  of plasmid DNA we used 3 or 4  $\mu l$  of viafect reagent. The mix was made in Optimem medium (11058021 ThermoFischer Scientific) as follow. In 200  $\mu l$  of Optimem medium (ThermoFischer 31985070), first we add DNA, mix then add viafect, mix again and let rest at room temperature for 10 to 20 minutes before dispersing this mix in 35mm wide culture dish containing the cell with  $800 \ \mu l$  of cell culture medium. On the next day the medium is renewed. Depending on the experiment, either the transfection is done after on cells adhering on a glass coverslip either on plastic culture dish. The latter requires an extra passage of the cell on the morning of the planned experiment to plate the cell on a fibronectin coated glass coverslip to allow observation and manipulation.

### 2.1.4 Stable cell lines

In order to improve our reproducibility by using more homogeneous cells we developed stable cell lines thanks to the help of F. Cayrac (BMBC platform). Other cell line were from K. Vaidziulyte working in the LOCCO lab. These cells were made by lentiviral infection. After an antibiotic selection the cells were expanded before being sorted using FACS. Magnetogenetic cell lines were clone sorted. As a result all cell in a petri dish are the result of the expansion of a unique cell having an homogeneous level of expression of the integrated construct. The table 2.1 that follows describe the cell line we used that were at our disposal for magnetic manipulation.

# 2.1.5 Coverslip coating

To improve the cellular adherence on the glass coverslip we used protein coating. Glass coverslips were cleaned in ethanol 70% for 3 to 5 minutes and rinsed in deionized sterile water two times. The coverslip was then covered by a solution of PBS with  $10\mu g/ml$  of

Cell line	engineered proteins	Reporter	done by
Hela	ITSN-mcherry-nbGFP	NWASP-IRFP	F. Cayrac
Hela	ITSN-GFP-Halotag	NWASP-IRFP	F. Cayrac
Hela	Tiam1-mcherry-nbGFP	WAVE-IRFP	F. Cayrac
RPE1	Itsn1-tgRFP-SspBnano + Ilid-mVenus-CAAX	none	K. Vaidziulyte

Table 2.1: List of stable cell lines used in this work

fibronectin (F1141 Sigma Merck) or collagen (C8919-20ML Sigma Merck). Incubation was about 30 minutes for fibronectin and 5 minutes for collagen. Coverslip was rinced with PBS and the appropriate concentration of cell was plated and left to adhere for at least two hours.

# 2.2 Micropatterning

# 2.2.1 Coverslip cleaning

Glass or PDMS coated coverslip was sonicated in a bath of absolute ethanol for 15 min, then in milliQ water for 15 minutes and finally dried with airflow (compressed air or laminar flow of a hood). Finally the coverslip was put in an air plasma cleaner (HARRICK PDC32G Plasma, 2005SD Alcated Primary pump) for 5 minutes. This final step was both cleaning and charging the surface making it more hydrophylic. It also allowed to form carboxyl groups at the surface of PDMS surface for further functionalization of the PDMS surface.

### 2.2.2 Passivating molecule: PLL-g-PEG

Following the work of Azioune et al. 2009 and Carpi et al. 2011 we used PLL(20)-g[3.5]-PEG(2) (PLL-g-PEG) from Susos to passivate glass and PDMS surface. The coverslip was first cleaned as previously described. Then we applied on the surface to passivate a solution of 0.1mg/ml of PLL-g-PEG in an HEPES (10mM pH 7.4) buffered solution. In the case of PDMS surface some extra steps were done, deep UV activation (UVO cleaner 342-220) for 5 minutes to form accessible carboxyl groups and incubation with EDC/Sulfo-NHS dissolved in an NaCl MES buffered(pH 6) solution for 15 minutes. This step converted the carboxyl groups in a Sulfo-NHS-ester ready to react with  $NH_2^+$  groups of PLL (Poly-Lysin) or the primary amine of the pMOXA polymer. Incubation with PLL-g-PEG on glass was done for an hour on glass coverslip and overnight for PDMS covered coverslip.

Finally passivated coverslip were stored at  $4^{\circ}C$  until use.

# 2.2.3 Passivating molecule: PAcrAm-g-(PMOXA,NH2,Si)

Following the aforementioned protocol and upon suggestion of Nicolas Carpi we derived the technique using a PAcrAm-g-(PMOXA,NH2,Si) (Susos, bought from 4DCell) (abbreviated pMOXA for the rest of this document) polymer (Weydert et al. 2017) for passivation. All steps are similar to PLL-g-PEG passivation. One difference to mention is that pMOXA polymer carry a Methyl Silane group that can react with Si-OH activated surface to produce a covalent bond instead of electric interactions.

### 2.2.4 Micropatterning: Quartz mask approach

After passivation the glass coverslip is applied on the quartz/chrome mask that was imprinted with the desired patterns. The masks are made of quartz to be permeable to deep UV (180nm-320nm) and a chrome layer to block UV light. The main advantage of Quartz mask is that it allows to "print" the desired patterns on many slides at a time.

A mask of 12 x 12 cm contain 16 slots that can be designed with a different set of pattern to print. If needed all of them can be used at the same time, allowing 16 slides to be done at the same time.

The process in itself consist in applying the passivated side of the coverslip again the mask. To avoid diffraction the coverslip has to be as close to the chrome layer as possible. Air in between the chrome layer and the mask can also be responsible for diffraction and decreasing the precision of the patterns. To fix this issue we are using a droplet of 2 to  $4\mu l$  of deionized water that fill the gap and maintain the coverslip close to chrome surface. When working with PDMS this is not always sufficient and pressure may be needed to be applied on the coverslip to maintain a close contact of the coverslip and the mask. Once in contact the mask is put in a UV hoven (UVO cleaner 342-220) for 5 minutes. The UV only passes through the pattern of the chrome mask and print them on the coverslip.



Figure 2.1: A) Patterning steps: 1) coating of the coverslip with a passivation molecule (PEG or pMOXA) 2) Printing of the patterns using deep UV illumation through a chrome mask 3) Incubation of the fibronectin which form a layer in the patterns. B) a patterned Hela CCL2 cell, in red a drawing is showing to what would look like the protein pattern

The coverslips are then stored at  $4^{\circ}C$  until further use.

# 2.2.5 Micropatterning: photopattering approach

Alternatively to chrome Mask patterning we tested the Primo (Alveole) photopatterning system. Using a photoactivator (PLPP) solution it allows to burn a PEG or pMOXA layer to print patterns of proteins using a UV 380nm laser. It has the advantage of allowing fast testing of new shapes of patterns if needed but require a long processing for one coverslip (20 to 30 minutes). In addition PLPP has to be carefully rinsed after processing (10 washes with PBS). If some testing has been done during this work none will be presented here. First because they did not provided significantly improved results compared to the other protocol, second because it is much slower we kept using the mask based technics for most experiments.

## 2.2.6 Incubation with protein

To favor the adhesion of the cells on the patterns we incubate a patterned coverslip with a solution of fibronectin. To do so we prepare a bicarbonate buffered solution of 100mM  $NaHCO_3$  (pH 8.4) in which we diluted the fibronectin stock solution to achieve a concentration of  $25\mu g/ml$ . A drop of 40  $\mu l$  of this solution is disposed on an hydrophobic parafin film (Parafilm) and the patterned surface is positionned on top of this droplet. Incubation for 30 to 45 minute is sufficient to achieve the adherence of the cells. Higher time lead to a decrease quality of the patterns. After incubation the coverslip is washed with milliQ water and stored at 4°C.

# 2.2.7 Cell platting

Cells were detached using Versene (EDTA 0.1%) for 10 to 15 minutes or Trypsin-EDTA (0.05%) (12605-010 ThermoFischer Scientific) for 3 minutes. They were then diluted in medium to obtain the appropriate concentration of cells and to inactivate the detaching agent.

Cells were diluted to a concentration of 100k cells/ml for patterned surfaces, 200k cells/ml when used on non patterned coverslip and 300k-450k cells/ml when used on non patterned coverslip for same day transfection. Volume of the medium were of 1ml for 35mm wells and of 0.5ml for micromagnet wells.

# 2.3 Molecular biology

## 2.3.1 Gene cloning

Engineering of gene constructs were done in house, some with the help of our Molecular biology plateform (F. Di Federico, Aude Batistella) or the engineers of the team (Laurence Vaslin, Gaetan Cornilleau). The design was done using the ApE software (by Wayne Davis, Website. Most enzyme were sourced from NEB (New England biolabs), Thermofischer Scientific, Promega and Clonetech.

We classically used the Phusion enzyme (M0530 NEB) for PCR. Insertion of DNA sequences in plasmid vectors were done using restriction enzymes and T4 DNA ligase

Construct	Vector	Source
Itsn1-mcherry-nbGFP	pIRESneo3	LOCCO lab
Tiam1-mcherry-nbGFP	pIRESneo3	LOCCO lab
Ish2-mcherry-nbGFP	pIRESpuro	LOCCO lab
AktPH-IRFP	-	LOCCO lab
NWasp-IRFP	-	LOCCO lab
Itsn1-tgRFP-SspBnano	pLL7	B. Kuhlman
Tiam1-LCF-linker-HCF-tgRFP-SspBmicro	pIRESneo3	Liße Lab, Osnabruck
Ilid-mVenus-CAAX	pLL7	B. Kuhlman
Ish2-mcherry-CRY2	pIRESneo3	LOCCO lab
CIBN-GFP-CAAX	pIRESneo3	LOCCO lab
GFP-HCF	pet21	LOCCO lab
Histag*6-GFP-HCF	pet21	LOCCO lab
Histag*14-GFP-HCF	pet21	LOCCO lab
streptavidin-mRaspberry-li	pIRESpuro	LOCCO lab

Table 2.2: List of plasmid used for transient expression of engineered proteins

(M0202 NEB). Alternatively we used the In-Fusion Cloning Kit (Clonetech).

Homemade constructs were subcloned in a pIRESneo3 or pIRESpuro vector. Amplification of the plasmids were done using antibiotic selection after transformation of TOP10 (ThermoFischer Scientific) or Stellar (Clonetech) bacterias.

DNA purifications were done using midi and mini prep kits from Macherey-Nagel.

## 2.3.2 Plasmid list

Bellow is the list 2.2 of plasmid we had access to during this work. As discuss in the introduction for manipulating signaling activity of the Rho GTPases only the DH-PH domain of the GEFs is needed. For the following construction when referring to a GEF, we refer to its DH-PH subdomain. For Ish2 plasmid, Ish2 corresponds to the activity domain of PI3K. Most constructs were subcloned from Plasmids source from Addgene plasmids bank. All plasmid sourced during this work were referenced below:

mRaspberry-N1 was a gift from Michael Davidson (Addgene plasmid 54645). Str-Ii was a gift from Franck Perez (Addgene plasmid 65312). tgRFPt-SSPB WT (Addgene plasmid 60415), Venus-iLID-CAAX (Addgene plasmid 60411) and hITSN1(1159-1509)-tgRFPt-SSPB WT (Addgene plasmid 60419) were gifts from Brian Kuhlman.

# 2.4 Microscopy

# 2.4.1 Olympus inverted microscopes

Most imaging were done on widefield epifluorescent olympus IX70/IX71 inverted microscopes.

The IX70 microscope (magnetic manipulation setup) is equipped of a fluorescent lamp olympus ULH100HG with a quadband dichroic mirror and two filter wheels equipped with different excitation/emission filter sets. The camera was a Photometric QuantEM 512SC. This system was used for magnetic manipulation and most tracking/targeting data of QDs-SB using laser Melle Griots 488nm set in epifluorescence using a homemade optical path. We used a 60X 1.25NA oil objective equipped with a phase contrast ring for particles manipulation inside cells. We used a 100X 1.49NA oil objective for particle tracking experiments.

The IX71 microscope is equipped of an Ilas2 (Roper Scientific) IRF/FRAP laser system with 488nm, 540nm and 640nm. The camera is a Photometrics Evolve. We used a 100X 1.45NA oil objective for particle tracking experiments of all commercial particles and some QDs-zwitterionic tracking. The FRAP was used for optogenetic testing using low power of the 488nm laser as an alternative the Optogenetic setup.

### 2.4.2 Nikon inverted microscope

Widefield Nikon Eclipse Ti inverted microscope was used with an Oxius laser system (488,532 lasers) equipped with a Tirf arm in epifluorescence configuration. The camera is a Hammamatsu Flash C13440 model allowing imaging of full cells at 5ms for the mapping datas.

# 2.4.3 Optogenetics: DMD equiped microscope

As explained in the introduction optogenetic relies on the light triggering of a conformational change in a cellular protein. To achieve a subcellular spatial control of these activation one need a specific setup to control pattern of light. The system is based on a digital mirror device (DMD) in epifluorescence configuration (DLP Light Crafter, Texas Instruments) illuminated with a SPECTRA Light Engine (Lumencor) at 440nm. The system is controlled by a combination of the Metamorph software for acquisition (Molecular Devices) and a MATLAB 2012B module to set the micromirrors to the appropriate configuration.

The microscope itself is an inverted microscope from olympus IX71 with a 60X objective with 1.4NA allowing a TIRF imaging with an iLas2 system (Roper Scientific). The camera is a Hammamatsu Flash C13440 camera.

### 2.4.4 Heating system

During experiments cell holder were put in a Thermocontroller 37-2 from Pecon. On the magnetic manipulation setup were done on an open configuration to enable for the micromanipulation injector/magnetic tips. Otherwise systems were closed with  $CO_2$  controlled systems (except for the Nikon Ti Eclipse).

# 2.5 Particles

### 2.5.1 Silica particles

Silica Core shell particles were provided by E. Secret (Phoenix, UPMC, Paris). This particles were improved version of Silica core shell particles (Georgelin et al. 2010) used in previous work (Fred Etoc, Vicario, et al. 2015). They are made of an Iron core of maghemite  $Fe_2O_3$  encapsulated in a silica shell. The magnetic cores were sorted to have more homogeneous population of particles. Also, two sizes of cores were made with diameter of 9nm (FFRS2) or 17nm (FFRS3). These cores are embedded in a silica shell of 15 - 20 nm thick. The final physical size of the particle beeing between 40 to 50 nm in diameter.

The silica shell incorporates on its surface PEG chains for passivation and  $NH_2$  group allowing later functionalization. Inside the shell a dye is also incroporated. We mainly used rhodamine Si-MNPS and used some cyanine Si-MNPs for specific testings. At the beginning of this work functionalization with the Halotag Ligand (HTL) was done in house, afterwards particles were provided to us ready to use. All reactions were done in buffers of 200mM HEPES pH8. Functionalization was done as followed first reaction of the  $NH_2$  group with a sulfo-NHS-ester-PEG(12)-DBCO with a ratio of reaction of NHS-PEG- DBCO to particles of 250:1 to 2500:1. The reaction was done at ambient temperature for 1 hour with continuous agitation of the particles. After that excess of non reacted molecule were removed using a desalting column (NAP5 Illustra GE Healthcare).

These previous steps allow the use of click chemistry in the following step to bind the HaloTag Ligand through an azide-DBCO reaction. We used an HTL-azide preparation (From Piehler lab, Osnabruck) mixed with the particles with a ratio of molecule:particles of 250:1. The reaction was done overnight at room temperature.

The final step consist in converting any residual  $NH_2$  group to COOH group which gives to the particles a negatively charge potential in order to protect it from unspecific interaction with mostly proteins and membranes. This is done by a reaction with succinic anhydride in large excess (100000:1). The particles are then passed through a desalting column using a gravity flow to eliminate non reacted molecules.

The final object is a highly magnetic nanoparticles that could be attracted over distance of about 70 to 80  $\mu m$  from the magnetic tip. The targeting system is using the Halotag system described in the introduction.

# 2.5.2 Ferritin

The second kind of particles is made of a protein cage filled with a magnetite crystal. The ferritin protein is endogenously the storage place for iron in the cells. This cage is formed by an hetero-multimeric assembly of two kind of protein subunit. The Heavy Chain Ferritin (HCF) which carries an enzymatic site for iron oxidation. And the Light Chain Ferritin (LCF) which has been described as being important as a helper for the activity of the HCF by helping electron transfer through the ferritin cage (Carmona et al. 2014).

The mammalian ferritin is a very good candidate to achieve the engineering of magnetic field sensitive mammalian cell, mainly because of the quantity of iron atoms it can carry (around 5000 atoms) that could provide a good magnetic response. But this has to be considered with caution. Mainly because in vivo the cage is storing the iron under a ferryhydrate form wich is way less magnetic than the magnetite or maghemite materials usually used in synthetic particles. That is why it is needed to use semi-synthetic particles. These semi-synthetic particles are made in two steps. First, the protein cages are produced by bacterias culture, then the core is synthesized in the cage in an in vitro controlled process. (Liße, Monzel, et al. 2017)

Our strategy is based on the purification of recombinant ferritin made of 24 subunit of GFP-HCF in Escherisha Coli followed by an in vitro loading of the cage by a magnetite core. This strategy first rely on a first step of purification of the protein followed by a pegylation to increase the stability of the protein. After what we proceed to the loading of the cage.

The process of production is described in the following subparts as well as some attempt of optimization.

The ferritin particles(fMNPs) was our main tool in order to work on the manipulation of signaling pathways. The GFP exposed at its surface allowed a fast and efficient targeting by fusion protein of nanobody-GEFs.

#### 2.5.2.1 Ferritin: Protein purification

The work on protein purification and core synthesis was mainly done by Cornelia Monzel and Maud Bongaerts (members of the LOCCO lab) with the help of John Manzi working in the biology platform of our unit. I also invested some time to test the purification using the histag (Method 2.5.2.4) and discussed with them the potential issues. I also participated to test different batches of produced particles in cell manipulation to validate their usability in cells(Results 3.2.1.2).

#### 2.5.2.2 Protein expression

B21 Codon Plus (DE3) RIPL bacteria were transformed with the plasmid expressing the subunit of GFP-HCF and a starter culture was done overnight.

On the next day the starter culture was used to seed 1L flask and bacteria were set to grow until OD600 = 0.8 was achieved. When reaching OD600 = 0.8 we added IPTG at 0.5mM to start the expression of the protein. Protein expression was done overnight at 16 °C.

On the next day the bacterias were harvested by centrifugation and re-suspended in 15mL of a buffer of 50mM HEPES at pH8 and 150mM of NaCl.

This preparation was frozen at -20°C until purification.

After thawing and before purification the suspension of bacteria was mixed with a protease inhibitor, 1mM of phenylmethylsulfonyl fluoride, DNase and lysozyme. Then the cells were lysated by sonication for 10 minutes, and centrifuged. The supernatant was collected for purification

### 2.5.2.3 Purification : protocol

Purification was done using the advantage of the stability to heat of the protein. First a heating to 70°C for 10 minutes was done. Then the sample was centrifuged and was further purified using ammonium sulfate precipitation of the undesired proteins. After dialysis the sample was passed through an FPLC column to further purify the sample by size exclusion. (Figure 2.2)

### 2.5.2.4 Purification : Histag

Using a histag protein was an attempt to improve the purity of the final product. Instead of relying on a purification process based on heat denaturation, salt precipitation and size sorting, the use of a tag could quickly eliminate some contaminant that are still present in the final product. The Histag is a classical system that rely on the affinity of histidine repeats for nickel or cobalt.

Two different size of histag were used and thus two constructs were designed either with 6 histidine repeats (6HIS) either with 14 repeats (14HIS). In both cases the tag was located in the N-terminal part of the construct to ensure that the histag is located on the outer part of the particle and remain accessible to interact with the purification column material. The later one is supposed to let use higher concentration of imidazole for the washing step by increasing the affinity of the protein for the Nickel-column used for purification. The first expression tests showed that the construct with 6HIS repeats had a better expression and that the affinity with the column was already high, necessitating concentration of more than 900mM of imidazole for the final elution of the protein. This is probably linked to the fact that the final cage is made of 24 monomers that are all carrying a 6HIS tag which already increases the affinity for the column material. For that reason the protocol for purification was established with the 6HIS-GFP-HCF construct.

A first step of purification was done using the advantage of the stability to heat of the protein with a heating to 70°C for 10 minutes was done. Then the sample was centrifuged and the supernatant was collected. The buffer was modified with a concentration of 20mM of Imidazole. The sample was first set to bind in the column overnight and washed with a buffer of 50mM HEPES, 150mM NaCl, 20mM Imidazole buffer at pH8 three times. We could elute the protein with buffer having a concentration of 300mM of Imidazole and we had to raise the concentration to 900mM to recover most of the proteins of the column. Still a fraction remained bound to it.

The sample was still passed through an FPLC column to complete the purification. The resulting products are shown in Figure 2.2 showing an improved purity of the final sample.



Figure 2.2: **Purification gels** A) Classical purification 2 and 3 are the final purification products B) Histag purification products; 2 is before FPLC purification; 3,4,5 are the first fraction out of the FPLC column. Histag purification allow to improve the purity of the sample (Adapted from John Manzi Datas)

Before discussing the core synthesis we point out that the particles with Histag were first intended for core synthesis without doping as the Histag is having an affinity for cobalt risking the initiation of core formation at the outer part of the protein.

### 2.5.2.5 Core synthesis

Once the cage of protein is produced we need to crystallize an iron oxyde core inside to render the particle magnetic. If one uses only Iron it will result in magnetite, but one can doped it with cobalt or zinc to improve the magnetic properties. The doping with zinc allow to increase the magnetisation of the magnetic core whereas the doping with cobalt allow the particles to have an hysterisis loop in its magnetisation/demagnetisation process and a higher anisotropy (J. M. Byrne et al. 2013; James M. Byrne et al. 2014). Cobalt doping at 5% is also having better magnetisation properties than no doping in Fantechi et al. 2014 but more than this percentage would decrease the magnetisation of the particles. This last criteria is specially of interest for hypertermia application. In our case we are interested in the magnetisation of the particle, but we used indefferently particles with Cobalt, Zinc or no doping. This mainly because our core synthesis protocol remained variable and it happened to have equivalent magnetic properties with either doping strategies as long as their magnetic properties allowed the manipulation of the particles inside the cell.

To synthesize a magnetic crystal we rely on a previously described reaction (Allen et al. 2002):

$$Fe^{2+} + H_2O_2 \to Fe_2O_3 + 4H^+2H_20$$
 (2.1)

The core synthesis was modified to improve the formation of the core. During that process both the concentration of iron and the speed at which the core is done is important. The objective is to get as close as possible of the maximum that can be loaded in the ferritin cage. The maximum evaluated is around 5000 iron atoms. When doping the particles it was done with 5% of cobalt or zinc. The entire process of core synthesis is done at 65°C under controled pH (pH=8.5). Thanks to the work of Cornelia Monzel and Maud Bongaerts the process was stabilized and allowed us to have access to magnetic particles with expected requirement even though we still observer variability between samples. (Figure 2.3) Still the best results were obtained with Co doped particles and pure magnetite particles.

Finally, the process was done using an autotitration system maintaining the pH around 8.5 by injection of NaOH solution to counteract the production of  $H^+$ . Two syringe are injecting in one a solution of  $H_2O_2$  and in another a solution of Iron amonium sulfate. The stochiometry has to be of 3:1 for  $H_2O_2$ : $Fe^{2+}$ .

### 2.5.3 Quantum Dots

Quantum dots are semiconductors that possess interesting optical properties that depend on their size and materials composition. They are used in biological application as



### Figure 2.3: SQUID measurements for fMNPs

Squid measurement made by E. Secret (UPMC, Paris) of the actually used ferritin made by Maud Bongaerts (UMR168, Curie Institut, Paris). Even if the protocol now allows us to get highly magnetic particles we still observe variability between samples. Each colors is a measurement for a different batches, but made with the same parameters for the core synthesis. (Plot was generated by Maud Bongaerts)

stable and bright fluorescent emitters. We worked in collaboration with the lab of T. Pons and N. Lequeux (ESPCI, Paris) to get access to quantum dots coated with various zwitterionic polymers offering high antifouling properties. Some of the QDs were produced by F. Dembele and M. Debayle (ESPCI, Paris).

Zwitterions are molecules that carries equal numbers of cationic and anionic groups. They have been found to have good antifouling properties for some of the reason we discuss in the introduction. The zwitterions that we tested were the phosphocholine(PC), the sulfobetain(SB) and the carboxybetain(CB). These different zwitterions were attached to a vinylimidazole chain allowing the binding to the inorganic surface of the quantum dots. This is done through the complexation of the vinylimidazole with the zinc cations present at the surface of the QDs.

### 2.5.4 Other particles

Other used particles are listed in 2.3

Particle	passivation	size	Source
QD655 ITK amino PEG	PEG	20nm	ThermoFischer Scientific
			Q21521MP
QDs Itk carboxyl	none	20nm	ThermoFischer Scientific
			Q21321MP
polystyrene/polymethacrylate NP	Streptavidin	35 + / - 8	Micromod 30-19-251
Ferritin-A647	PEG	16nm	D. Liße

Table 2.3: Methods : list of particles for tracking experiments List of particles used in tracking experiments

# 2.6 Magnetic configuration

### 2.6.1 Iron tips

As explained in the introduction two parameter are needed in order to manipulate the particles. First we need to magnetize the particle (ie: to align their magnetic moment with the field). Second we need to generate a gradient that will produce the attraction. The artisanal way to achieve such characteristic was to pull iron strings over a flame producing fine iron tips of 10 to  $30\mu m$  that were cut  $\approx 2mm$  behind the tip and magnetized using one mm size magnet followed by a centimeter long magnetic cylinder of 0.5cm of diameter.

## 2.6.2 Micro array

Made by Koceila Aizel and on inspiration from the work of Kunze et al. 2015 we used microarrays for the development of a parallelization technique of manipulation of particles.

First they were designed using numerical simulation of many size and shape of micromagnets. For producing gradients of  $10^3$  to  $10^4$ T/m over a few ten of  $\mu m$  it appears that square micromagnets of  $100\mu m$  were ideal. These micromagnets were extensively characterized (Toraille et al. 2018) to confirm their magnetic properties.

The process rely on photolithography processing of a resin and electroplatting of the material using a solution of nickel and iron ions.

# 2.7 Internalization of particles

### 2.7.1 Microinjection

Microinjection was done using a microinjector (Femtojet Eppendorf) and a micromanipulator (Injectman NI2 Eppendorf). The micromanipulator allow micrometer position with slow movement (less than  $1\mu.s^{-1}$ ). The microinjector is a pump that allow stable presure from 0hPa to thousands of hPa. This pressure is pumped in a plastic tube connected to a capillary holder. These capillaries from Harvard Apparatus are made in borosilicate. We are using two different capillaries with an outer diameter of 1mm (G100TF-4) and 1.2mm (GC120TF-10). They have an inner diameter of respectively 0.78 and 0.94. The capillaries are pulled before use using P2000 laser puller from SUT-TER INSTRUMENT with following parameters: Heat:410; Fil:2; Delay: 240 Velocity: 19; Pulling:120. This opening size have been characterize using the work of (Schnorf et al. 1994). Resulting in diameter of opening after pulling of 400nm to 500nm.

During an experiment, we approach the capillary from the cell and use a pressure from 15hPa to 80hPa depending on the size of the opening of the capillary and the type of material we were injecting. Too low pressure led to needle clogging and too high pressure led to overinjecting the cell who will not be able to recover and will enter apoptosis shortly after.

After microinjection a recovery time is let to the cell to evaluate if the cell will survive. After ten minutes cell which are too damaged will dye or will show morphological sign of damage. Cell who succeed to recover the damage due to injection in that range of time is considered usable for the experiment.

### 2.7.2 Electroporation

The electroporation was done using a Biorad genepulser 2 with the capacity expender module. Parameters used were 150V,  $0.975\mu F$ . Electroporations were done in 2mm wide cuvettes filled with 200  $\mu l$  of mixture of cells with medium and particles. The expected time constant (Tc) was between 15ms to 20 ms. Electroporation of QDs were usually giving a Tc of 17ms.

Electroporation was usually done with 500000 cells in the electroporation wells but due

to lack of materials some experiments were sometimes done with 250000 cells with no significant differences.

Concerning MNPs electroporation, attempts were made both with fMNPs and SiMNPs. fMNPs could be internalized using electroporation but due to the recovery time necessary after electroporation and the viability time of the particle this way of internalization of the particles were stopped.

The SiMNPs could not be internalized using electroporation even though we tested alternative parameters for the electroporator as well as a different electroporating system (Nepagene electroporator) with no more success.

### 2.7.3 Pinocytic loading

Another way of internalyzing the particles is to use a pynocytic loading. This process relies on the use of an hypertonic solution of sucrose and PEG diluted in the medium of the cells. We add to this solution a few  $\mu l$  of particles solutions and we incubate the cells in that mixture. After ten minutes the medium is replaced by an hypotonic solution made of deionized water and cell medium without FBS with a ratio of 3:2. This result in the bursting of the recently formed endosomes due to the osmotic shock, freeing the particles in the cytoplasm. The exposure to the hypotonic solution has to be limited to about 2 minutes otherwise the cell death is getting too important.

This technique is efficient except it is difficult to obtain high concentration of particles in the cells. For that reason we limited that technique to tracking experiments.

# 2.8 Micromanipulation: attraction of the particles

### 2.8.1 Magnetic tips

After internalization of the particles the cells were left to recover for about 10 minutes. Using the Eppendorf micromanipulator NI-2 we approached the magnetic tip close to the cell (gradient from  $10^3 to 10^4 \text{ T/m}$ ) which will attract the particles in the direction of the tips. As soon as the attraction started the position of the tip is adapted to maintain the localized concentration of particles without pulling the particles out of the cells. As the particules accumulate, one should observe the colocalization of the targeted protein. During a manipulation a movie is recorded by taking images every 3 to 10 seconds. Attraction was maintained during multiple ten of minutes to monitor the colocalization of the downstream reporter and the eventual formation of a protrusion.

### 2.8.2 Micromagnets

Similarly we can manipulate particles using the micromagnets. As a reminder, these micromagnets are made of a soft magnetic material and thus need to be magnetized in order to produce a magnetic field gradient. The micromagnets are magnetized using large NeFeB magnets to homogeneously magnetized the sample. This strategy exposed the complete sample to field of the order of 100mT to 200mT. From the saturation curves of both the particles and the micromagnet we are near magnetization saturation for the particles fMNPs and completely saturate the micromagnet. This conditions allow us to be in a predictable situation were simulation should fit with the produced gradient and potential induced forces.

To stop the attraction either the large magnet is removed or we rotate it at 90 degree so that the magnetic field lines get perpendicular to the cell/magnet axis.

# 2.9 Analysis and quantifications

### 2.9.1 Intensity measurements

For measuring the variation of intensity during the attraction of MNPs a ROI (region of interest) was drawn around the location to measure and the sum of intensity of the points was plotted over these points over the full length of the video.

### 2.9.2 Protrusion growth

To plot the protrusion growth over time we first made a kymograph of the intensity of fluorescence of an intracellular fluorescent marker over the course of the experiment. Each line of the kymograph is the maximum intensity projection of a rectangle covering the protrusion.

The kymograph is first blurred using a gaussian filter of 2px large to smooth noise related high intensity pixels. Then the kymograph are threshold and the progression of the protrusion is measured. (Figure 2.4)



Figure 2.4: Kymograph of membrane

Kymograph with plot of the protrusion for magnetic manipulation, and optogenetic manipulations

### 2.9.3 Single Particle tracking

After acquisition the data were analyzed using Slimfast a software developed by Christian Richter and based on MTT (Multi Targeet Tracing) algorithm (Sergé et al. 2008). Slimfast perform the localization of the particles and reconnect the trajectory steps. Localization parameters were adapted to the sample to achieve the detection of the maximum number of particles. Most data were analyzed using an error rate of  $10^{-6}$  but in some cases were the signal to noise ratio was degraded the error rate was increased to  $10^{-5}$ . Regarding the tracking of the particles the main parameter to define is the expected max diffusion coefficient for a trajectory. Fast particles were analyzed with max value of 9 to  $12 \ \mu m^2/s$  slower particles were analyzed with max D of  $4 \ \mu m^2/s$ .

Another important parameter is the off frame parameter. It allows to take in account the blinking for quantum dots but also allows to skip a frame were the particle would be lost for the localization software. Thus it allows to directly reconnect with the following frames. This parameter was usually left to 2. Only in rare occasion with dense number of particles that were not blinking the off frame was set to 0.

Once the trajectories were obtained the trajectory were first controlled by a simple overlay

of the trajectory on the raw data using a Python script relying on the scipy and skimages and other modules (Stéfan van der Walt et al. 2014; Hunter 2007; S. van der Walt et al. 2011; Jones et al. 2001).

### 2.9.4 SPT: analysis

The analysis of trajectories mainly relied on the computation of the mean squared displacement (MSD) for each trajectory to then extract the D and *alpha* coefficient from fitting using MATLAB 2017b and 2018b.

The MSD was computed using the following formula:

$$\langle MSD \rangle_{\tau} = \langle (x(t+\tau) - x(t))^2 + (y(t+\tau) - y(t))^2 \rangle$$
 (2.2)

As this calculation is done on single trajectories where  $\tau$  is the time step length, the more  $\tau$  increase the less data points are available for the calculation. As we evaluate the D coefficient on the first 6 points of the MSD and the  $\alpha$  on the third of the available points we keep only trajectories that have at least 40 points so that the minimum number of points on which is calculated the last MSD point is about 27.

D is evaluated using a fit of the 1st to the 6 point following considering that the tracking is done in two dimensions.

$$D = \frac{\langle MSD \rangle}{4\tau} + b \tag{2.3}$$

b should be equal  $4\sigma$ ,  $\sigma$  being the localization error. The average over the different particles allows to evaluate the localization precision of our setup.  $\alpha$  is evaluated considering 1.4 as

$$\alpha = \log(MSD)/\log(t) \tag{2.4}$$

by a fitting of the datas over the third of the points of the MSD.

We present the data as an  $\alpha D$  map. In y-axis is the  $\alpha$  value, in x-axis is the D coefficient in log scale. The map is colored as function of the density normalized to 1 of the number of trajectory present in the sample.

Figure 2.5 presents one sample with according histograms of the respective value. It shows that these particles have mostly a brownian behavior, but with a fraction of the



Figure 2.5:  $\alpha \mathbf{D}$  maps

particles which are displaying a confined behavior. Similarly most of the particles have a D coefficient between 0 and 5  $\mu m^2/s^{-1}$ .

As we extract the D coefficient even for anomalous particles, it is not in that case corresponding to the classical D coefficient. But rather we consider it as an instantaneous D coefficient which gives information on the motility of the particle in a short  $\tau$  time step. Indeed we evaluate the D coefficient on the beginning of the MSD curve for  $\tau \leq 6$ . It corresponds to the red spot on the map. A sample that would have the red region located on left-down part of the map would be mostly composed of confined particles.

# 2.9.5 Cell mapping

We decided to map spatially the D coefficient means over a cell. To do so trajectories were first recovered from Slimfast. Then from the list of the localization points a K-means algorithm is used to clustered the points. The centroid of this cluster is used for a Voronoi algorithm to create a mesh delimiting areas spread all over the cell. This allows a the mesh to be generated without any supervision.

From the widefield image of the cell the border of the cell is delimitated manually to create a mask who will eventually restrict the mesh produced.
Each trajectory is then attributed to one area and spitted in case it crosses a border. For cell mapping as the trajectories are cut when crossing a border and to keep a reasonable amount of trajectory we lowered the threshold to keep a trajectory to 30 steps. The MSD and D coefficient are recovered similarly to previous section.



Figure 2.6: Cell mapping process Mapping of cytoplasm process. Step:1 acquisition and tracking of the particles. Step:2 delimitation of the cell Step 3: meshing and and attributing the trajectories to areas Step:4 Processing of the trajectories

## Chapter 3

# Results

# 3.1 Exploration of cytoplasm environment and particles diffusion properties

As discussed in the introduction, to improve the manipulation of magnetic object we need to know are the required properties for an object to move freely in the cytoplasm. As it was shown previously the size of the object is limited by the size of the pores of the meshwork of the structures of the cytoplasm.

The first part of the results is discussing the behavior of different particles possessing different passivated layer and will present result using emerging new polymers the zwiterrions.

### 3.1.1 Behaviors of particles below pore size

This work was initiated by Fred Etoc and Chiara Vicario by analyzing the diffusion of various particles below the pore size in Hela cells. But as it is known that there is differences in the proteomic profiles in between cell lines these results had to be expended to other cell type. We choose to reproduce these results in near primary RPE1 hTERT cells and with primary hMSC cells as they are better representatives of cells of an organism. Another cell line HDFa was tested by Davide Normanno who provided to us the raw data that we analyzed similarly to our samples.

The particles tested were an attempt to represent a broad variety of particles from simple particle suspension (stabilize from their  $\zeta$  – *potential*) to semi-synthetic particles coated

with 2000kDa PEG. They were the following QD-Itk-COOH (QDs-COOH), negatively charge but with no coating; QD-Itk-PEG-NH2 (QDs-PEG), with more positive charge but globaly negatively charged with antifouling coating; PMMA-Streptavidin (RhoD), negatively charged with protein coating; and Ferritin-A647 a protein cage coated with antifouling coating. All these particles are about 20nm in diameter so well below the estimated pore size in a cell (Size table 1.3.0.2).

As it could be expected the QDs-COOH that have no antifouling layer diffuse poorly. If one look at the particles trajectories they move slowly and only few are diffusing, most of the particles are stuck at various location of the cells. The  $\alpha$  coefficient is about 0.2 to 0.3 (hMSC cells and RPE1 respectively) confirming the confinement of the movement of the particles. The diffusion coefficient is about 0.04  $\mu m^2/s$ . QDs-Itk-PEG-NH2 have slightly better characteristic but the diffusion coefficient is still low even if these particles are nearly three time faster that the QDs-Its-COOH. They are also higly polydisperse in behavior with an  $\alpha$  average value of 0.6 which hides particles with brownian behaviors to highly confined or immobilized particles.

The RhoD particles that for reminder are coated with a streptavidin protein are much better. Most of the particles display a brownian profile. Ferritin-647 have a D coefficient which is even higher. In hMSC the  $\alpha$  is rising up to 0.72 and 0.76 respectively. The D coefficient rises to 0.82 and  $2.17 \mu m^2/s$  respectively.

Comparing the RhoD and Ferritin-A647 to QDs is striking. Even if the size of RhoD and Ferritin-A647 are higher or similar respectively to the QDs they diffuse much faster. The Stokes-Einstein equation would indicate that an increase of radius would decrease proportionally the D coefficient. But the Ferritin-A647 are only 4nm bigger in diameter than the QDs but the D coefficient is 1 to 2 order of magnitude higher, confirming the crucial role of the passivation layer to avoid unspecific interactions in the cell.

If one can observe differences in between cell lines the trends are all the same. Particles below the pore size can diffuse in a brownian fashion to the condition that their passivation layer protect them from the proteins and lipids of the cell.

These datas also confirm the good results using PEG passivation but they also show that it is not a perfect strategy to avoid all unspecific interactions. It is thus interesting to test other passivation surfaces.



Figure 3.1:  $\alpha \mathbf{D}$  maps for different surface passivated particles  $\alpha \mathbf{D}$  maps.  $\alpha$  is representing the behavior of the particles. D is in log scale. Hela cells results were previously obtained in the lab by Fred Etoc. HDFa data were obtained from Davide Normanno and analyzed in our lab. RPE-1 and hMSC results were done in the lab. From Fred Etoc, Balloul, et al. 2018

### 3.1.2 Zwitterionic Quantum dots

#### 3.1.2.1 Diffusion of zwitterionic coated particles in the cytoplasm

As discuss earlier zwiterrionic polymers seem a promising alternative to PEGylation. We worked in collaboration with the lab of Nicolas Lequeux and Thomas Pons to test the behavior of Quantum Dots coated with a zwitterionic polymer inside the cytoplasm. Because of their photo stability the quantum dots are useful to probe the intracellular environment as they allow long term imaging without photobleaching.

In a first attempt, three different QD coated with three different zwitterionic polymers (described in 2.5.3) were tested to assess their antifouling properties and to test them as probes for biological applications. All of these particles have a hydrodynamic diameter of about 14nm (measured via FCS) (Debayle et al. 2019). Way below the pore size of a mammalian cell.

The three polymers tested were sulfobetain, phosphocolin and sulphocarboxybetain. In

vitro testing were already demonstrating the superiority of the sulfobetain to get rid of the formation of an hard corona of protein when mixed with BSA. We tested the behaviors of the particles in Hela CCL2 cell. The particles were internalized using an electroporation protocols (Methods 2.7.2). The results show that the bests polymers SB (sulfobetain Figure 3.2A)( $< \alpha > \approx 0.89$  and  $< D > \approx 2.68$ ) and PC (phosphocholine Figure 3.2B)( $< \alpha > \approx 0.92$  and  $< D > \approx 2.57$ ) have a diffusion coefficient close to the Ferritin-647 that we tested previously. If one exclude the few particles with anomalous behavior (clear blue on the graphs) they both exhibit an homogeneous behavior of brownian diffusing particles. The carboxybetain polymer is less efficient even though part of the analyzed trajectories exhibits a D coefficient as high as the other polymers but they also display a broader variation of behavior with an immobile subpopulation and a subdiffusive one. ( $< \alpha > \approx 0.73$  and the  $< D > \approx 0.98$ )(Figure 3.2C).



Figure 3.2: **QDs zwiterrionic**  $\alpha$ **D maps**  $\alpha$ D maps.  $\alpha$  is representing the behaviour of the particles. D is in log scale. Experiments were done using Hela cells. QDs were internalized using electroporation.

#### 3.1.2.2 Efficient targeting using Biotin-Streptavidin strategy

It is also interesting to point out if adding a biomolecule for targeting a biological protein would affect the behavior of the particles and how efficiently and specifically we can target a cellular structure. In a first step a primary amine was added (SB-APMA particles). Even though the particles are keeping an overall negative  $\zeta - potential$ , the increase of positive charge at the surface of the particles is increasing the number of unspecific interaction with surrounding proteins.

The coefficient of anomalous behaviors drops to 0.6 in average with two population. One that even if slow down can still diffuse and one that is immobilized (Figure 3.2D).

This phenomenon is fully reversed by converting the primary amine to carboxyl group with a diffusion coefficient raising up to 3.5  $\mu m$  in mean and an  $\alpha \approx 0.97$  (Figure 3.2E). Alternatively the conjugation of the QD to a neutral biotin ligand provide the similar effect with the quasi disappearance of the immobile fraction (Figure 3.2F).

Finally we targeted the endoplasmic reticulum (ER) of Hela CCL2 cells using a strepta-



Figure 3.3: QDs zwiterrionic: ER targeting

Two cells expressing a streptavidin anchored to the endoplasmic reticulum. Before injection with no QD no signal is visible. After injection the signal of the QDs overlap the signal of the anchored streptavidin visualized thanks to a fused mRaspberry fluorescent protein.

vidin anchored to the ER membrane. In seconds time scale after inject of QDs-SB-Biotin the ER is decorated with quantum dots. The targeting is confirmed by the colocalization of the fluorescent protein included in the anchor with the signal of the QDs. (Figure 3.3)

## 3.1.3 Exploration of intracellular cytoplasm

#### 3.1.3.1 Diffusion in full cells

All previous results are based on sampling trajectories of particles diffusing in the periphery of the cell. But the cytoplasm is not homogeneously constituted. Some organelles like the endoplasmic reticulum and the Golgi apparatus are often located around the nucleus. Which location vary between cells. The cytoskeleton itself doesn't have the same structure with filament allowing transport from inner to outer part of the cell in the center, and filament along the membrane at the periphery to maintain the cellular shape. We wondered how this could affect the diffusion of the particles. To answer this question we proposed to map diffusion coefficient over whole cells. In order to do so we design an experiment to track particles in two dimensions in cells plated on patterns to have reproducible shapes and similar internal organization (Schauer et al. 2010). This results in a general front to back orgnisation as follow. The nucleus is located at about two third of the cell from the front, and the endomembranes are located at about a third of the cell from the front with some around the nucleus. To initiate this part of the work we relied on the QDs-SB that have displayed stable fluorescent properties as well as low unspecific interactions with intracellular elements.

We first evaluated for how long the particles were stable in the cytoplasm of the cell. We electroporated cell with the particles and let them grow in culture plate for at least a day before starting imaging. We could collect data at day 1 (+ 24 hours post electroporation ) and day 3 (+72 hours post electroporations). Whole cells were imaged to avoid subselection of any subregion. Figure 3.4 presents the results at day 1 and day 3 and compare it with the original results obtained with electroporation and imageing on the same day with tracking in peripherical cytoplasmic regions only.

These datas shows the relative stability of the particles over time. One can see slight differences but one should ensure that they are time related and not simply sample related as shown in Figure 3.5A. A sample image at day 2 (+48 hours) was showing confined behavior even though we could observe freely diffusing particles in other samples at day



Figure 3.4: QDs zwiterrionic : stability over time

 $\alpha$ D maps.  $\alpha$  in y axis is representing the behaviour of the particles. D is in log scale for x axis. Experiments were done using Hela cells for D0 (periphery only), with RPE-1 for D1 and D3. QDs were internalized using electroporation.

3. Also, over one sample confined population of particles vary from cell to cell (Figure 3.5B,C,D,E ). These differences may be explained by different reasons that may be hard



Figure 3.5: **QDs zwiterrionic : differences between samples**  $\alpha$ D maps.  $\alpha$  in y axis is representing the behaviour of the particles. D is in log scale for x axis. Experiments were done using with RPE-1 for all samples. QDs were internalized using electroporation. Intra sample and inter-sample variation in particles behaviors.

to identify when one is facing a samples presenting highly different characteristic than usually encountered. But we can hypothesis different reasons that don't imply the cytoplasm structure but rather side effects of the internalization process. Electroporation is inducing the formation of clusters that may enter the cells and the cells may endocyte particles if rincing is not done fast enough. These endocyted particles will stay confined in endosomes. I made the choice of considering samples behaving very differently than most experiment would be discarded for now. Meanwhile we pursued to improve the reproducibility by trying to keep track of various parameter as trying to keep electroporation time in a close range and rincing the cells after electroporation quickly. This allowed to get similar samples to the ones previously observed with particles freely diffusing as in experiments based on periphery only.

Once having a proper sample we finally imaged cells on pattern to evaluate potential differences through cytoplasm space. Because we wanted to get the best spacial coverage we overlapped the cell after aligning them on top of each others. (Method 2.6 and Figure 3.6A) We allowed this process because of the described similarities in the positioning of the organelles of the cells RPE1 when plated on crossbow fibronectin patterns. (Schauer et al. 2010)

The obtained results are displayed in Figure 3.6. We highlight that in some regions only few trajectories are evaluated and that the regions having the slowest D coefficient seems to have the higher number of trajectories. These regions located are mainly around the front of the peri-nuclear region, where it has been described to be rich in endomembrane (Schauer et al. 2010). This could be related to endocyted particles that would be stored in that region or to an increased number of unspecific interaction with specific endomembranes.3.7

These lower D regions are not necessarily linked with a higher unspecific interaction rate with all diffusing particles in that region but rather linked to a confined population that is over represented because of their low D coefficient that would bias the average D of these regions. Filtering the trajectories by D coefficient shows that particles diffusing above  $1\mu m/s$  are homogeneously distributed without any easy pattern to visualize (Figure 3.7A,C).

The pattern of slow population being located in the front part of the cell appear again when visualizing the particles with a D<1 $\mu$ m/s (Figure 3.7E).

This suggest that particles can diffuse freely anywhere in the cytoplasm but that trapped particles are concentrated near the endomembrane rich region localized at the front of the perinuclear region ((Schauer et al. 2010)). This could be explained with a fraction of the particles that would be endocyted by the cell during the process of internalization ending up in endosomes instead of being free in the cytoplasm or as said ealier with an increased number of unspecific interaction in that region.

For the moment any further conclusion could not be made, and additional experiment



Figure 3.6: Cell mapping: results

A)We overlapped 7 cells data after rotating and aligning them B)Maps of D coefficient in RPE1 cells C)Plot of the D coefficient mean (x-axis) with the number of trajectories (y-axis). D)Map of number of trajectory per area. At the middle of the first third of the cell from the front one can see a region where the D mean is lower. It correlates with the location where the number of trajectories is the highest.

with a different internalization method to avoid potential endocytosis would be required to confirm this proposition.

### 3.1.3.2 Exploring intracellular space: what's next?

These results indicate that for small particles diffusion is not affected even in regions crowded with organelles. It would be of interest to see if and how the inhomogeneity of the structures of the cell would affect the diffusion of bigger particles or with different passivations.

Meanwhile these datas suggest that particles can move easily anywhere in the cell if properly passivated which confirm the possibility of manipulating small magnetic particles over the full cell.



Figure 3.7: Cell mapping: mapping with filtering per D coefficient A,C,E)Maps of D coefficient in RPE1 cells B,D,F)Map of number of trajectory per area. One can see that the low D coefficient region is not in contradiction with fast diffusive population of particles everywhere.

## 3.2 Stability of magnetic particles

## 3.2.1 Behaviors of magnetic particles

#### 3.2.1.1 Si-MNPs

The previous work done in the lab both in Vicario 2016 and Fred Etoc, Vicario, et al. 2015 used Si-MNPs with success. With the improvement of synthesis techniques we first decided to continue using this type of MNPs.

The newer generation of Si-MNPs with their core ranging from 9 to 17nm were very

promising in term of magnetic performance. As a particle moment scale with its volume, a doubling of the diameter multiply by 8 it's magnetic moment. It's nearly one order of magnitude more that allows either sharper attraction profiles or to attract over larger distance (as the gradient decrease with the distance respective to the magnet).

But attempt to use them in single cell experiments were not straightforward. While we could achieve manipulation of protein using the Halotag strategy the particles suffered of instability inside the cells. After a time ranging from 30 to 60 minutes particles are forming round structure when injected in cells. It has to be highlighted that the particles can stay as a stable colloid suspension for weeks in a salt buffer or even mixed with FBS or BSA solution.

In a first step we studied the variation in the behavior of the particles over time. Single particles tracking demonstrates that particles are quickly switching from a near brownian behavior to an anomalous behavior. (Figure 3.8) As discuss earlier this can be because of



#### Figure 3.8: SiMNPs stability : diffusion maps

(a) Diffusion map after 10 minutes post injection (b) Diffusion map after 30 minutes post injection. Trajectories are getting anomalous as their diffusion rate decreases. This suggest that the particles interact in between them or with their environment getting more and more confined.

unspecific interaction with proteins or because particles interact together increasing their hydrodynamic diameter which induce their confinement in the cytoplasm.

In a second step we wanted to understand if the observed "clusters" are linked to any recognition mechanism that would target the particle to the degradation machinery. We attempted to control if the particles were colocalizing with autophagosomes marker LC3 (Hansen et al. 2011) as this was shown to be one of the reason of degradation of other kind of particles (Liße, Richter, et al. 2014).

We injected particles in cell expressing an LC3 fused to GFP and waited for the particles to cluster. One can clearly see that there is no overlapping between the clusters of particles and the autophagosomes (Figure 3.9 ).



### Figure 3.9: SiMNPs stability : autophagomes testing

A) LC3-GFP image B) Rhodamine signal from SiMNPs C) Overlay of the two channels show distinct localization of the two signal (green LC3, red SiMNPs and that the phenomenon is not related to cell recognition of the particles.

Finally based upon the microfluidic works to induce particle clusterization in microchannel we can postulate that the injection process is not neutral for the stability of the particles. To confirm this suspicion we produced droplet of magnetic particles inside an mineral oil



#### Figure 3.10: SiMNPs stability : droplet test

SiMNP were injected in mineral oil to form droplets to test if the injection process is having effects on the particles stability

phase.(Figure 3.10)

Figure 3.11(A) shows that particles already form small cluster after passing through the fine tips of the needle. Moreover it seems that this clustering once initialized is quicker when exposed to an external magnetic field (Figure 3.11(C,D)).



#### Figure 3.11: SiMNPs stability : In vitro clustering

Epifluorescence imageing of droplet of particles in their buffer. The droplet are surrounded by mineral oil. A) particles few minutes after gowing through the needle B) drolet of particles mixed with high BSA solution (560ml/ml) deposited with a large pipette C) Clusterization on the border of the droplet when exposed to a magnetic field D) After exposing A with a magnet clusters gets larger

Meanwhile exposure to high concentration of protein like BSA (560mg/ml has no effect 3.11(B). We conclude that the clustering is mostly the product of particle-particles interaction initiated by the shear stress during the injection process.

As injection remained the main tool for internalizing high amount of magnetic particles we decided to stop the use of SiMNPs for further manipulation of proteins. They were only used for short testing, less than 30 minutes to benefit of their high magnetization to assess the functionality of our magnetic tools.

### 3.2.1.2 fMNPs

As developed in the material and methods parts the ferritin particles required a long optimization to achieve a good magnetic properties. As SQUID measurements are not available in house we had many time to evaluate if the particles were useful for our applications by practical experiments. After the production of fMNPs, we needed to ensure the quality of the protein and of the passivation layer so we tested the diffusivity of the fMNPs inside Hela CCL2 cells.

One can remark that the diffusion coefficients are lower than the Ferritin-647N but similar to Rho Particles. The  $\alpha \approx 0.83$  and  $D \approx 1.09 \mu m^2/s$ .(Figure 3.12A,B) Compared to



Figure 3.12: **fMNPs : diffusion** A)  $\alpha$ D map just after injection B) Map after 40 minutes

Si-MNPs the behaviors is stable over the first 40 minutes. After more than one hour the particles can also be harder to move in the cytoplasm but no cluster can be observed. Immobilization is thus probably the results of unspecific interaction with proteins of the cell.

Finally we wanted to assess that the particles can reach the plasma membrane. We used cell transiently expressing an aGFPnb-mcherry-CAAX protein thus localized at the membrane thanks to the CAAX peptide and targeting the GFP of the particles thanks to the nanobody. The figure 3.13 is showing that the particles colocalized with the CAAX



### Figure 3.13: fMNPs : membrane localization

Epifluorescence images of Hela cells expressing aGFPnb-mcherry-CAAX. A) transmission image B) Ferritin-GFP particles C) aGFPnb-mcherry-CAAX B,C: red rectangle is showing the localization of the particles at the membrane confirming that they can reach protein localized on the plasma membrane.

anchor and can thus reach the membrane. It is an important confirmation than the particles can interacts with proteins at the membrane as our targets the Rho GTPases are also anchored at the plasma membrane.

### 3.2.2 Parallelization

An interesting progress for particle manipulation was the development of reproducible micromagnets allowing reproducible magnetic field gradient to be produced. Instead of producing handmade random shaped magnetic tip we could reliably expose the MNPs to a known field. But as the gradient is variable in space if one wants to expose different cell to similar fields, one would have to position them at a defined distance from the micromagnet.

The micromagnets were produced in our team by Koceila AIZEL based on previous work done by Kunze et al. 2015 as explained in section 2.6.2. The produced magnets can be either covered by a glass layer using sputtering of Si atoms or by a PDMS layer.

As we started our work using patterning with a mask we decided to pattern a PDMS because it allows a much thicker layer to be deposited covering the gaps in between the micromagnets. The PDMS layer is deposited on the coverslip and is spin coated to be flattened which produce a layer of PDMS of approximately the same height than the micromagnets ( about 10 micrometers). This surface is rather concave which makes it difficult to apply against the mask for printing patterns. Nevertheless we could achieve some success in patterning round cells close to micromagnets and showed the possibility to manipulate particles on patterned cells using the micromagnets.

We first tested PLL-g-PEG passivation of PDMS, but it gave us inconsistent results. Thus we tested a new polymer based on pMOXA chains which displayed a better stability. We coupled it to the PDMS surfaces using an EDC/NHS activation of the PDMS surface allowing it to react with the NH2 group as previously described.

After passivation we printed the patterned using a mask tightly pressed against the coverslip. We plated Hela CCL2 cells and injected them with Si-MNPs.

As displayed in figure 3.14 we could obtained many cell at a similar distance from the micromagnets (3.14B) and we could as expected manipulate the particles of the cell by



#### Figure 3.14: Parallelization : micropatterned cells

A) fluorescence of 488 fibrinogen mixed to fibronectin to visualize the patterns B) Patterned cell on a micromagnet coverslip coated with PDMS C)off mode of micromagnet either by removing the magnet either by turning it perpendicularly to the direction of the cell allow to let the particles disperse in the cell; on mode the magnetizing magnet is aligned with the direction of the cell and the particles are attracted toward the micromagnets D) a patterned cell next to a magnet (black part on the top) E) micromagnet is off F) micromagnet is on

exposing the whole sample to an homogeneous field using a permanent micromagnet. (3.14C,D,E,F).

Using this technique seem advantageous at first because of the potential reproducibility of the conditions to which the cell would be submitted to. But because some strategy choices had to be made we temporary stopped the patterning because of the following reasons. To print the pattern using a chrome mask we had to use a PDMS layer which in addition of being more complicated to pattern, is also putting further away the cell from the magnet. Because of the former the gradient to which the cell is exposed is less than what we could achieve without the PDMS layer.

Because of the previous reason and because we had to stop to use the Si-MNPs and had to refocus on the fMNPs, we had to get the most of the gradient produced by the micromagnets because the core of the ferritin particles are only up to 7nm compared to the 9 to 17 nm we can have from the synthetic cores.

Finally because we had to use a different polymer than PEG to achieve a correct passivation of the PDMS which was not available at the moment with an azide functionalization prevented us to eventually have access to the BCN-RGD coupling to free the cell from its pattern. These would have been necessary to assess the possibility for the cell to move in the direction of the cell if it had to move.

Thus we succeeded to assemble the techniques that would allow parallelization. But because of the requirement of our experiment and the availability of the techniques in the lab we had to go a step back and use the micromagnets without a PDMS layer and thus without patterning.

#### 3.2.2.1 Note on the development of parallelized manipulation

Some note have to be made on potential development that could be made for developing a patterning at the level of the micromagnet without the need of using a chrome mask from printing the patterns.

The technique relies on the setup of Alveole for photopatterning using a UV laser described in the methods. The technique was use to nicely align micropattern with the micromagnet in Toraille et al. 2018 in supplement of our micropatterns done with the chrome mask.

Since with this system there is no risk of diffraction as it relies on the focus plane of the objective it become plausible to attempt patterning of a glass surface at the level of the micromagnet and to benefit from the high gradient and the technique using the combination of azide-PEG/BCN-RGD to release the cell to monitor cell migration. If preliminary work was attempt during the project no result could be produced yet. But if one requires the fulfillment of parallelized manipulation with reproducible localization of cells this could be the perfect match to solve the previously described issues.

## 3.3 Magnetic manipulation of intracellular signals

This is the core of the project. Using magnetic nanoparticles (MNPs) biofunctionalized in vivo we wanted to manipulate the activity of the RhoGTPases located at the membrane. We monitored the results on two aspects. First, we looked at the colocalization of downstream effectors and second we measured the formation of protrusion where the Rho GTPases were supposed to be activated.

The particles were manipulated both using magnetic tips and micromagnet arrays. Most of the experiments were done using a cloned selected cell line expressing Itsn1-magneto and the NWasp-Irfp Reporter. The particles even if similar were the products of different production of fMNPs.

The preliminary work started on both the study of both Tiam1 and Itsn1 DH-PH domain manipulation. But the observable results showed that Itsn1 DH-PH domain manipulation were the more promising in term of success, and thus we focused on the manipulation of this signal in most of this present work.

## 3.3.1 Manipulation of ITSN1 DH-PH domain

Here we present the work on the activation of Cdc42 using the Itsn1 DH-PH domain fusion protein described in methods 2.2. The DH-PH domain fused to a nanobody can target the fMNPs when we inject them in the cytoplasm. From there we can manipulate them. In the following part Itsn1-magneto will refer to this fusion protein (Figure 3.15) We could in a repeated manner activate the Cdc42 using Itsn1-magneto manipulated us-



#### Figure 3.15: Itsn1-magneto

A) Itsn1 protein domains B) Fusion protein, nanobody::mcherry::Itsn1DHPH that we call Itsn1-magneto C) Itsn1-magneto target the fMNPs anchoring itself on the GFPs

ing fMNPs (Figure 3.16). The results were a direct continuation to what was observed in



Figure 3.16: Manipulation of Itsn1-magneto to activate Cdc42 By bringing the MNPs close to the plasma membrane using a magnetic tip Itsn1DHPH domain can activate Cdc42 which can then recruits the Wasp complex.

the work of Vicario C. (Vicario 2016). The Cdc42 activity visualize thanks to the colocalization of NWasp-Irfp reporter was observed multiple time and it was often link to small displacement of the membrane here referred as protrusions even though their morphological appearance may not be homogeneous in between them. Also we could observe some cell retraction at the rear of the cell while these protrusions were observed.

#### 3.3.1.1 State of the technic: a successful manipulation

The best case obtained during this work was obtained with the Hela cell line stably expressing Itsn1-magneto and the NWasp reporter. For one hour and 30 minutes we attracted fMNPs toward a micromagnet. We report here the signal fluctuation observed at location of the protrusion during the first 30 minutes (Figure 3.17). First from 3.17A we showed (blue symbols) the protrusion toward the magnet and the retraction at the rear of the cell. Second we confirmed is a tight correlation between the attracted particles, the localization of the Itsn1-magneto (3.17A,B). And finally we could observe the colocalization of spikes of signal of the NWasp reporter colocalizing with the two aforementioned elements (3.17A,C). This confirmed that by manipulating Itsn1-magneto we could observe downstream signalling activity.

From this first presented result we can conclude that our technique was reproducibly



Figure 3.17: Itsn1 manipulation : signal quantification

A) Manipulation of particles at 4 minutes and 34 minutes, one can observe the formation of a protrusion while the cell retracts at the back. B) The dynamics of the intensity of the fMNP and Itsn1-magneto are similar showing that the particles allow the manipulation of the local concentration of GEFs C) NWASP-IRFP reporter is having spikes of activity overlapping with increases of concentration of GEFs.; Notes:there is a gap of about 4 minutes while setting up the field on;

working to manipulate a protein, and to induce some downstream activity by the visualization of the reporter. Over about 30 minutes we observe that the signal of NWasp is having spikes of activity. These spikes seems aligned with the phase if the increase of the concentration of Itsn1-magneto at the membrane. But even though the attraction is sustained the activation of signal is not.

This is a clear issue if one wants to control the behavior of a cell. A first step to understand any issue of manipulation of signal was to study the dynamics of protrusions with another method of manipulation of intracellular signals, the optogenetics.

#### 3.3.1.2 About the dynamics of the observed events

If one looks to other activations it is important to notice that the balance between attraction of the particles at the membrane and the pulling on the membrane is sometimes subtle as one may observe in some situation we tear the membrane on the magnet. But by adjusting the distance from the tip of the magnet to the cell we can maintain a focalized concentration of particles over an hour or more.

This raise a first point, the particles can apply a force on the membrane in addition to the molecular activation. We can hypothesized that it would increase the growth of the protrusions. On the opposite we are not simply moving individual proteins but we move many proteins at once with a rather big object that is roughly ten time the size of a single signaling protein. This could affect the signaling activity by reducing the chances of the downstream complexes to assemble correctly.

In a first attempt to visualize these potential effects we compared the protrusion formation of the magnetic manipulation with a manipulation using the Itsn1-tgRFP-SspBnano construct that will bind to the membrane upon blue light illumination thanks to a membrane anchor (Ilid-Venus-CAAX). These is referred as Itsn1-opto on the following section. In all cases the only the same Itsn1 DH-PH domain was used. We highlight that optogenetic experiments were all done in RPE1 cells which may result in slight differences compared to the Hela that were used for the magnetic manipulation.

In optogenetics between the start of the recruitment and the protrusion start there is a delay of minutes.

About the dynamic in magneto-Itsn1, we can observe that the movement seems to be also triggered in minute time scale. Even it appears that in some magnetic manipulation a range of tenth of second is sufficient. Although we don't possess the first second of stimulation for all our experiment it seems that the speed of formation with our magnetically induced protrusion are compatible with the timescale of a signaling activation.

Thus in optogenetic the protrusion continues to expand with a mean speed of  $1\mu m/1min$  with no limit and will eventually trigger cell migration. In magnetic manipulation, we only got one experiment where the protrusion reached more than ten micrometer of extension.(figure 3.18B blue color for magnetic manipulation, magenta is showing optogenetic activation) This protrusion was formed only in a few minutes just while setting up the magnetic field on the protrusion progressed of nearly 10 micrometers and 4 more while imaging over the ten first minute of experiment. The time of the protrusion expansion fits with the time of the spikes of activity of NWasp discussed in previous section (figure



Figure 3.18: Comparison of the protrusions between techniques A) Plot of the protrusion growth over time, blue: magnetic manipulation green: opto-Itsn1 B) Over a longer time protrusion due to magnetic manipulation is not progressing. It shows that we cannot induce more than the formation of a protrusion.

3.17) For this experiment, or others where the protrusion is much smaller it seems important to highlight that after starting to protrude, the protrusion stops. Then it even tends to retract.

As the force being exerted on the particles is maintained this raise the possibility that the particles are perturbing the intracellular machinery necessary for the protrusion to form. Even though their size is not an obstacle to activate membrane signals at first. There is a second difference in addition to the size with the magneto-Itsn1, the signaling proteins once bound to the particles are bound definitely whereas in optogenetic particle can bind and unbind thus providing some renewal of the Finally we can still remind a few facts. Manipulation of magnetic particles inside the cytoplasm to control the spacial location of a protein was repeated successfully as it was done in the passed by C. Vicario and F. Etoc. We could obtain similar results but also start some comparison with pre-existing manipulation tools confirming some of the potentiality of our tool but also highlighting some limitation that may be intrinsic to the technic.

For these reasons we started to design different experiment to get a glimpse of understanding of the reasons of these limitations.

# 3.4 Exploring the limits of magnetic intracellular manipulation

As explained different issues could affect our technic. (Some are presented on the figure (3.19).

The first is related to the process of activation. In optogenetics the molecules are continuously renewed with unbinding and rebinding of proteins. In the case of magnetic manipulation as we used a very high affinity binding system we can assume that unbinding is unlikely and thus that binding of a protein is permanent. To circumvent that issue we tested a low affinity binding using CFP mutated variants produced by the lab of J. Piehler and D. Liße (Osnabruck University). We took at the same time advantage of a newly developed particle using synthetic magnetic cores coated with an iron binding peptide protein fused to a mEGFP or an ECFP mutated to have different binding affinities with the nanobody. We denominate this particle p-MNPs in the document. (More information cannot be disclosed about this particle)



#### Figure 3.19: Issues of the magneto-molecular approach

No turnover of the GEFs on the particles: There is no renewal of the GEFs on the particles, which may result in a reduced activation of the signalling proteins; Activation: We may be below the threshold of activation to get a proper resulting behavior from the cell; crowding: The particles may disturb the internal organization of the cell and preventing the correct assembly of the complexes that would make the protrusion grow;

Also we wondered if the signal activation could be too low to produced a sufficient activation to increase the size of the protrusion, polarize the cell and induce migration. This led us to attempt using PI3K activity domain Ish2 which allow to convert PIP2 in PIP3 and favor the localization of GEFs at the membrane. This is thus an attempt to benefit from the amplification of signal that occurs along the pathways.

Finally we discussed and tried to test how the size and the presence of a high number of particles may affect the activation of the signal due to a steric effect. We thus decided to see if we could perturb an optogenetic activation of the intracellular signals using magnetic particles attracted at the same location.

#### 3.4.1 Low affinity

In a first attempt we tested low affinity particles. Instead of having a definitive binding, the mutation of a CFP protein allows a degraded binding of the nanobody. It allows the renewal Itsn1-magneto protein at the surface of the particles (Figure ??).

Two versions of this protein coating were designed by D. Liße, and produced by M. Kappen and A. Budke-Gieseking (Osnabruck University). They were assembled on a synthetic



## Figure 3.20: Low affinity strategy

By using a low affinity binding between the particle and Itsn1-magneto we expect to get more renewal of the protein at the surface of the particle. The activating protein could also get free from the particles and stay bound to its target to improve the efficiency of signal activation.

maghemite core by E. Secret (UPMC, Paris).

Two variant of this particle were made, having an affinity lower of a least 1000 times compared to our classical constructs. The resulting particle are denominated midAp-MNPs and lowAp-MNPs. The lowAp is having even lesser affinity for the nanobody.

In both cases (Figures 3.21 and 3.22) we could manipulate Itsn1-magneto and achieve



## Figure 3.21: midAp-MNPs manipulations

Manipulation using the midAp-MNPs low affinity; A,E Transmission; B,F midAp-MNPs; C,G ITSN-nbGFP; D,H NWasp signal; ABCD Before manipulation; EFGH After attraction with a magnetic tip



#### Figure 3.22: lowAp-MNPs manipulations

Manipulation using the lowAp-MNPs very low affinity; A,E Transmission; B,F lowAp-MNPs; C,G ITSN-nbGFP; D,H NWasp signal; ABCD Before manipulation; EFGH After attraction with a magnetic tip

activation of the NWasp signal at the attraction point. For these experiments we decided to use a magnetic tip instead of a micromagnet in order to avoid a blind period during the setting of the magnetic field. After short testing it appeared that the lowAp-MNPs seemed promising in terms of intensity of the activation reported by NWasp-IRFP and in terms of magnetic properties.

But measurement of the produced protrusion result in similar results than what was observed using fMNPs. Similarly to what was previously observed they allow the formation of a protrusion before the cell membrane retracts. Still a remarks about the measured cell is that the whole front of the cell is retracting and not only the protrusion. But this also shows that the signal activation is not sufficient to counteract other cellular mechanisms.

After further testing these particles appeared to have some drawbacks. First because of their synthetic cores these particles have much stronger magnetic properties and they can be attracted more easily. It seems interesting at first but one has to be careful as they can be pulled out from the cell. It also increases the chances to generate forces on the membrane that may result in uncontrolled events.

Also their stability over time is not high as after 30 minutes after injection they start to be impossible to manipulate. This limits their use to short experiment for now.



Figure 3.23: Comparison of low affinity versus other manipulations Comparison of the manipulation with lowAp-MNPs with the previously described magnetic and optogenetic manipulations. As for fMNPs after more than 10 minutes the cell retracts.

### 3.4.2 Upstream signal activation: Ish2

In order to increase the amplification of signal we can generate, we tried to use a protein more upstream in the pathway of signaling. We choose to use PI3K and more specifically its subdomain Ish2 who is responsible for the conversion of PIP2 to PIP3. PIP3 serves as an anchor for GEFs to activate the Rho GTPases. The intent is that one Ish2 domain could convert many PIP2 allowing the recruitment of more GEFs that we could do by magnetic manipulation of the GEFs themselves.

We based our construct on a previously described optogenetic construct that was inducing cell spreading when targeted to the membrane. The functionality of this optogenetic construct was confirmed in our hand previously to building a magnetic version of this construct. When activated with optogenetics it allows the ruffling and the spreading of the cell membrane.

In a first attempt 3.24 we used an IRFP-AktPH fusion protein to report the conversion of PIP2 to PIP3. But no signal of the reporter could be clearly observed. Since this reporter was not tested previously in our hand, it may be that the reporter is faulty. So



#### Figure 3.24: Ish2 manipulations

Ish2 domain manipulation; A,E Transmission; B,F MNPs; C,G Ish2-mcherry-nbGFP; D IRFP-AktPH signal; H IRFP-Nwasp signal; Ish2 domain can be brought at the membrane but no clear reporter signal can be highlighted. A,B,C,D lowAp-MNPs used in RPE1 transfected cells ; E,F,G,H fMNPs used in Hela cells.

similarly with Nwasp-IRFP as a reporter we attempt another time to activate signaling using Ish2. Still no clear signal of the reporter could be observed even though we observed small fluctuation at the plasma membrane and of intensity of the reporter. These two could simply be related to small fluctuation in the cells or be induced by the force applied on the cell membrane as we were using lowAp-MNPs.

This is still be a point to be made clear.

The absence of clear effect could be related to the non activity of our construct when docked on a magnetic particle either because of the conformation once assembled on the particle or because the time of residence of the particle close to the membrane is too short as the particle still diffuse and are not blocked at the membrane.

## 3.4.3 Crowding

Another steric effect can be due to the crowding produced by the large amount of particles we are bringing at the membrane.

We assume that the particles are free to diffuse in the free space of the cytoplasm and that they would not occupy completely this free space. But one could imagine that because we densely concentrate them that they end up making a blockade preventing some necessary signaling complexes to assemble as they should to allow protrusions to grow.

We thus designed an experiment combining on one hand optogenetic to activate the



#### Mixing optogenetics with magnetic manipulation

#### Figure 3.25: Crowding strategy

By attracting particles at the same time we activate the recruitment of Itsn1-opto at the membrane we want to assess if the accumulation of particles can perturbe the signal cascade. We illuminate with blue light the full cell triggering recruitment of Itsn1-opto (in green) at the membrane; at the same time we attract the particles (in gray); (red represent the CAAX-iLID)

signaling at the membrane as we know that this technique is functional. On the other hand, magnetic manipulation is used to attract particle at a location of activation to prevent the signal activation to occur (Figure 3.25).

This experiment has revealed to be more tricky to be realized and only one cell could assemble both techniques. This is just a preliminary result that may open discussion on the topic. As one can observe in figure 3.26, in front of the accumulation of the particles it seems that no protrusion has formed. Rather the cell growth from each side to circumvent the area where the particles are located.

These experiments should be repeated to assess if this explanation is correct and if it can be overcome with a different type of particle.

## 3.4.4 Discussion on magnetic manipulation

The conclusion of these results is ambiguous. On one side we seem to have a tool that allows signal manipulation but on the other side, the progress toward an effective manipulation tools are limited by many unknown factors.



## Figure 3.26: Crowding test

A) mVenus fluorescence of fMNPs and Ilid-mVenus-CAAX B) RFP signal of Itsn1-RFP-SspB C) The cell has been activated with blue light indicing the recruitment of Itsn1 to the membrane. Blue arrow indicates an empty space in front of an accumulation of particles attracted by a micromagnet located at the bottom of the picture.

More and more proofs are accumulating showing that there is a protein activity linked to the location of our particles bound to proteins but the effects are very limited. The question to know if it results from crowding, residency time at the membrane, quantity of input signal we can generate or simply not appropriate conformations of our constructs (specially of Ish2) remain open (Figure 3.19)

We conclude the results to open a discussion on the perspectives if one wants to develop a tool to manipulate intracellular signals but also if one wants to get a better picture of the intracellular space.

## Chapter 4

## **Conclusions and perspectives**

# 4.1 Manipulation of intracellular mechanisms with the magneto-molecular approach

The cytoplasm is a complex environment were many processes are taking place. A signal integrated from a receptor on the plasma membrane will cross a pathway made of protein regulating each other. These cascades from which members are mostly known still need to be untangled to understand how a signal gets modulated through them. One way of doing so rather than observing, is to interfere with these pathways. Many tools were developed over the last decades to bypass part of this signal transmission and to study " submodules " who compose these pathways.

They demonstrated it was a valuable approach to improve our knowledge. And we can also expect than further development of these techniques may help regenerative medicine to go one step further. Indeed if one want to rebuild some complex tissues that were set during the embryonic development, there is a need to provide information to the cells for them to know what to do. If one wants to achieve that in vivo " whispering " these information directly to the regenerative cells would be the most secure option to avoid disturbing neighboring cells.

Magnetic manipulation development is still ongoing. We chose to develop a magnetomolecular approach, and repeatedly we produced many proof that the concept is valid for activating intracellular signals with subcellular precision. But one has still to reach a so called " industrial " stage were we can ensure a certain level of efficiency and reproducibility. The path to develop a new technique is sinuous. About reproducibility, even if not perfect we could obtain the colocalization of downstream effectors at the desired location in many occasion. But concerning efficiency we are still far from our goal. In the best case the protrusion form over about ten micrometers and the cell retracts from the rear. But it never goes further. Biologically one should try to better characterize the observed events. This could be done by monitoring activity levels of other RhoGTPases to see if the cell polarize. But the experiment remain an heavy task to reproduce, and it is hard to say how difficult adding layers of complexity for producing these datas are possible.

From our results we can also wonder if the particles are disturbing the signal processing inside the cell, this could also bring valuable information of how much free space is necessary for a cell to operate correctly. But would probably put an end point to this manipulation method. But we are still not clear about the other issues that could limit the technic, one of which clearly rely in particles instabilities who prevent to produce long term experiments.

Being able to hijack a signal but ensuring that we are not perturbing other cell mechanism should be the target for the development of our technic.

So what could be done to make of this technique a potential tool that could be used daily and not only as an experimental approach?

#### 4.1.1 Magnetic particles

We tested many type of MNPs, but rare are the particles who possess all the requirement. The stability of the particles we had access to is from 30 minutes to 1 or 2 hours. This is far from allowing long experiment over one day or more. This may also prevent any transfer to in vivo manipulation who will probably require longer manipulations. This is one of the main bottleneck of the technic.

Still great progress for highly stable particles could be made with the zwitterionic polymers development. QDs passivated with this type of polymer were stable for days. Once this chemistry will be adapted to magnetic particles one may hope to keep these properties at least for small nanoparticles which have few risks of particle-particle interaction due to their magnetic properties. This could also provide a big improvement in the signal manipulation results. Because we cannot exclude that the cell is not sensitive to long manipulation because of unstable MNPs that quickly loose their capability to move freely and that loose their capability of activating signals at the membrane.

Answering the last point could be done by attempting to correlate the diffusivity with the time window in which we can actually actuate on signaling pathways with the MNPs.

### 4.1.2 Magnetic manipulation

Concerning single cell manipulation the magnetic tools are now sufficiently developed. We have two different way of producing the necessary magnetic field, the magnetic tips and the micromagnets arrays. The second can be reproducibly produced and well characterized. They even opened the door to parallelized experiment on the long run. Nevertheless magnetic manipulation may also be on of the trickiest issues that may result prevent transition to *in vivo* models. To manipulate nanoparticules one can use micromagnets to manipulate them overs hundred of micrometers in the best conditions. For in vivo manipulation this would require the implantation of micromagnets at the vicinity of the cell to manipulate. It is a complicated task as once in the oxidative environment the magnet will soon start to loose its magnetic properties. Also if one wants to guide a process over the long distance one should find a way to deactivate magnet one by one let the cell sense the next one. A complicated task to foresee.

Micro electromagnets mostly developed in the context of MEMS still suffer of high dissipation and don't produce the required field intensity we need to magnetized our particles. On that aspects the Magneto-mechanical actuation have probably a brighter future when it come to in vivo manipulation. Synthetic particles of a bigger size can compensate the loss of gradient that can be achieved on longer distance. Similarly magneto-thermal manipulation is much less limitated in the distance of actuation.

## 4.1.3 Signaling proteins

Some progress could finally be achieved with differently engineered proteins. The first case that we started to explore is the low affinity targeting to increase the renewal of the signaling protein at the surface of the particles. The second case is to use different targets who may be better fitted for this kind of manipulation.

The structure of the engineered protein may also be modified to increase for instance the degree of liberty of the proteins at the surface of the particle that may be a limitating factor as discuss earlier.

#### 4.1.4 A clearer view on the cytoplasm

Outside of molecular biology aspect we still have a lot to explore on the physical environment of the particles. Particle tracking could bring us useful and precise information on the spacial organization and crowding of the cell. Are the pores of the intracellular meshwork uniform or is there some differences across a cell? It could highlight some asymmetries that may be interesting to relate with different cell behaviors. Magneto-molecular actuation could also be used to disrupt the intracellular organization to obstruct some location of the cell. It could highlight how much the cell function as whole and how much some sublocation can work independently from the rest of the cell.

If isolation of subpart of the cell is feasible then this would help dissecting the many processes that are cooperating in the cell. With the idea that we may add some understanding in how biological system compute information, how from a very noisy environment they organize and maintain themselves.

From the manipulation of particles to control intracellular signaling proteins many fundamental questions have arise. Are our objects innocuous to the cytoplasm? When unspecific interaction are affecting the particles is this an homogeneously distributed process in the cytoplasm? One should probably continue to study how unspecific interaction arise and can be avoided.

We could expand our results to map the cell for the diffusion of larger object over the cell to answer the first questions. Also, as we mostly averaged our results on different cells and one could be interesting to know how much differences there may be from a cell to another. If one wants to manipulate objects in different cell type what has to be expected? Our first results suggests that there is few or no differences but the range of tested cells remain narrow.

## 4.2 Final words: Controlling cells mechanisms

The way to build an ubiquitous manipulation technique to control all intracellular mechanisms is still far from reach. The ideal system would also be endogenously expressed. It would also have to be reliable and easily adaptable to in vivo systems. It is still an ideal to pursue.

Optogenetic remain one step ahead for most applications but remains difficult to transfer to in vivo models. The need of implantation of light emitters at the vicinity of the cells to activate or the need to manipulate only transparent biological sample is a clear limitation toward translational transfer of the techniques.

Magnetic manipulation on it side offer a clear advantage but many limitation appears for magneto-molecular approach. Concerning other magnetic approach there is an interest for magneto-thermal or magneto-mechanical approaches. In the first case one need to produce radiowaves which heat a particles to activate a channel, in the second either pulling either clustering of receptors is possible over larger distance using large magnetic particles. But these two cannot provide directionality to the stimulation which in case of neural growth is a requirement.

The solution may come from the study of magnetobacterias or from the understanding of magneto sensing in specific vertebrate like birds. But the later mechanism is very unclear, and only magnetobacterias start to be understood. As magnetobacterias are sensing the orientation of the field because of the alignment of their magnetosomes with the earth magnetic field one may imagine that genetically engineered cells could produce magnetosomes to sense the directionality of an external homogeneous field. But the way to achieved such transfer from bacteria is long. If one ever succeed it would a powerful strategy. For translational aspect it would go with a lot of ethical, practical and safety question as it would require heavy genetic modification of the cells that could be implanted in patients.
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