



Challenges and Methods for the Study of CPP Translocation Mechanisms

Astrid Walrant, Françoise Illien, Sandrine Sagan, Nicolas Rodriguez

► To cite this version:

Astrid Walrant, Françoise Illien, Sandrine Sagan, Nicolas Rodriguez. Challenges and Methods for the Study of CPP Translocation Mechanisms. Cell Penetrating Peptides, 2383, Springer US, pp.143-152, 2022, Methods in Molecular Biology, 10.1007/978-1-0716-1752-6_9 . hal-03443598

HAL Id: hal-03443598

<https://hal.science/hal-03443598>

Submitted on 10 Apr 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Challenges and methods for the study of CPP translocation mechanisms

Astrid Walrant, Françoise Illien, Sandrine Sagan and Nicolas Rodriguez

Sorbonne Université, École normale supérieure, PSL University, CNRS, Laboratoire des biomolécules, LBM, 75005 Paris, France

Corresp. author: sandrine.sagan@sorbonne-universite.fr

Running head: Evaluation of membrane crossing by a fluorescent peptide

Abstract

Fluorescence-based methods are widely used to detect crossing of peptides across model or biological membranes. For membrane-active peptides, i.e. peptides that have strong membrane tropism, fluorescence experiments must be accompanied by relevant controls, otherwise they can lead to inconsistent interpretation and underestimation of their limitations. Here we describe how to prepare samples to study fluorescent peptide crossing droplet interface bilayer (model membrane) or cell membrane (biological membrane) and the pitfalls that can affect observational qualitative and quantitative data.

Keywords Droplet interface bilayer, fluorescence spectroscopy, flow cytometry, peptide, membrane translocation, model membrane, biological cell membrane, internalisation, quantification

1 Introduction

An intriguing aspect of cell-penetrating peptide internalisation is the great diversity of routes they can employ to enter cells. A clear distinction is made between active transport mechanisms i.e. endocytic routes and passive crossing of the membrane i. e. direct translocation, and still, CPPs are able to use both types of pathways concomitantly [1]. The detailed molecular description of CPP internalisation pathways is the main focus of many studies, one of the objectives being to understand the key molecular features that direct a CPP towards one entry route versus another [2]. There are many biochemical, pharmaceutical or biotechnological

tools to study endocytosis, whereas direct translocation remains more elusive. One of the most powerful approaches to study CPP internalisation is fluorescence. Fluorescently-labeled peptides are easily accessible by peptide synthesis and are compatible with many experimental setups. The relative contributions of endocytosis and direct translocation can be quantified on large populations of cells using flow cytometry, provided certain precautions are taken, as discussed in the next paragraphs [3, 4]. Another approach to quantitatively study peptide internalisation on a large population of cells is fluorometry, i.e. the quantification of total fluorescence in solution [3]. This method is described in details in this chapter. With flow cytometry and fluorometry, direct translocation is indirectly evaluated by playing on the experimental conditions such as temperature or ATP depletion. Internalisation is evaluated on a large cell population in a single experiment but with no information on the cellular distribution of the peptide following its internalisation. Fluorescence microscopy, on the other hand, allows a sub-cellular tracking of the peptide, on fixed [5] or live cells [4]. Direct translocation can be evaluated as it is usually associated with diffuse cytosolic labeling or direct labeling of the nucleus, whereas endocytosis is associated with punctuate labeling corresponding to CPPs trapped in endocytic vesicles. Again, as detailed below for flow cytometry, fluorescence microscopy is prone to experimental artifacts and precautions have to be taken to accurately evaluate the contributions of endocytosis and direct translocation.

Direct translocation is a passive mechanism and only requires a lipid bilayer to cross. Several studies have evidenced direct translocation using a fluorescent peptide and artificial or semi-artificial cell membrane mimics. Direct translocation in large unilamellar vesicles was studied using NBD-labeled peptides [6–8]. Reduction of NBD by dithionite allows the quantification of translocated peptide by fluorometry. Translocation in purely synthetic giant vesicles [9–12] or giant vesicles derived from plasma membrane (GPMV) [13] can be directly visualised by fluorescence microscopy. More recently,

direct translocation was studied using droplet interface bilayers (DIBs), a model system that allows to mimic the lipid asymmetry of the plasma membrane [14]. This method is described in details in this chapter.

Fluorescence based techniques are diverse, widespread and powerful. However, they have some pitfalls which must be taken into account and, if possible, overcome [15]. For example, fluorescence microscopy of fixed cells has been used in the nineties to assess fluorescently labelled CPP translocation before discovering that cell fixation could lead to the redistribution of the peptides and misinterpretation of their internalization pathways [4]. The problem has been solved by working since then with live cells. A more difficult challenge is the impact of the fluorophore attached to the CPP on its internalization properties. Indeed, the fluorophore can significantly affect several physicochemical properties of the fluorophore-CPP complex such as charge or hydrophobicity. And these modified properties will in turn modify the interactions of the complex with the cell membrane during membrane adhesion, insertion or crossing. This can lead to changes of the amount of internalized complex and use of different routes of entry for example modifying the endocytosis/direct translocation ratio. Several studies have shown the impact of solely changing the fluorophore on cytotoxicity, depth of insertion in the membrane, final distribution inside the cell mirroring routes of entry ,...[16–18]. It seems that the best way to take this fluorophore influence into account is by varying the fluorophores and developing studies to identify or design fluorophores with minimal Interference properties. Crosstalk with non-fluorescence based techniques, for example mass spectrometry, is also desirable [3, 19]. Another difficulty when studying the internalization of fluorescently labelled CPPs by fluorometry or flow cytometry is to distinguish CPPs which have reached the interior of the cell or are at least deeply inserted in the membrane from peptides which are adhering on the outer surface of the cell. A simple wash of the cells by use of saline buffer is not enough to remove these adhering

CPPs. For this purpose two kinds of solutions have been proposed: enzymatic treatments to remove membrane bound peptides [1, 3, 4] or use of a fluorescence quencher to remove their contribution to the fluorescence signal [3, 6]. Finally, a possibly misleading property of the fluorophores is the sensitivity of their optical properties (absorbance, quantum yield) to the physicochemical properties of their environment (which can differ notably between the aqueous cytosol and the hydrophobic core of the bilayer) and to the presence of close other fluorophores which leads to quenching and fluorescence extinction. Both causes make difficult a quantitative interpretation with deduction of the amount of internalized peptide from the measured fluorescence intensity. As it is likely that with many experimental conditions, CPPs do not enter as isolated peptides and can form aggregates at different stages of their entry inside cell, the quenching possibility must be carefully considered. One way to overcome these difficulties is to quantify the fluorescence of the internalized fluorescently labelled peptide in a standardized environment which would also prevent aggregation. This can be obtained with fluorometry by quantifying the fluorescence of the internalized peptides (after membrane bound peptide removal) in cell lysed with detergents [3, 20]. Fluorescence correlation spectroscopy can also give hints about the aggregation states of the CPPs interacting with cells [21].

The following sections will present in details two fluorescence-based techniques: translocation with DIBs which are versatile model membranes which enable the formation of asymmetric bilayers to study CPP translocation and translocation with cells which use the detergent lysed cell protocol to make the translocation measurement quantitative.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents in a refrigerator.

2.1 Translocation with DIBs

1. Aqueous phase is generally Phosphate Saline Buffer (PBS) plus 10 mM MgCl₂. Peptides are added only in aqueous phase A at typical concentrations in the micromolar range.
2. Oil phase is made of squalene plus 15% v/v of chloroform and 8⁰/₀₀₀ (w/w) of lipids. If an asymmetric bilayer is prepared, lipids are different in oil phases A and B. Peptides will be initially on the side of the monolayer composed with oil phase A lipids.
3. Glass coverslip used for observation of the translocation are covered with a thin (~ 1 mm) layer of PDMS (polydimethylsiloxane). This transparent layer does not prevent observation and avoid spreading of the droplets.

2.2 Translocation with cells

All reagents, buffers and solutions to incubate with living cells are sterilized and kept in a refrigerator.

1. Fluorescent-labeled peptides (1 mM in water) are stored at -20°C in the dark. Concentrations from weighted peptides can be checked by NMR.
2. Low-binding microtubes are preferentially used to prevent peptide binding to plastic.
3. Peptide dilutions for incubation with cells are prepared in the culture medium without serum.
4. Trypsin/EDTA (commercially available) or pronase are used at 0.05% (w/v) in PBS.
5. One tablet of Complete Mini (Roche) inhibitors is dissolved in 2.5 mL PBS.
6. Bovin serum albumin is prepared at 1 mg/mL in PBS.
7. Trypan Blue is prepared at 0.2% in PBS from a commercial solution.
8. Cell lysis solution is 50 mM Tris pH7.4, 1M NaCl, 1% Nonidet P40.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Translocation with DIBs

Fluorescently labelled peptide translocation assay through droplet interface bilayers (Fig. 1):

1. Mix (gentle vortexing) 2 μ L of aqueous phase A (with peptide) with 20 μ L of oil phase A (droplet population A).
2. Mix (gentle vortexing) 2 μ L of aqueous phase B (without peptide) with 20 μ L of oil phase B (droplet population B).
3. Deposit population A on a glass coverslip covered with a thin layer of PDMS. Deposit population B sample on top of population A to allow their gentle mixing. No need for further mixing.
4. Observe the sample with an epifluorescence microscope and look for a spontaneously formed pair of adhering droplets (*see Note 1*). The two droplets must come from both populations as evidenced by their fluorescence difference.
5. Record the fluorescence changes of both droplets to assess a possible translocation from the peptide from its initial droplet to the other one. The typical translocation time is of the order of a few minutes.

3.2 Translocation with cells

For semi-quantitative flow cytometry analyses, follow steps 1 to 16 to measure translocation of fluorescein-labeled or fluorophore-labeled peptides (*see Note 2*). For quantitative fluorescence spectroscopy analyses (Fig. 2), follow steps 1 to 20 to measure translocation of fluorescein-labeled fluorophore-labeled peptides (*see Note 2*).

Perform experiments independently at least three times (flow cytometry) in triplicates (fluorometry).

1. Wash adherent cells (*see Note 3*), cultivated in 100 mm plates with complete (with serum) medium, 3-folds with PBS (same volume as the culture medium), and aspirate the liquid under vacuum or with a pipette without touching cells.

2. Add 2 mL trypsin to cells and incubate at 37°C for 2-3 min until the cells detach (detachment step followed under microscope).
3. Add 8 mL culture medium with 10% serum to inhibit trypsin.
4. Count cells using for example a Malassez counting chamber.
5. Dilute cells if necessary and seed them in 1 mL culture medium (plus 10 % SVF) per well of 12-well plates, to obtain one million cells 24-hours later (*see Note 4*).
6. Leave cells in multi-well plates at 37°C and under 5% CO₂ and humidified atmosphere for 24 hrs.
7. Put the multi-well plate in a basin containing crushed ice mixed with water, so that the bottom of the plate is kept at low temperature ($\leq 10^{\circ}\text{C}$) during the whole experiment (*see Note 5*).
8. Aspirate gently the culture medium and wash cells 3 times with cold culture medium.
9. Add the fluorescein-labeled peptide at different concentrations (*see Note 2*) in 1 mL cold culture medium to the multi-well-plate for 60 min (plateau of the internalization kinetics) (*see Note 6*).
10. Aspirate carefully the liquid above cells and wash cells 3 times with PBS.
11. Add 500 μL cold pronase (0.05%) for 10 min or until cells detach (*see Note 7*).
12. Add 100 μL (Complete Mini) enzyme inhibitors and 100 μL bovin serum albumin (1 mg / mL).
13. Transfer cells into a microtube in ice.
14. Rinse the well with 1 mL cold Tris buffer (50 mM, pH 7.4) containing 0.1% BSA and add it to the microtube.
15. Centrifuge cells gently at 500-2000g (depending on the fragility of the cells), discard the supernatant, wash the cell pellet with 1 mL cold Tris buffer (50 mM, pH 7.4) containing 0.1% BSA and centrifuge cells once more at 500-2000g.
16. Skip this step for fluorescence spectroscopy. For flow cytometry analysis, discard the supernatant, add 400 μL

**Stop here for flow
cytometry**

PBS and proceed for analysis of at least 20,000 cells for example with a FACSCalibur flow cytometer.

16. Alternatively, for fluorescence spectroscopy, discard the supernatant and add 200 μ L cold lysis solution (*see Note 8*).
17. Sonicate the cell pellet in the microtube in a cold ice-water bath for 15 min.
18. Centrifuge 10 min at 16,000g and collect the supernatant.
19. Monitor the fluorescence intensity, for example with a MOS 200M fluorimeter, in the supernatant at the maximal intensity wavelength of the fluorophore (for example at 525 nm for carboxyfluorescein).
20. Calculate the quantity of internalized peptide using the calibration curve run in parallel (*see Note 9*).

4 Notes

1. If the size of the droplets is inappropriate (not enough ~100 μm diameter droplets), the strength and duration of the gentle vortexing must be adjusted. If there are not enough droplet pairs due to droplet coalescence several parameters can be modified: Increase of lipid and/or chloroform proportions, change of the chains of the lipids.
2. This method can be adapted to any fluorophore (rhodamine, alexa 488 etc...) provided the appropriate controls are performed. For instance, if flow cytometry is going to be used with cells growing in suspension, a quencher specific of the fluorochrome labeling the peptide should be used (for instance trypan blue absorbs the fluorescence emitted by green-emission fluorochromes only). For fluorometry, the choice of detergent for cell lysis should be confirmed by fluorescence assays showing that the detergent permits the recovery of total fluorescence, similarly with or without cell lysates [3].

Fluorescein- or fluorophore-labeled peptides have first to be tested in cytotoxicity assays since it has been reported by many groups that addition of a fluorophore to a peptide sequence may endow the peptide with cytotoxic properties [12, 16–18]. It is therefore necessary to test short-term (1-2 hrs) and long-terms (24-48 hrs) cytotoxicity effects according to the concentration (1-50 μM) of fluorophore-labeled peptides compared to the unlabeled membrane-active peptide. If the labeled peptide has cytotoxicity which is not observed with the unlabeled one, one should consider different internalisation pathways e. g. membrane permeation or a different balance between the internalisation pathways for the labeled and unlabeled peptide.

Regarding the choice of fluorometry or flow cytometry, the two methods can give different information. Flow cytometry is a non-invasive method as is fluorescence microscopy. If one considers that the translocated fluorescent-labeled peptide arrives directly into the cytosol of cells, it can be either free, bound to a macromolecule or

an organelle. Depending on the local concentration or environment of the fluorescein-labeled peptide or its interaction with intracellular molecules, the fluorescence signal can be quenched or decreased. This is not the case with fluorometry for which cells are lysed and all types of intracellular interactions destroyed by the detergent and high salt concentration. Therefore, when comparing different peptides or different conditions for a single fluorescent peptide, using fluorometry measurements that permit absolute quantification of the peptide should be run in parallel to flow cytometry or fluorescence microscopy.

3. Cells in suspension can also be used. In that case, washing steps 8 and 10 are followed by centrifugation (instead of liquid aspiration) at 5-7°C and low speed (500-2000g according to cell fragility) to gather cells as a pellet without mechanical damage. In addition, omit steps 10 and 11 (enzyme treatment). Instead, at step 16 add 0.2% Trypan Blue/PBS to cells in order to quench the remaining free and membrane-bound fluorescein-labeled peptide and detect only intracellular peptide by flow cytometry.
4. Seed cells in wells according to their division time, which is generally between 10 and 30 hrs. When starting the experiment, one million cells should present in each well. Set aside one or more wells in the 12-well plate to check the cell number.
5. Although decreasing the temperature below 10°C alters the fluidity of the membrane lipid bilayer, internalisation by translocation is measured by incubating the fluorescent peptide with cells at 4-10°C, since all energy-dependent endocytosis pathways are inhibited. Furthermore, it has been reported that endocytosis inhibitors have to be extensively studied with each cell line before using them [22]. It is very important to keep cells and solutions at low (4-10°C) temperature during the whole experiment, including flow cytometry analysis. Alternatively, for

fluorescence spectroscopy, it is important to stay at low temperature up to the lysis step 16.

If one wish to study internalisation in general, including endocytosis and translocation, the whole experiment is run at 37°C and pronase can be replaced by trypsin at the step 11. In that case, preliminary experiments are needed to check whether the fluorescent peptide (containing arginyl and lysyl residues) is indeed sensitive to degradation by trypsin (for example degradation can be followed by HPLC and/or mass spectrometry). It is important to use short time (3-5 min) incubation of the peptide with the enzyme to fit the better with the protocol with cells.

6. The incubation is generally determined in kinetic experiments. The plateau of the kinetics is the preferred incubation time since it will give more reproducible results.
7. Pronase is an enzyme working at 4°C. In preliminary experiments check whether the fluorescent peptide is indeed sensitive to degradation by pronase (for example degradation can be followed by HPLC and/or mass spectrometry). It is important to use short time (5 min) incubation of the peptide with the enzyme to fit the better the protocol with cells.
8. The lysis solution contains the detergent NP40 to lyze cell membranes and was found to unaffected the fluorescence signal of the fluorescein-labeled peptide.
9. The amounts of translocated peptide is calculated by comparing the fluorescence intensity of the sample with a calibration curve. The calibration curve is done in parallel of the samples by adding 10 different amounts (1 to 500 pmoles for example) of the fluorescent peptide to one million cells in 200 µL cold lysis solution. Proceed to steps 16 to 19.

References

1. Jiao C-Y, Delaroche D, Burlina F, Alves ID, Chassaing G, Sagan S (2009) Translocation and endocytosis for cell-penetrating peptide internalization. *J Biol Chem* 284:33957–33965
2. Walrant A, Cardon S, Burlina F, Sagan S (2017) Membrane Crossing and Membranotropic Activity of Cell-Penetrating Peptides: Dangerous Liaisons? *Acc Chem Res* 50:2968–2975
3. Illien F, Rodriguez N, Amoura M, Joliot A, Pallerla M, Cribier S, Burlina F, Sagan S (2016) Quantitative fluorescence spectroscopy and flow cytometry analyses of cell-penetrating peptides internalization pathways: optimization, pitfalls, comparison with mass spectrometry quantification. *Sci Rep* 6:36938
4. Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ, Chernomordik LV, Lebleu B (2003) Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J Biol Chem* 278:585–590
5. Derossi D, Joliot AH, Chassaing G, Prochiantz A (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* 269:10444–10450
6. Drin G, Cottin S, Blanc E, Rees AR, Tamsamani J (2003) Studies on the internalization mechanism of cationic cell-penetrating peptides. *J Biol Chem* 278:31192–31201
7. Swiecicki J-M, Bartsch A, Tailhades J, Di Pisa M, Heller B, Chassaing G, Mansuy C, Burlina F, Lavielle S (2014) The efficacies of cell-penetrating peptides in accumulating in large unilamellar vesicles depend on their ability to form inverted micelles. *Chembiochem* 15:884–891
8. Terrone D, Sang SLW, Roudaia L, Silvius JR (2003) Penetratin and related cell-penetrating cationic peptides can translocate across lipid bilayers in the presence of a transbilayer potential. *Biochemistry* 42:13787–13799
9. Bárány-Wallje E, Keller S, Serowy S, Geibel S, Pohl P, Bienert M, Dathe M (2005) A critical reassessment of penetratin translocation across lipid membranes. *Biophys J* 89:2513–2521
10. Moghal MMR, Islam MZ, Sharmin S, Levadnyy V, Moniruzzaman M, Yamazaki M (2018) Continuous detection of entry of cell-penetrating peptide transportan 10 into single vesicles. *Chem Phys Lipids* 212:120–129
11. Persson D, Thorén PEG, Herner M, Lincoln P, Nordén B (2003) Application of a novel analysis to measure the binding of the membrane-translocating peptide penetratin to negatively charged liposomes. *Biochemistry* 42:421–429
12. Walrant A, Matheron L, Cribier S, Chaignepain S, Jobin M-L, Sagan S, Alves ID (2013) Direct translocation of cell-penetrating peptides in liposomes: a combined mass spectrometry quantification and fluorescence detection study. *Anal Biochem* 438:1–10
13. Säälík P, Niinep A, Pae J, Hansen M, Lubenets D, Langel Ü, Pooga M (2011) Penetration without cells: membrane translocation of cell-penetrating peptides in the model giant plasma membrane vesicles. *J Control Release* 153:117–125
14. Gehan P, Kulifaj S, Soule P, Bodin JB, Amoura M, Walrant A, Sagan S, Thiam AR, Ngo K, Vivier V, Cribier S, Rodriguez N (2020) Penetratin translocation mechanism through asymmetric droplet interface bilayers. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 183415
15. Patel LN, Zaro JL, Shen W-C (2007) Cell penetrating peptides: intracellular pathways and pharmaceutical perspectives. *Pharm Res* 24:1977–1992
16. Birch D, Christensen MV, Staerk D, Franzyk H, Nielsen HM (2017) Fluorophore labeling of a cell-penetrating peptide induces differential effects on its cellular distribution and affects cell viability. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1859:2483–2494
17. Cavaco M, Pérez-Peinado C, Valle J, Silva RDM, Correia JDG, Andreu D, Castanho

- MARB, Neves V (2020) To What Extent Do Fluorophores Bias the Biological Activity of Peptides? A Practical Approach Using Membrane-Active Peptides as Models. *Front Bioeng Biotechnol* 8:552035
18. Hedegaard SF, Derbas MS, Lind TK, et al (2018) Fluorophore labeling of a cell-penetrating peptide significantly alters the mode and degree of biomembrane interaction. *Sci Rep* 8:6327
 19. Burlina F, Sagan S, Bolbach G, Chassaing G (2005) Quantification of the cellular uptake of cell-penetrating peptides by MALDI-TOF mass spectrometry. *Angew Chem Int Ed Engl* 44:4244–4247
 20. Seisel Q, Pelletier F, Deshayes S, Boisguerin P (2019) How to evaluate the cellular uptake of CPPs with fluorescence techniques: Dissecting methodological pitfalls associated to tryptophan-rich peptides. *Biochim Biophys Acta Biomembr* 1861:1533–1545
 21. Vasconcelos L, Lehto T, Madani F, Radoi V, Hällbrink M, Vukojević V, Langel Ü (2018) Simultaneous membrane interaction of amphipathic peptide monomers, self-aggregates and cargo complexes detected by fluorescence correlation spectroscopy. *Biochim Biophys Acta Biomembr* 1860:491–504
 22. Vercauteren D, Vandenbroucke RE, Jones AT, Rejman J, Demeester J, De Smedt SC, Sanders NN, Braeckmans K (2010) The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. *Mol Ther* 18:561–569

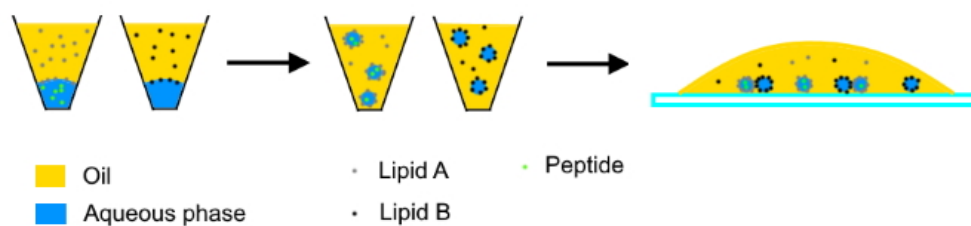


Figure 1: Translocation assay across droplet interface bilayers (see text for details).

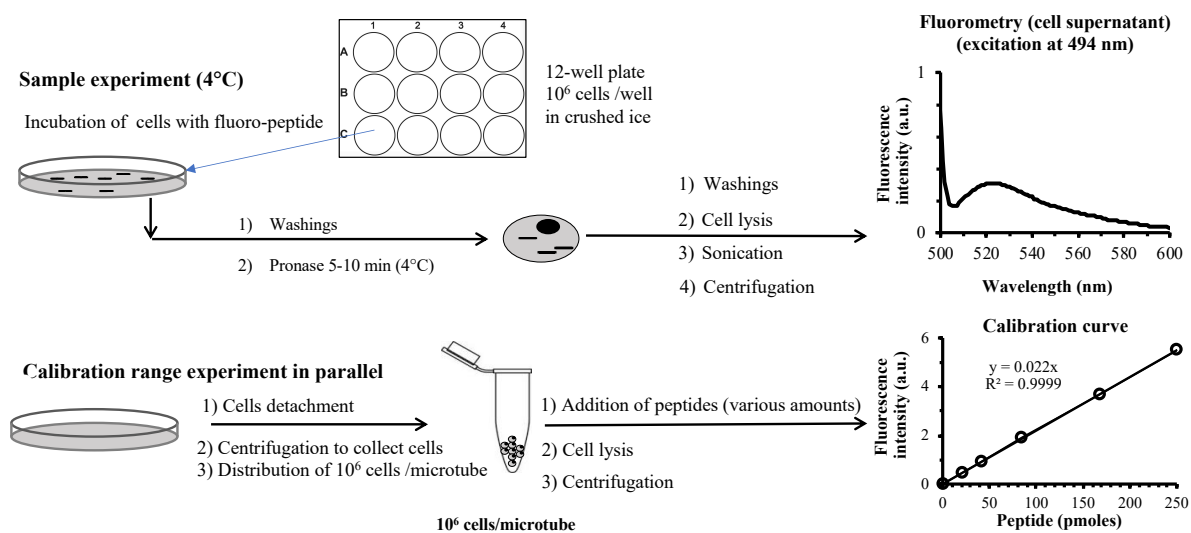


Figure 2: quantitative fluorescence spectroscopy protocol to measure translocation of fluorescein-labeled fluorophore-labeled peptides (see text for details).