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Fluorescent imaging analysis of dextran leakage across large sized pores brain microdialysis probes

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Abstract

Microdialysis (MD) is a minimally invasive diffusion based method for a time continuous sampling of drugs and endogenous compounds present in extracellular fluid and for the drug delivery directly at the site of interest. Large molecular weight cut off (CO) MD is a new area of application of MD focused on sampling large biomolecules e.g. cytokines and other proteins. Large CO probes have a high risk of the convective transmembrane transport that would lead to a not negligible perturbation of the external microenvironment. One way to inhibit the convective flow is to adjust the transmembrane pressure by adding dextrans as osmotic agents to the perfusion fluid. It is crucial that when dextrans are used they do not leak out into the tissue since it would alter again the sampling microenvironment and might cause physiologial responses of the organism. The declared by the constructor CO seems from the previous studies to be not reliable to predict the behavior of different type molecules. Therefore, the behavior of dextrans is important to study. This project performed a comparison of leakage of different molecular weight dextrans (40 kDa, 70 kDa, 250 kDa, 500 kDa) through the large sized pores membrane (100 kDa CO) of the brain MD probe. This was done by fluorescent imaging analysis, in the attempt to validate the fluorescence microscopy (FM) as a tool for qualitative and quantitative study of the MD. A MD probe holder device was designed and manufactured with micromachining techniques, mainly up of polidymetilsyloxane and glass. Qualitative image analysis showed MD process and MD probe details. A method for quantitative imaging analysis was developed. The method consisted in use of Matlab code to perform the sum of pixel brightness values over the normalized image of dextrans leakage pattern. The measurement resulted to be repeatable. Probes of the same type resulted to have different leaking properties. Although the MWCO of the membrane was

100 kDa some of 250 kDa dextrans leaked out anyway. There was no leakage of 500 kDa dextrans. Concentration loss during the perfusion was also assessed from pictures of inflow and outflow tubes and was consistent with leakage data.

Estratto

Microdialisi (MD) è un metodo ben affermato per il campionamento a tempo continuo di farmaci e composti endogeni dal fluido extracellulare. Inoltre MD è usata per somministrare farmaci direttamente in situ. MD consiste nell'inserimento nel sito di interesse di un catetere costruito in modo da imitare un capillare sanguigno. Il principio di lavoro ideale di questo metodo minimamente invasivo è il trasporto transmembrana esclusivamente di tipo diffusivo; cioè senza perdita o guadagno di fluido durante la perfusione. L'uso tradizionale della MD si focalizzava sul campionamento di piccole molecole idrofiliche come glucosio, glutammato, lattato, urea da differenti matrici biologiche. Con l'introduzione di membrane di MD con peso molecolare di cut-off di 100kDa e superiore nuovi studi, orientati a sviluppare metodi di utilizzo di MD nel campionare grandi biomolecole come citochine e altre proteine, sono stati attivati. Un esempio di applicazione della MD ad alto CO è il monitoraggio neurochimico del cervello in pazienti in terapia neurointensiva. Le membrane con alto cut-off hanno un alto rischio di perturbazione del microambiente esterno a causa di trasporto transmembrana di tipo convettivo. Un modo per inibire il flusso convettivo consiste nel regolare la pressione transmembrana aggiungendo nel fluido perfusore molecole di dextran con funzione di agente osmotico. E' importante preservare i dextran dalla dispersione verso il tessuto per evitare l'alterazione del microambiente di analisi e possibili risposte fisiologiche dell'organismo. Studi precedenti mostrano che il cut-off dichiarato dal produttore non sembra predire in modo affidabile il comportamento di trasporto di varie molecole attaverso la membrana. Per questa ragione è importante studiare il comportamento dei dextran. Un obiettivo del seguente progetto è stato quello di studiare la dispersione dei dextran di diverso peso molecolare attraverso la membrana del catetere di microdialisi cerebrale con

grande cut-off verso il microambiente esterno. Lo studio è stato fatto attraverso l'analisi di fluorescence imaging nel tentativo di validare la microscopia a fluorescenza come strumento per studio qualitativo e quantitativo della micordialisi. Al momento, infatti, nessuno ha applicato la microscopia a fluorescenza alla microdialisi. Per farlo, un dispositivo per il contenimento del catetere è stato progettato e costruito con tecniche di micromachining. Polidimetilsilossano (PDMS), materiale molto usato nella fabbricazione dei chip microfluidici, e vetro sono stati i principlali materiali usati. L'analisi qualitativa delle immagini ha mostrando i dettagli del processo di microdialisi e del catetere. Un metodo di analisi quantitativa per confrontare la capacità di dispersione dei dextran di diverso peso molecolare attraverso la membrana di MD con il cut-off di 100 kDa è stato sviluppato ed applicato. Il confronto è stato fatto tra dextrans di seguente peso molecolare: 40 kDa, 70 kDa, 250 kDa, 500 kDa. La valutazione delle immagini è stata fatta per mezzo di un codice Matlab che ha effettuato la somma dell'intensità luminosa di ogni pixel. Le immagini sono state opportunamente normalizzate per fare in modo che i dati contenessero solo informazioni relative alla capacità di dispersione attraverso la membrana e non altre informazioni variabili durante gli esperimenti (p. es. concentrazione, parametri ambientali). La misurazione è risultata essere ripetibile. Seguenti conclusioni sono state fatte. Dispositivi MD dello stesso tipo possono avere capacità di dispersione verso l'esterno diversa. Nonostante il cut-off della membrana è stato di 100 kDa alcuni dextran di 250 kDa sono stati lo stesso dispersi all'esterno. Nessuna dispersione invece è stata rilevata per i dextran di 500 kDa. Una stima di riduzione di concentrazione dei dextran nel liquido di dialisi rispetto a quello nelliquido perfusore è stata fatta dalle immagini dei tubi di ingresso e di uscita. Tale stima è risultata di essere consistente con i risultati di dispersione dei dextran.

1. Introduction

1.1 What is Microdialysis

Microdialysis (MD) is a well established method for a time continuous sampling of drugs and endogenous compounds present in extracellular fluid and for the drug delivery directly at the site of interest. MD is the only technique available that explicitly provides time resolved data on the extracellular space [**Chaurasia et al., 2007**]. MD has become one of the most important and promising in vivo method and has been exploited and developed for more than 20 years in clinic and research.

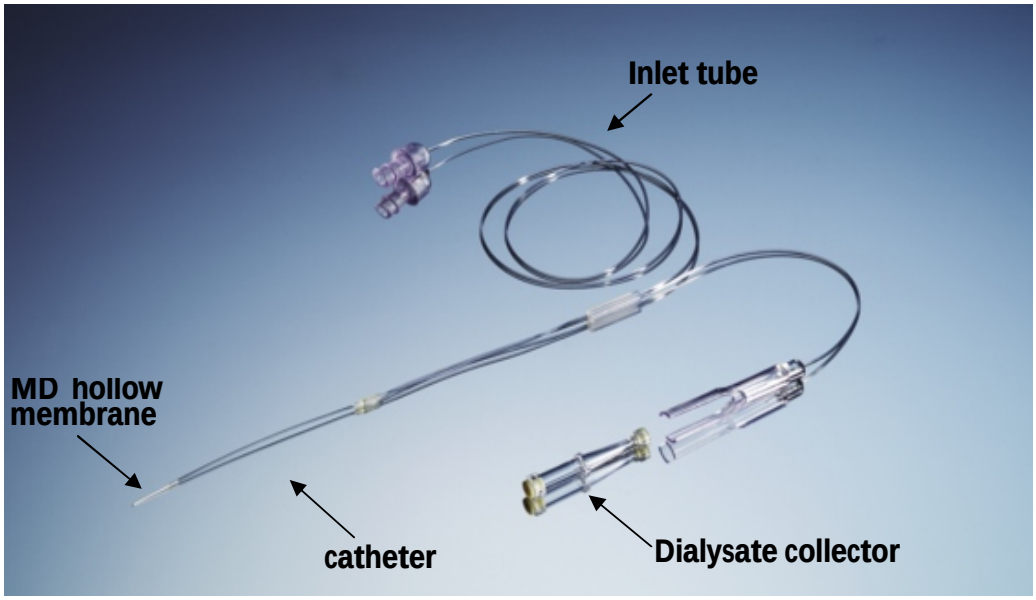


FIG. 1.1. | *Microdialysis kit. It consists of inlet tubing perfused by a perfusion fluid, an MD catheter implanted into the tissue matrix of interest and a microvial where the dialysate is collected*

The MD probe consists of inlet tubing perfused by a perfusion fluid, an MD catheter implanted into the tissue matrix of interest and a microvial where the dialysate is collected (**Figure 1.1.1**). The catheter is a structure made of two concentric tubes (see the **Section 1.2**). The outer tube is connected at one side with the inlet tube while the other side of it ends with a hollow semipermeable membrane sealed at the tip. The inner tube is connected at one side with the outlet tube while the other side, unsealed, reaches the tip of the catheter allowing for the dialysate fluid to flow out of the probe.

The MD membrane on the catheter mimics a body capillary with regard to the exchange of compounds processes. The ideal work condition of the MD is to sample the outer microenvironment only through the diffusive transport; without any loss of body fluid, and to remove only small quantities of analytes from the tissue environment, that its concentration remains in first approximation constant. In this way the measurement preserves the physiology of the tissue where the catheter is implanted in. Accordingly, MD could potentially be used in any animal or human tissue. Indeed, MD was first used in brain tissue [**Ungerstedt et al., 1974**] but its application has widely spread on other organs and tissues such as eyes [**Atluri, H., Mitra, A. K., 2003**], bones [**Stolle et al. 2003**], heart [**Mantovani et al., 2002**], liver [**Nowak et al., 2002**], skin [**Blunk et al., 2004**] and many other tissues [**Plock et al., 2005**].

An important field of application of MD is the monitoring of severely injured patients, e.g brain trauma patients [**Vespa et al., 2003; Marklund et al., 2001; Plock et al., 2005**]. MD has also been used for the simultaneous pharmacokinetics studies at multiple sites, e.g in both brain and blood or brain and bile in rats [**Tsai et al., 2000; Cheng et al., 2002; Tsai et al., 2003**]. Besides the therapeutic application, this technique might provide useful data to enhance the understanding of the physiology and pathophysiology of tissues and of a whole organism [**Plock et al., 2005**]. The use of MD is not restricted to

in vivo applications, it has been applied to different in-vitro systems including cells cultures. [**Maas et al., 1992, Stenken et al., 2003**].

Several alternative techniques are available for the determination of tissue compounds concentrations such as blood sampling, biopsy, saliva sampling, ultrafiltration [**Plock et al., 2005**]. However, the distinguishing features of MD offer excellent advantages over the other sampling techniques relatively to a specific purpose. A summarized comparison between different techniques is shown in **Table 1.1**. The ultrafiltration technique is very similar to the MD but with the difference that it is based on the convective transmembrane transport and not only on diffusion. The convection generates a bulk movement of the tissue fluid into the perfusate. Such continuous loss of fluid makes the technique less appropriate for continuous long time sampling. This statement is particularly valid on small animals such as mice and rat, which have small body fluid volumes. The possibility to operate directly in the tissue of interest is not offered by blood or saliva sampling. They present high probability of the over- or under-estimation of analytes at the site of interest [**Lee et al.,1996; Muller et al., 1998a**]. The capacity to differentiate between the intracellular, extracellular, intravascular spaces is not offered by the biopsy that often analyzes the sample after a homogenization processing. Moreover, the biopsy does not allow for the continuous sampling at the same site.

TABLE. 1.1 | *Comparison between different sampling techniques where "1" means apply and "0" means not apply.*

	MD	Biopsy	Blood	Saliva	Ultrafiltration
Collection at site of action	**1**	**1**	**0**	**0**	**1**
Delivery to target tissue	**1**	**0**	**0**	**0**	**0**
No fluid loss	**1**	**0**	**0**	**0**	**0**
No or minimal invasiveness	**1**	**0**	**0**	**1**	**0**
Simple sampling technique	**0**	**1**	**1**	**1**	**1**
Continuos sampling at the same site	**1**	**0**	**0**	**1**	**0**
Multiple site sampling	**1**	**0**	**0**	**0**	**1**
No calibration necessary	**0**	**1**	**1**	**1**	**1**
Differentiation between different tissue compartments	**1**	**0**	**-**	**-**	**1**

MD has also important limitations. The continuous flow of the anayte-free perfusion fluid inside the probe makes it impossible to attain thermodynamic equilibrium between the outer and the inner environments. For this reason, the relative recovery that is the ratio between the analyte concentration in the dialysate ($\boldsymbol{C}_{\mathbf{d}}$) and that in the external microenvironment ($\boldsymbol{C}_{\mathbf{e}}$), never reaches the value of 1. In general, a lower flow rate ($\boldsymbol{F}$) generates a higher relative recovery

(***RR***), as shows the **Equation E1**, derived in case of no flow loss/gain across the membrane [**Li et al, 2007**]

$$RR = 1 - \exp\left(-\frac{kA}{F}\right) \qquad \textbf{[E1]}$$

$$RR = \frac{C_{\mathrm{d}}}{C_{\mathrm{e}}} \qquad \textbf{[E2]}$$

where **A** is the surface area of the membrane and **k** is the mass transport coefficient [**Plock et al., 2005; Kjellstrom et al., 1998**].

In order to assess the actual analyte concentration of the outer environment a calibration technique should be applied to the probe before to sampling.

Several calibration methods have been developed. In the "method of flow rate variation" the in vivo procedure is accomplished plotting the flow rate **F** against **Cd** and extrapolating to the zero flow using **Equation E1, Equation E2** [**Jacobson et al., 1985**]. This calibration method requires the knowledge of the mass transfer, in tissue. Another method that does not require the presumptions about **k** is the method of no-net-flux [**Lonnroth et al., 1987; Chaurasia, 1999**]. This in vivo calibration technique uses interpolation. Different analyte concentrations are added to the perfusate. If $C_{\mathrm{e}} > C_{\mathrm{perfusate}}$ then $C_{\mathrm{dialysate}} - C_{\mathrm{perfusate}} > 0$. If the concentrations C_{e} and $C_{\mathrm{perfusate}}$ are vice versa then $C_{\mathrm{dialysate}} - C_{\mathrm{perfusate}} < 0$. In case of $C_{\mathrm{dialysate}} - C_{\mathrm{perfusate}} = 0$ the $C_{\mathrm{dialysate}}$ value exactly represents the actual analyte concentration in the external environment independently on the flow rate. By plotting the $C_{\mathrm{dialysate}} - C_{\mathrm{perfusate}}$ against $C_{\mathrm{perfusate}}$ and interpolating it is possible to extract $C_{\mathrm{perfusate}}$ value corresponding to $C_{\mathrm{dialysate}} - C_{\mathrm{perfusate}} = 0$. The obtained $C_{\mathrm{perfusate}}$ value corresponds to the actual analyte concentration C_{e}. Other in vivo calibration techniques have been

proposed such as that uses an endogenous substance as a reference for the relative recovery value. This technique requires the actual concentration in the tissue site of the reference substance to be known. Urea has been used for this scope in several studies [**Deguchi et al, 1991; Strindberg, L., Lonnroth, P., 2000**]. Instead of measuring the recovery of an endogenous substance the Internal Standard Method estimates loss of the compound added to the perfusate by measuring it Relative Loss (RL). The RR is then give by RL:

$$RR = RL = \frac{C_p - C_d}{C_p} \qquad \textbf{[E3]}$$

Where C_p and C_d are perfusate and dialysate concentrations respectively.

In brain MD the cerebrospinal fluid may be sampled from the extravascular brain compartment. This actual study is focused on large sized (100 kDa molecular weight cut off) brain MD probes. From the very beginning, low cut-of membranes (<20 kDa) has been used to sample small molecules [**Chefer et al., 2009**]. The availability of large cut off probes opened new areas of study leading to an increasing interest in the exploitation and optimizing of the large MWCO microdialysis [**Dahlin et al.; Clough et al., 2005;Wang et al., 2006;Stenken et al., 2005; Rosenbloom et al., 2005; Trickler et al., 2003;Li et al , 2007**].

1.2 Principle of Microdyalisis and use of the Osmotic Agents

In our MD probe the catheter consists of an inner tube surrounded by a semipermeable hollow membrane. The outer diameter of the hollow membrane is 590 µm. **Figure 1.2.1** could help to visualize the MD process.

The perfusion fluid is pumped through the inlet tubing by a syringe pump. It reaches the membrane where the exchange of the molecules between the perfusate and the tissue microenvironment occurs. Only the molecules with the molecular weight (MW) lower than the molecular weight cut off (MWCO) of the membrane pass through in the sense of the following definition. MWCO is the molecular weight at which 80% of the molecules are prevented from passing through the dialysis membrane [**CMA, www.microdialysis.se**]. The dialysate is then collected in a microvial.

The perfusate composition plays an important role in the transport through the membrane process. In general, it mimics the outer microenvironment in order to avoid high osmotic pressure differences between the perfusate and the external fluid, which would generate a high loss of the perfusion fluid [**Plock et al., 2005**]. As mentioned earlier the ideal work condition of MD is the molecules exchange exclusively through the diffusive transport. In such a case, no bulk fluid movement through the membrane could occur, leading to 100% fluid recovery, i.e. the pumped flow equals the dialysate flow. This feature gives to the MD most of the advantages described in **Table 1** over the alternative techniques. Nevertheless, it is not so straightforward to achieve 100% diffusive transport since other transport processes, such as convective transport caused by hydrostatic and osmotic pressures differences, are

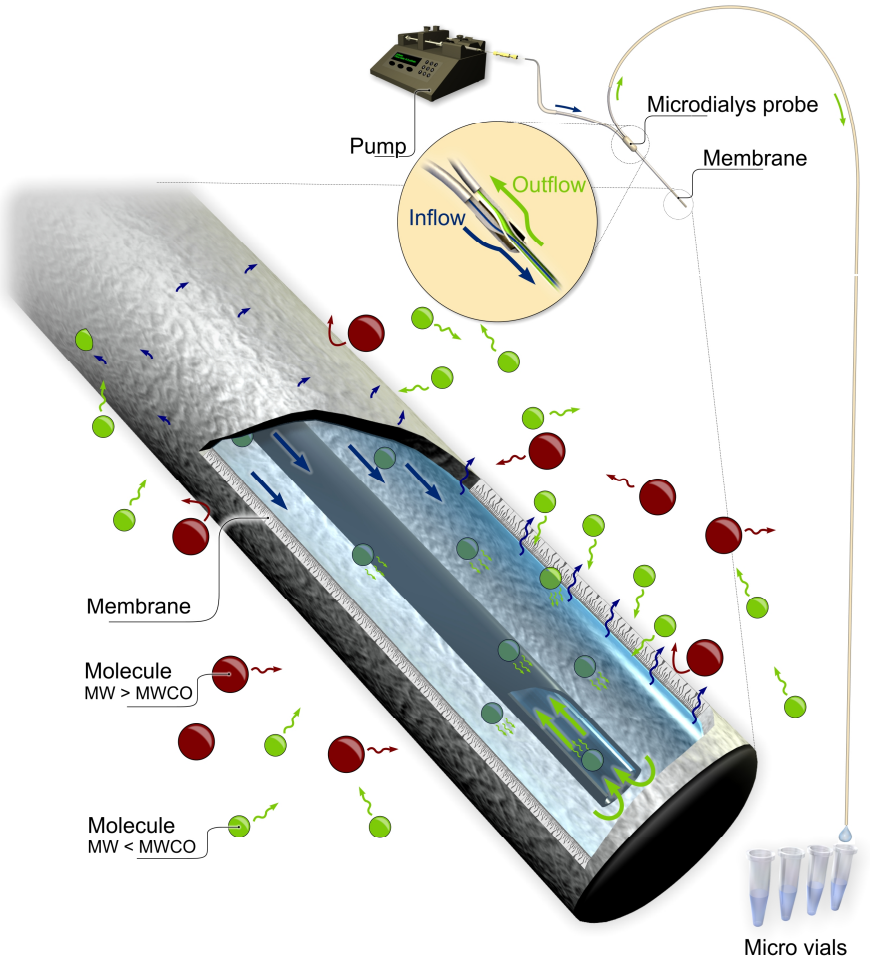


FIG. 1.2.1 | *Microdialysis detailed schematic. The perfusate flows in contact with the membrane (blue arrows). The thin blue arrows show the fluid loss in case of positive transmembrane pressure. The molecules with MW<MWCO are sampled from the external microenvironment and carried out of the probe by the central tubing unsealed at the tip (green arrows). The dialysate is then collected into the microvials*

generally involved in the MD as well. This difficulty is even higher in the large sized pores MD membranes (≥ 100KDa MWCO) with respect to the traditional low MW cut off probes (≤ 20KDa MWCO) since the resistance on the transmembrane fluid flux is lower. The inhibition of unwanted convective processes is possible by adding osmotic agents (OA) that regulate the osmotic pressure of the perfusion fluid, but there are more ways of controlling the fluid convective processes [**Trickler et al., 2003, Li et al., 2007, Rosenbloom et al., 2005**].

Diffusion.

The transmembrane concentration gradient of the molecules with MW<MWCO is maintained different from zero by the continuous flow of the perfusate. This condition generates a continuous diffusion of such molecules through the membrane. The analytes move into the perfusion fluid from the tissue microenvironment. In case of drug delivery they move out of the perfusion fluid to the tissue. No bulk movement of fluid occurs. The diffusive solute transport in an ideal solution is described by Fick's first law:

$$\vec{F} = -D \cdot \mathrm{grad}(C) \qquad \textbf{[E4]}$$

The diffusion flux vector $\vec{F}$ ($mol \cdot m^{-2} \cdot s^{-1}$) is the amount of solute moving through a unit area in a unit time interval. C ($mol \cdot m^{-3}$) is the concentration of solute at point at certain spatial position. D ($m^2 \cdot s^{-1}$) is the diffusion coefficient of the solute (or diffusivity). According to the Einstein-Stokes equation for non charged spherical particles it is approximately:

$$D = \frac{k \cdot T}{6 \cdot \pi \cdot \eta \cdot r} \qquad \textbf{[E5]}$$

where T (K) is the absolute temperature, η (Pa·s) is the viscosity of the medium the solute is suspended in, r is the particle radius (m), k is the Boltzmann constant. In case of microdialysis the substances diffuse across three distinct medias i.e. tissue, MD membrane, perfusate, having different diffusivities. An expression of diffusive flux (toward radial direction) that allows for the consideration of diffusive transport of analyte molecules across the three media is presented in **Equation E6** [**Friedman, 1986**] :

$$F = -\frac{\Delta_C}{R_a + R_b + R_c} \qquad \textbf{[E6]}$$

where the Δ_C is the overall concentration difference across the three media and R_i are the resistances to diffusion for the three media. For the membrane, $R = K_m^{-1}$ is the inverse of the permeability K_m of the membrane. The membrane permeability is in general an empirical property that could be found by measuring the flux F_m across the membrane caused by a concentration difference Δ_{Cm} between the two sides of it i.e. $K_m = F_m / \Delta_{Cm}$. For the other two media $R_i = \delta_i / D_i$ is the radial thickness δ_i of the i-th medium, across which the diffusion occurs, divided by its diffusion coefficient as comes from the **Equation E4** [**Friedman, 1986**]. Several models have been proposed to describe the analyte transport occurring during MD [**Chefer et al., 2009, Bungay et al 1990**]. These models enrich this elemental description, taking into account the physiological processes of the tissue of interest such as metabolic processes, transport between the extracellular fluid and plasma, transport between the extracellular space and intracellular space [**Bungay et al., 1990**].

Ultrafiltration.

The transmembrane pressure difference ***TMP*** enforce a convective transport through the membrane leading to a net gain (for negative ***TMP***) or loss (for positive ***TMP***) of fluid across it. If the composition of the perfusion fluid is the

same as that of the external fluid then the ***TMP*** is the difference between the pressure built up by the pumping inside the probe and the external hydrostatic pressure. Several pumping methods exist including push, pull-push and pull [**Li et al., 2007**]. In the push method the perfusate is pumped by a syringe pump. In case the pressure of the external fluid is the atmospheric pressure (***P_{atm}***) this method raises the pressure inside the probe above P_{atm} leading to a positive ***TMP***. In the pull method the dialysate is pulled by a pump leading to a negative ***TMP*** instead. Both pumping methods are used simultaneously in the so called push-pull method. In all pumping methods the pressure damps along the axis of the hollow membrane consistently with the hydraulic theory. The flux loss ***F*** ($L \cdot m^{-2} \cdot min^{-1}$) across the membrane could be estimated with the **Equation E7** [**Li et al., 2007**]:

$$F = \frac{TMP}{\eta \cdot R} \qquad \textbf{[E7]}$$

where fluid loss is directly proportional to the ***TMP*** and inversely proportional to the hydraulic resistance ***R*** ($m^2 \cdot L^{-1}$) and viscosity of the fluid η (Pa·min).

The push-pull method could reduce the fluid loss/gain by conveniently adjusting the pressure inside the probe [**Li et al., 2007**]. However, a zero ***TMP*** could be achieved only at one section of the hollow membrane since the pressure is not constant in the hollow membrane axis direction.

In case of push the method, the ***TMP*** could be tailored by adding Osmotic Agents in order to achieve no net fluid loss [**Trickler et a., 2003; Li et al., 2007**]. These agents regulate the osmosis process across the MD membrane. The driving force of the osmosis process is the transmembrane difference in osmotic pressure (Π), Δ_{Π}. In case of no transmembrane hydrostatic pressure present the fluid moves to the zone of higher Π to reach equilibrium. The osmotic pressure is a colligative property of a solution. Therefore, it depends on the quantity of

the solute dissolved in the solution and not on the chemical nature of it. According the Morse equation the osmotic pressure is:

$$\Pi = i \cdot C \cdot R \cdot T \qquad \textbf{[E8]}$$

where C is the molar concentration, R is the gas constant, T the absolute temperature of solution, i is the Van't Hoff factor, that is the ratio between the concentration of particles produced in the solution after the dissociation of the solvent and the concentration of the solvent as calculated from mass. For instance, for NaCl that dissociates in water in two ions, $i = 2$.

If the perfusate is pure water the osmotic pressure of the perfusate is lower than that of the tissue fluid generating the loss of the perfusate fluid if no transmembrane hydrostatic pressure is present. In order to lower this osmotic pressure differences the perfusate used in the MD is in general a Ringers' solution containing different salts that mimics the tissue environment. Moreover, by adding specific molecules that could further raise the osmotic pressure it is possible to shift ***TMP*** to have zero net flow across the membrane [**Li et al., 2007; Rosenbloom et al., 2005**] :

$$F = \frac{TMP - \Delta_{\Pi}}{\eta \cdot R} \qquad \textbf{[E9]}$$

where ***TMP*** is the TM pressure before to add the OA. If $\Delta_{\Pi} > TMP$ there would be an inward ultrafiltration even in case of push pumping method [**Li et al., 2007**]. It is important to notice that the damping over the axis of the hollow MD membrane of the osmotic pressure is generally not equal to that of the hydrostatic pressure. Therefore, by adding of the OA, the transmembrane flow of the perfusion fluid can not, in general, be avoided totally even in the presence of 100% flow recovery. Indeed, at one section of the hollow membrane there could be an outward ultrafiltration and at another section an inward

ultrafiltration. It is also worth to note that by using the OA a net zero flux has been achieved [**Trickler et al., 2003**].

Other effects could be present in the transport through the membrane process (e.g. electric migration). Such effects are negligible for what concerns this actual work.

1.3 Aim of project.

It is crucial that the osmotic agents used do not leak out to the tissue since it would alter the sampling microenvironment and might cause allergic responses of the organism [**Dahlin et al., 2010**]. Several different osmotic agents have been used in MD including albumin and dextrans [**Rosenbloom et al., 2005; Trickler et al., 2003**] but also micro beads [**Pettersson et al., 2004**]. Different studies demonstrated the declared MWCO could be not useful for determining the size of molecules that will reliably pass through the probe membrane [**Schutte et al., 2004; Rosenbloom et al., 2005**]. At present, no one has studied the leakage behavior of different molecular weight dextrans.

Fluorescent microscopy is a well established technique mostly exploited in cell and molecular biology. It is based on selective imaging of the objects of interest, that are required to fluoresce. To our understanding this microscopy technique has not been applied to study the microdialysis process yet and could therefore provide useful information about the MD process.

The aim of this project was to validate the fluorescence microscopy as a tool for qualitative and quantitative study of the MD process and to study the different molecular weight dextrans behavior relatively to the leakage through large sized pores membrane of MD probes. Therefore a method for quantitative fluorescent imaging was developed. In order to apply the fluorescence microscopy to MD a probe holding device was designed and fabricated. This was the first attempt of this group for a quantitative fluorescent imaging study of the MD.

2. Experimental set-up

The MD probe (CMA Microdialysis, Solna, Sweden) was inserted into the chamber of the probe holding device (**Figure 2.1**). The microdialysis was performed using a push method with a syringe pump Harvard PHD 2000 (Harvard Apparatus, Holliston, MA, USA). The device was placed on the mechanical stage of a Nikon inverted fluorescence microscope "Eclipse TE2000-U" (**Figure 2.2**). The filter set (filters block) used in all the experiments was the Nikon "B-2E/C FICT". The B-2E/C block represents the correct choice relatively to the fluorophores used in all the experiments [**Nikon, http://www.microscopyu.com/**]. A 100 W high pressure mercury arc lamp (HBO OSRAM 103 W/2) was used as excitation light source. FICT (fluoresceinyl isothiocyanate) dextrans, purchased from Sigma Aldrich (St.Louis, MO, USA) were added into the perfusion fluid. The perfusion fluid consisted of an in-house made aqueous solution of 147 mM NaCl, 2.7 mM KCL, 1.2 mM $CaCl_2$ and 0.85 mM $MgCl_2$, (all chemicals from Sigma Aldrich, St. Louis, MO, USA). The declared maximum of the excitation spectrum of FICT-dextran was 490 nm (**Sigma Aldrich, 1997**). The declared emission maximum was 520 nm. The filter block was composed of the excitation filter, dichroic mirror and a barrier filter. The excitation light coming from the lamp was filtered by the excitation filter having its pass band (465-495 nm) matched with the excitation spectrum of the fluorophore. The transition from reflection to transmission of the dichroic mirror resided between the emission and the excitation maxima of the fluorophore; at 505 nm. The barrier filter was a band pass filter transmitting only the emission light (515-555 nm). Two objectives with different magnification M and numerical aperture NA were used. A cooled CCD camera (Nikon E4500, Tokyo, Japan) was used to transduct the image that

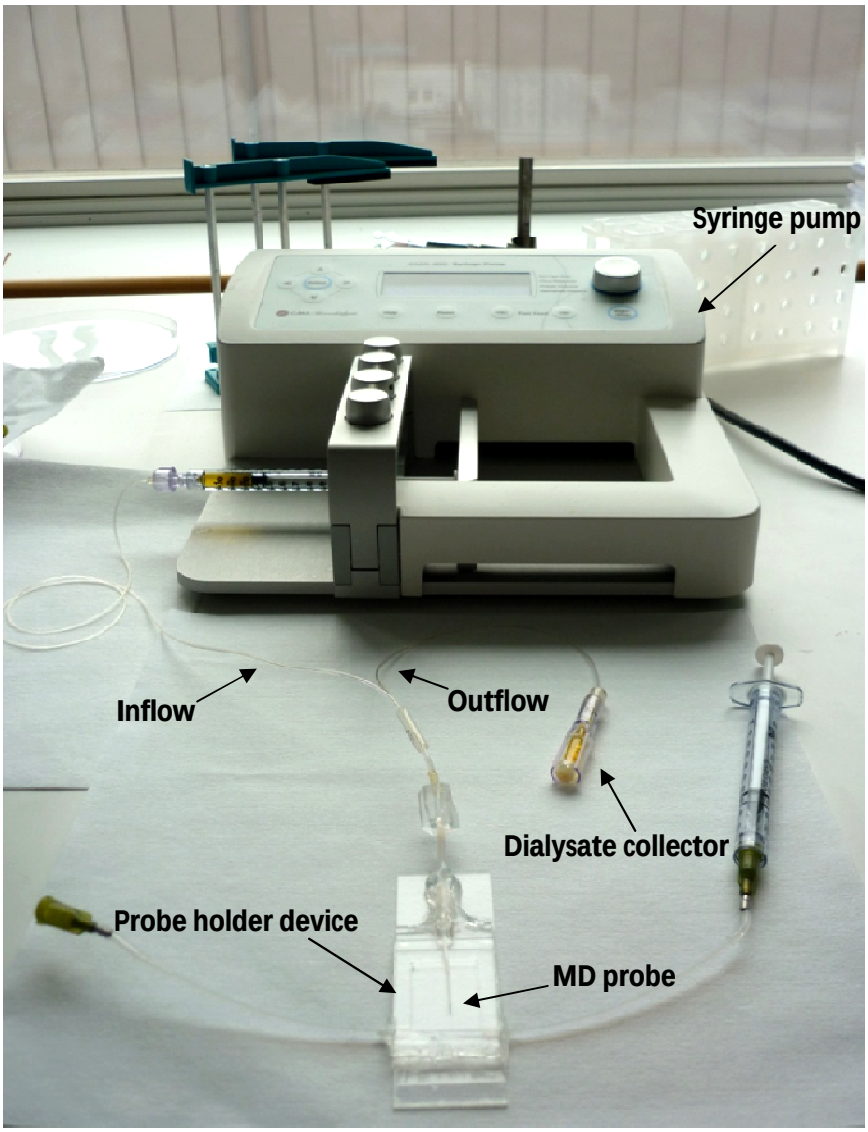


FIG. 2.1 | *Push pumping method. The picture shows a CMA syringe pump, often used for cleansing of the membranes after perfusion, and the holder device fabricated within this project. Notice the MD-probe inside the chamber of probe holder device.*

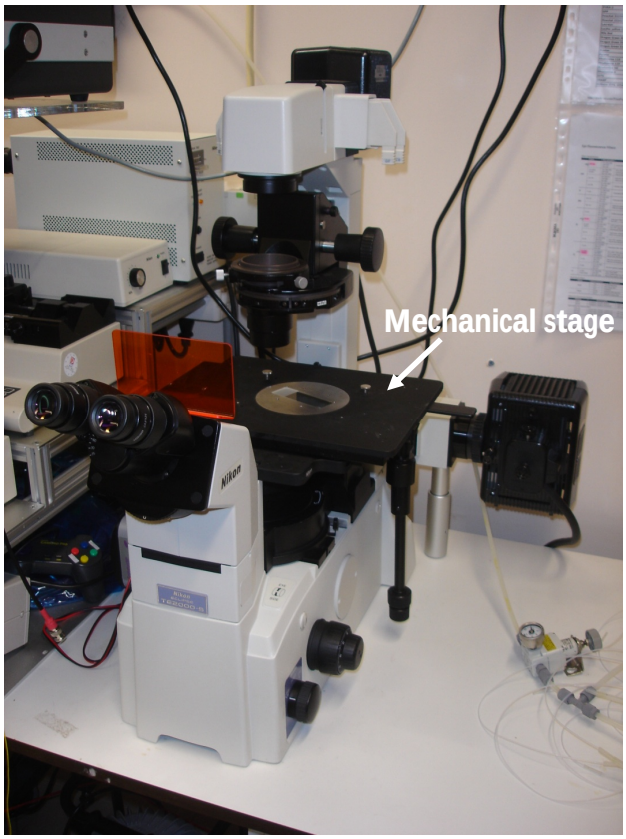


FIG. 2.2 | *Nikon epi-fluorescence Eclipse TE2000-U microscope. Mechanical stage where the device holding the MD probe was placed is shown. The acquired data was processed by a personal computer interfaced to the microscope.*

was then delivered to a personal computer for storage. The experimental procedures regarding the quantitative analysis are discussed in the **Section 5**.

3. Probe holder device fabrication

The purpose of the holder device was to contain the microdialysis probe while being processed by the fluorescent microscope. The functional specifications were:

1. **Mechanical specifications.** The device should be steadily coupled to the mechanical stage of the microscope.
2. **Optical specifications.** The device should suit the imaging purpose. Distorted images of the probe could be generated in case of an improper device.
3. **Fluidic specifications.** No leaking is tolerable. Moreover, the filling process introduces air bubbles into the chamber that could alter the image. Therefore, the bubbles should be easily removable. The device should allow also for the effective cleansing.
4. **Fabrication specifications.** An easy processing is preferred.

Requirement 2 constrained the chamber to have two optically transparent plane faces. Two normal microscope glass slides were used for this scope. This choice fitted also the specification 1 since the microscope glass slides could be steadly coupled to the mechanical stage of the microscope in excellent manner. It was decided to use the cross-linked **PDMS** (Polydimethylsiloxane) (Wacker-Chemie GmbH, München, Germany) as the other main material of the chamber. The PDMS is a well established material in the Lab-On-Chip fabrication techniques. It allowed an easy and rapid processing since no clean room techniques were

needed. Such a choice fitted the requirement 4. Several attempts to construct and validate the device lead to the final project shown in **Figure 3.1**.

The morphology of the chamber and the position of the channels were designed to fit the fluidic functional specification. The channel for the MD-probe insertion was located in the middle of one side of the chamber. The input and output channels for the filling of the chamber were placed on the corners. The number and position of the channels was particularly favorable for an easy filling; leading to no visible air bubbles inside the chamber and an easy cleansing procedure. The top and the bottom are glass slide as discussed before. The thickness of the chamber should be small enough to collect toward the in-focus plane all the FICT-dextrans leaked in all the possible directions over 360°. But obviously it should be not too thin so to be sure the hollow probe is not throttled.

In order to prepare the cross-linked PDMS one part of curing agent (Elastosil RT 601 A) was mixed with 9 parts the elastomer base (Elastosil RT 601 B) weight to weight. The fluid was placed in -20°C freezer for 30 minutes in order to allow the air bubbles to come to the top of the mixture. The bubbles were then removed by a nitrogen gas stream. In order to create channels plastic wires were inserted into plexiglass blocks conveniently arranged on the Petri dish. Then, the liquid PDMS was cast into the dish. The structure was placed again in the -20°C freezer for 30 minutes and the top bubbles were removed. The structure was then cured for 40 minutes in a convective oven at 70°C . The solidified cross-linked PDMS was removed then from the Petri dish (the solidified PDMS act mechanically as an elastic solid, similar to the rubber). Subsequently, in order to create the channels, the plastic wires were removed from the so prepared structure. Afterwards, a rectangular frame was cut out from the channeled PDMS slice with a surgical blade (BS 2982, Swann Morton LTD, Sheffield, England).

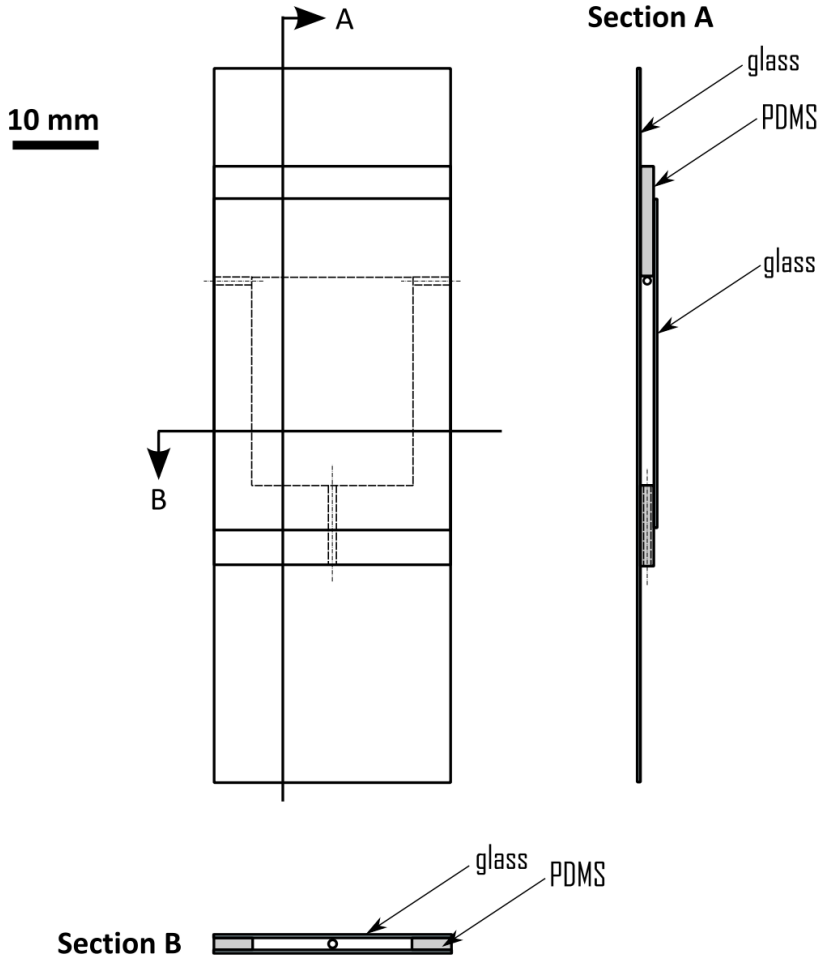
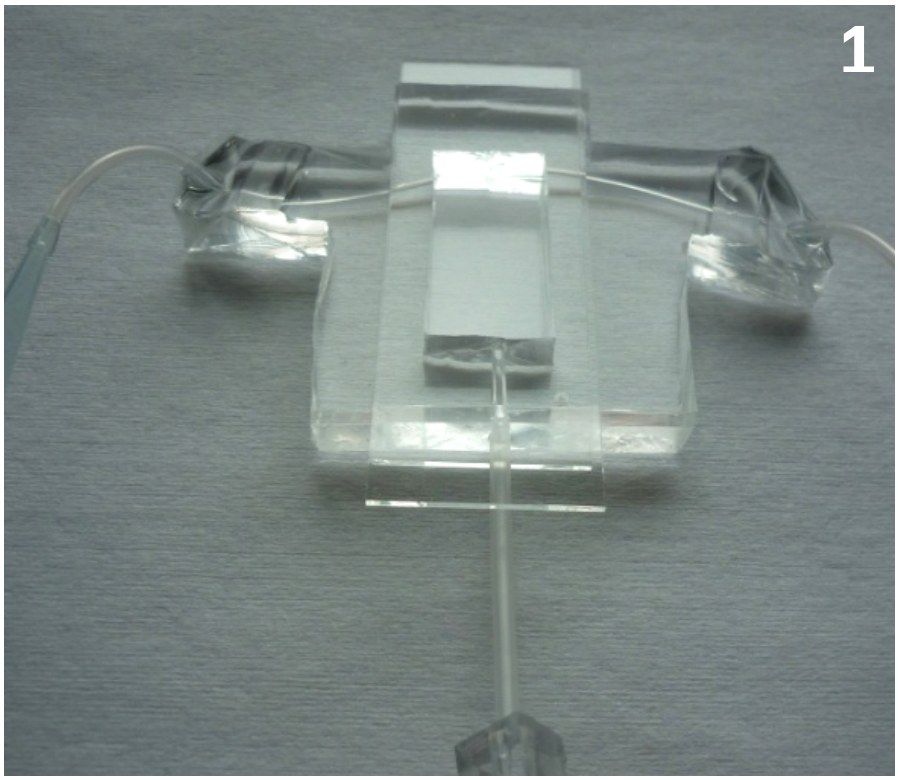
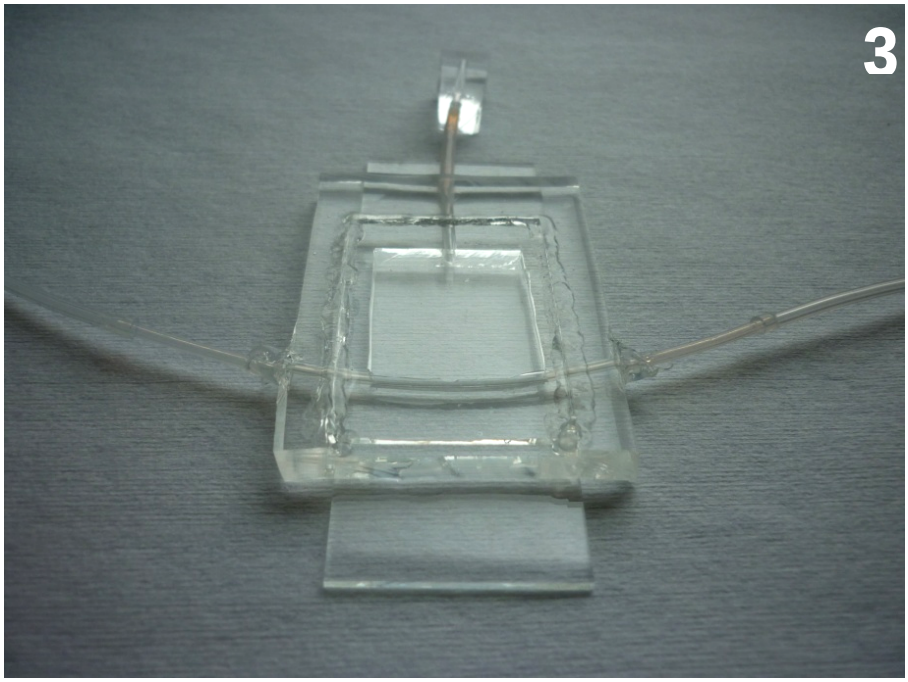


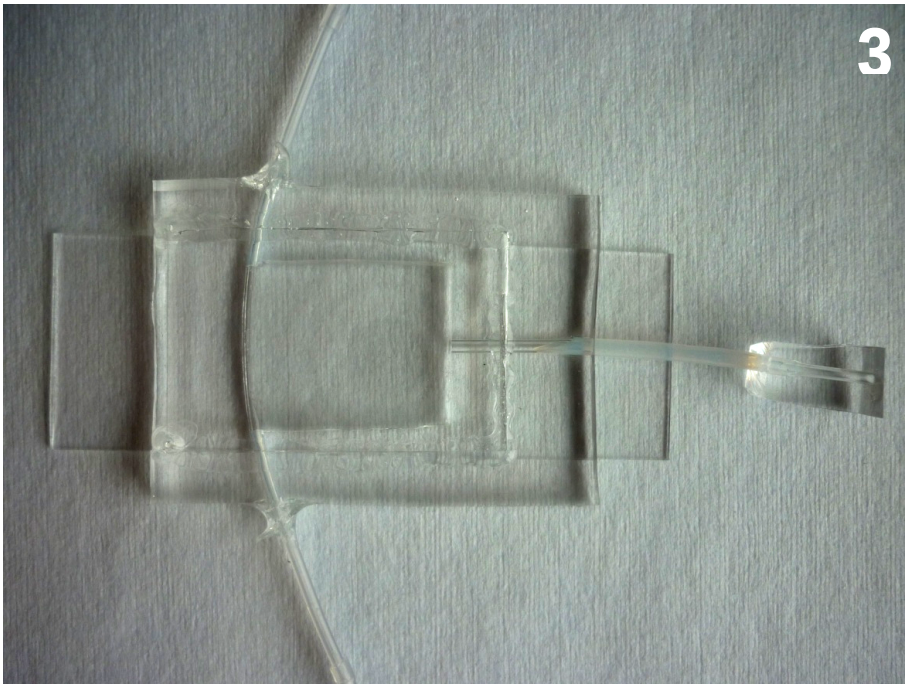
FIG. 3.1 | *Probe holder device. The two cross-sections show the layered structure materials. The structure was provided with three tubes. Three cylindric channels for the tubes are shown.*

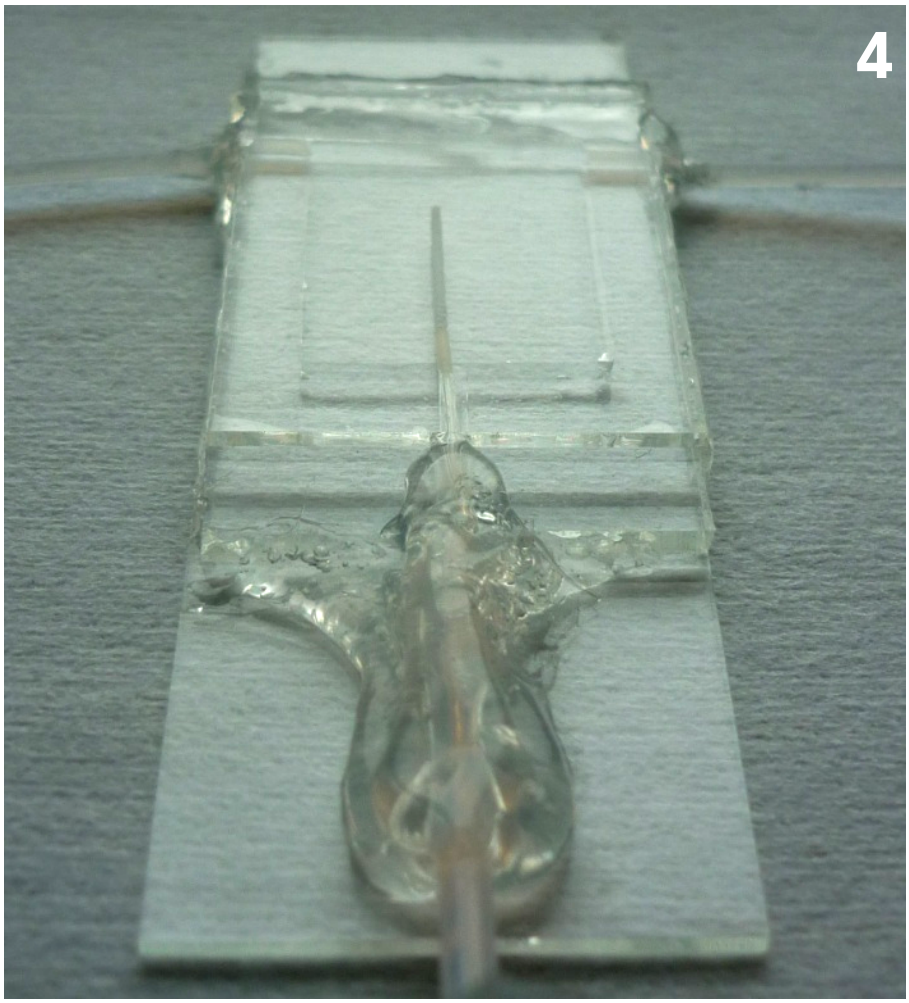
Two rectangular scraps were cut out from a normal glass microscope slide to function as the top and the bottom of the device. One face of each slide and both the top and the bottom faces of the PDMS structure were processed with the plasma surface treatment provided by a Tesla coil generator (KOJAIR TECH ORION S-95, Vippula, Finland). In order to make all the parts to hold together, the elements were joined then together and cured for 40 minutes in 70°C in a convective oven. Afterwards, three silicon tubes were inserted in the channels (inner diameter 0.7 mm, outer diameter 0.8 mm). The two corner channels were fitted with screwable Luer Lock syringe needles in order to allow a good coupling with the syringes that also had Luer Lock connections. Channeled PDMS was used as a coupler between the MD-probe channel and the probe itself. The realized device was validated with regard to functional specifications listed before (Page 23). The device fitted all the specifications. **Figure 3.2** shows the evolution of the device that leaded to the final device number 4. Device 4 was used for the quantitative studies.


1


2


3


3


4

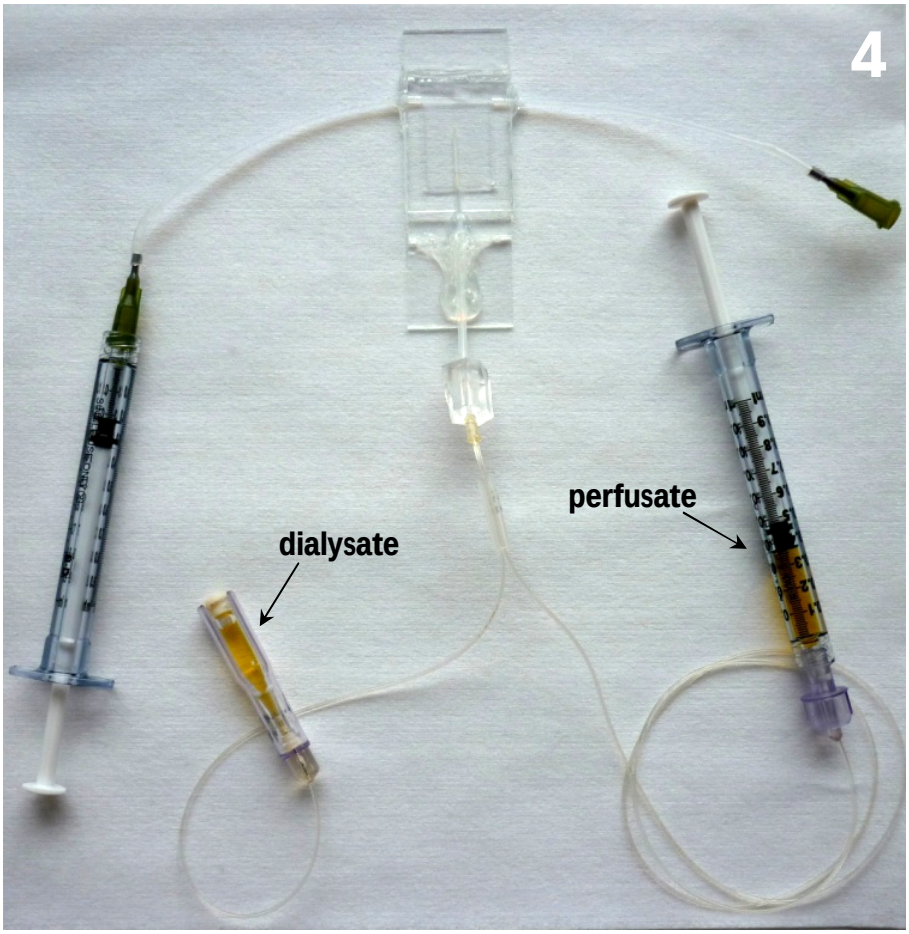


FIG. 3.2 | *The figure shows some fabricated devices. The device 4 was used for the quantitative section. This device was constructed as described above. The fabrication process of the other devices presented few differences but the main sequence was similar. The chamber 1, as the intuition could suggest, is the first attempt of fabrication.*

4. Qualitative analysis

The fluorescence microscopy is far from being a novel technique but it has, to our knowledge, never been applied to microdialysis even though several imaging techniques have been used for that by the constructer company, CMA Microdialysis (Solna, Sweden). The following pictures show that the fluorescence microscopy could be a convenient imaging method for in vitro studies of the MD probe relatively to specific purposes.

Several pictures were taken with different experimental parameters. Probes with different MWCO were perfused with different weight dextrans added. The first images **Figure 4.1** and **Figure 4.2** represent a 100 kDa MWCO probe in the beginning and after 8 minutes of perfusion with the Ringer's solution and 40 kDa FICT-dextrans added (5 µL/min flow rate). The magnification M of the objective is 4 (NA=0.2). The homogeneous brightness pattern outside the probe is due exclusively to leaked dextrans since the diffused light contribution is negligible. Indeed, in the beginning of perfusion, the probe that reached the stationary brightness state did non present any brightness pattern outside the probe edges. The same argument is valid for all images and will be developed further on in quantitative section. The edges of the membrane are brighter than other zones. This optical artifact is due to the fact that the edge is perpendicular to the image plan leading to more fluorophores contributing to a pixel with respect to other zones of the membrane. This effect is always present in the cells fluorescent imaging [**Lichtman et al., 2005**]. **Figure 4.2** shows also a magnified part of an image relative to the same probe but with another focus able to show the pore details since the depth of field did not allow for the whole probe to be in focus. The scales of the whole probe picture and that of the detail are the same.

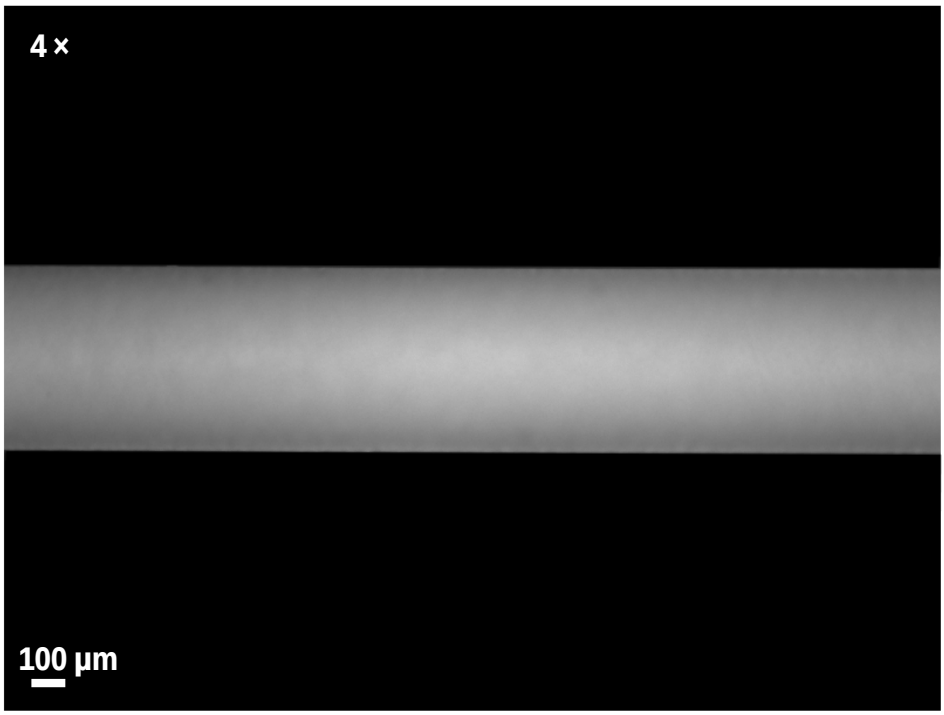


FIG. 4.1 | *100 kDa MD probe in the beginning of perfusion with 40 kDa FICT dextrans solution. The flow rate was 5 µL/min. There is no diffusion light contribution since no brightness is present outside the probe.*

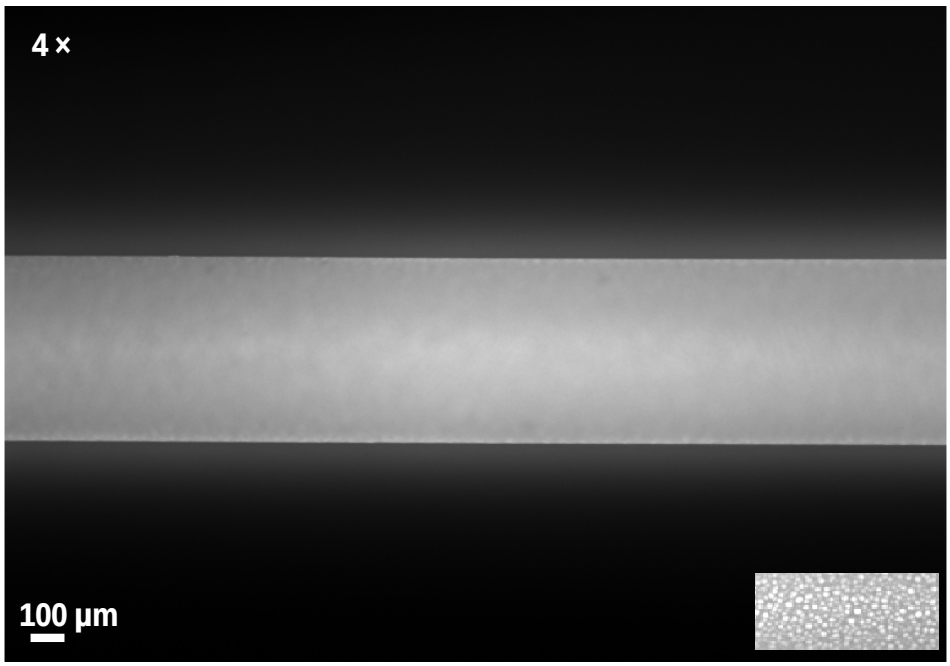


FIG. 4.2 | *Same experiment as in previous figure. The probe after 8 minutes perfusion with 40 kDa FICT dextrans solution. Leaked dextrans form homogeneous brightness pattern outside the probe. The figure shows also a magnified part of an image relative to the same probe but with another focus able to show the pore details.*

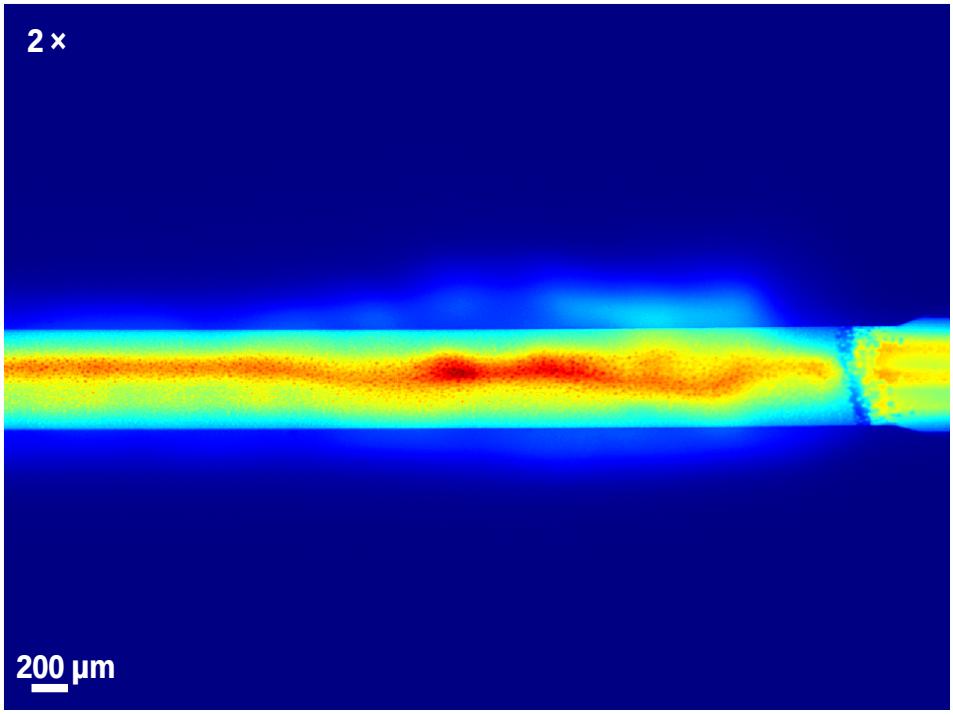


FIG. 4.3 | *300 kDa MWCO probe perfused with 250 kDa dextrans perfusate (M=2) for 10 seconds (F=5 µL/min). The image is visualized with a Matlab colormap giving a more sensible perception of fluorescence intensity variations.*

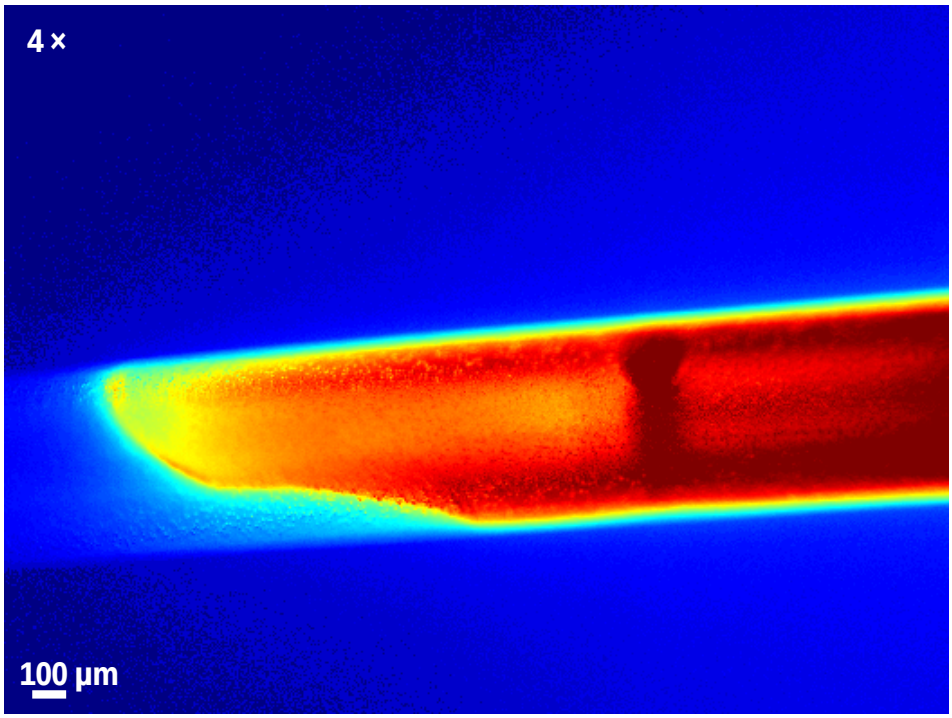


FIG. 4.4 | *20 kDa MD probe perfused with 40 kDa FICT dextrans solution. The figure clearly depicts the hole in the inlet tube where the dialysate flows in. Leakege pattern is present despite the declared MW to be higher than the declared MWCO.*

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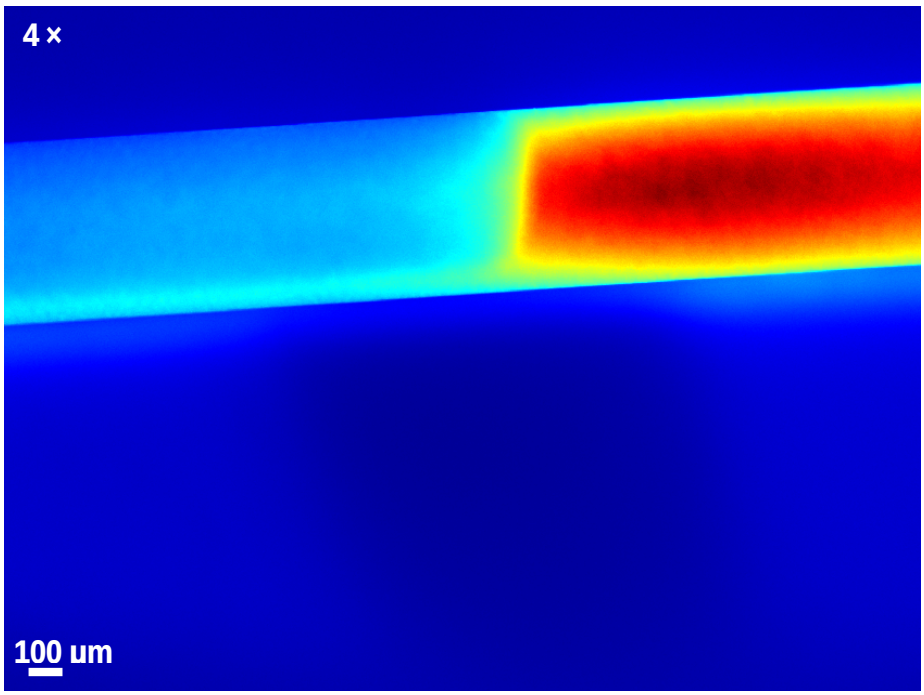


FIG. 4.5 | *100 kDa MD probe perfused with 40 kDa FICT dextrans solution. The fluorescent pattern of the lost dextrans clearly shows an alteration from normal sampling condition in the sense of the dialysis principle discussed in* ***Section 1.1*** *due to the presence of air bubbles inside the probe.*

Figure 4.3 shows 300 kDa MWCO probe perfused with 250 kDa dextrans perfusate (M=2) for 10 secondes (F=5µL/min). The image is visualized with a Matlab (MathWorks, Natick, USA) colormap giving a more sensible perception of fluorescence intensity variations. **Figure 4.4** shows a 20kDa MWCO probe perfused with 40kDa dextrans solution (M=4, NA=0.2). The figure clearly depicts the hole in the inlet tube where the dialysate flows in. It is worth to notice the picture shows the leakage of the dextrans with MW >> MWCO of the membrane. However, we do not know exactly the range of MW of the leaked dextrans since no study has been done in that sense. In addiction, the MW dispersion of the dextrans sample is unknown since the dextrans provider has not performed studies of measuring the MW dispersion around the declared MW mean value. The following, **Figure 4.5**, showing a 100 kDa MW cut off probe perfused with the same, 40 kDa dextrans solution, (M=2,NA=0.2) gives an example of how the sampling process could be perturbed from normal conditions if some air bubbles are present inside the probe altering the pressure and the flow of the perfusion fluid. Indeed, the fluorescent pattern of the lost dextrans clearly shows an alteration from normal sampling condition in the sense of the dialysis principle discussed in **Section 1.1**. **Figure 4.4** shows a qualitatively different leakage pattern with respect to smoother pattern of **Figure 4.5**. This is due to the fact that the amount of the leaked dextrans is much less in case of **Figure 4.4** since it deals with a 20 kDa MWCO membrane and 40 kDa dextrans. **Figure 4.4** shows more defined membrane pores. This is mostly due to the focus plane being not in the middle of the membrane but slightly moved toward the top of it and also due to the specific morphology of the 20kDa MWCO membrane. Indeed, a 20kDa MWCO membrane always showed more defined pores with respect to a 100 kDa MWCO membrane and even more markedly with respect to a 300kDa MWCO membrane, in pictures taken within the same experimental set up.

To summarize, the fluorescent microscopy was able to show details of the MD process and that of the MD probe and could provide a valid qualitative information for specific studies.

5. Quantitative study

This attempt for a quantitative fluorescent imaging analysis is focused on comparison of the leakage of 40 kDa, 70 kDa, 250 kDa and 500 kDa dextrans through a 100 kDa MWCO MD probe. A quantitative study of the MD process using the set up described in **Section 2** is an ambitious goal and not straightforward to achieve and many challenges had to be overcame. The goal of the following study was to develop a method capable to find out the behavior of different types of dextrans and to perform a comparison between different weight dextrans. This was the first attempt for a quantitative fluorescent imaging study of the MD.

5.1 Challenges

The quantitative analysis was performed through a numerical summation of the pixel brightness values over the, captured by the images, leaked fluorescent dextrans pattern. For that purpose the data had to be sensible only on the variation of the capacity of different dextrans to leak through the MD membrane and not on the variation of other information carried by the fluorescent signal. Since the interest is focused on a comparison the absolute values of the leaked dextrans concentration were not required to be determined.

The brightness of the leakage pattern is sensitive to several factors:

1. **The FICT-dextran concentration.** The number of fluorescent groups per unit of solution volume is proportional to the

concentration of FICT-dextran. Therefore, the fluorescent signal increases as the dextran concentration in the solution increases.

2. **The type of the FICT-dextrans** i.e. the molecular weight. Indeed, different weight dextrans solutions have different fluorescent signal, even when the mass concentrations is equal.

3. **The exciting light source intensity**. As stated by the provider, after 30 minutes from the initiation the lamp intensity reaches a steady state but there is a continuous exponential and then approximately linear damping over the lamp lifetime. After 300 hours the lamp loses 50% of the initial intensity. The experiments are performed over 40 hours leading to an approximately -8,8% of variation of the intensity assuming an exponential damping.

4. **Temperature of the perfusate and that of the chamber**. The absorption or emission of a photon is accomplished by a transition of the molecule system between two different vibrational-electronic states (within the Born-Oppenheimer approximation). The variation of the temperature could change the vibrational state of the system altering the fluorescence properties of the solution. Therefore, the room temperature changes between the different experiments and the chamber heating due to the mercury lamp light have to be taken into account.

5. **Degradation of flurophore** affects the fluorescence intensity of the perfusion fluid .

6. **The photobleaching of FICT-dextrans.** Since there was no possibility of automatic time-gating of the excitation light source, that is a valid way to minimize the bleaching, the photobleaching

process could not be negligected for our set-up. Moreover, the FICT Dextrans used in the experiment, are prone to a strong bleaching with respect to other fluorescent dextrans available on the market [**Lichtman et al., 2005**].

7. **The diffused fluorescent light noise signal** coming from the MD probe. In case it is present, it should be removed from the images. It depends on the source intensity (here the MD probe emitting the fluorescent signal) that will be different for different type dextrans.

8. **Autofluorescence**, i. e. is the natural photon emission of the chamber materials in the spectral range of FICT emission spectrum.

9. **Background noises**. Background noises could be present on images, e. g. dark current CCD sensor noise.

10. **Shading**. Some areas of image could be brighter than others despite the same amount of fluorophore because of inhomogeneous illumination or non uniform sensor sensitivity.

As stated before, we were interested in the capacity of the certain type dextrans to leak through the membrane. Therefore, the other information carried by the fluorescence signal that could be changing between the different experiments had to be removed from the recovered data. In order to do so, it was necessary to sample that information. For what concerns the points from 1 to 5 this was done by taking pictures of the perfusate fluid. All test pictures were taken using the same exposure time of the CCD sensor. Two factors were taken into account for the choice of the optimal exposure time: the sensitivity of the image acquisition system and the saturation of the image. Indeed a too low exposure time leads to low image intensity or black image. Therefore, the exposure time had to be high

enough to have a good signal even for the less fluorescent dextran type. On the other side, a high exposure time leads to an overexposed image. The exposure time of 150 ms. was chosen as a compromise between these two factors. Moreover, for what concerns point 4 it is worth to notice that the temperature of the tested perfusate, and that of the solution filling the chamber were exactly the same before perfusion, but at the shooting time the leaked perfusate has run through the inlet tube and through the membrane thereby raising its temperature. Therefore the temperature of the tested perfusate and that of the leaked dextrans forming the leakage pattern of the image could be slightly different. The method deployed here neglects this aspect and works under the reasonable assumption that the flow rate and the increase of the temperature are low enough to preserve the temperature of the whole outer environment of the probe. This is possible since the leaked volume should be negligible with respect to the chamber volume. In addiction, the room temperature was monitored to take into account the variations of the diffusion coefficient.

The brightness of the resulting perfusate test picture was then used as a normalization factor for the same dextran type leakage pattern picture. In this way, the sum of the pixel intensity values over the whole leakage pattern divided by the normalization factor contains only the information of the capacity of a certain type dextrans to leak through the MD probe membrane.

Lets now examine challenges 6 and 7. For what concerns the photobleaching, the specific experiment performed in the same conditions as for the main experiments of the quantitative comparison revealed that exposing the chamber even over ten seconds the dextrans loss pattern does not have any qualitative change (**Figure 5.1.1**; **Figure 5.1.2**). The leakage pattern is relative to 40 KDa dextrans and it is similar to what then observed for the final comparison experiments.

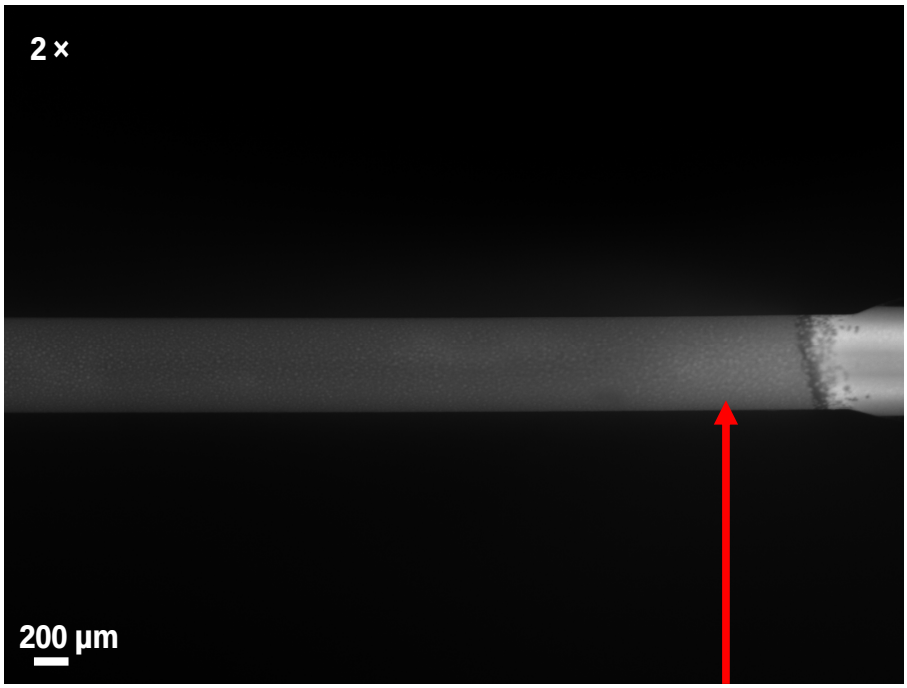


FIG. 5.1.1 | *The red axis where the one-dimentional leakage pattern was extracted for the bleaching assessment showed in the following figure. The qualitative result showed in the following figure is not dependent on the position of the red axis.*

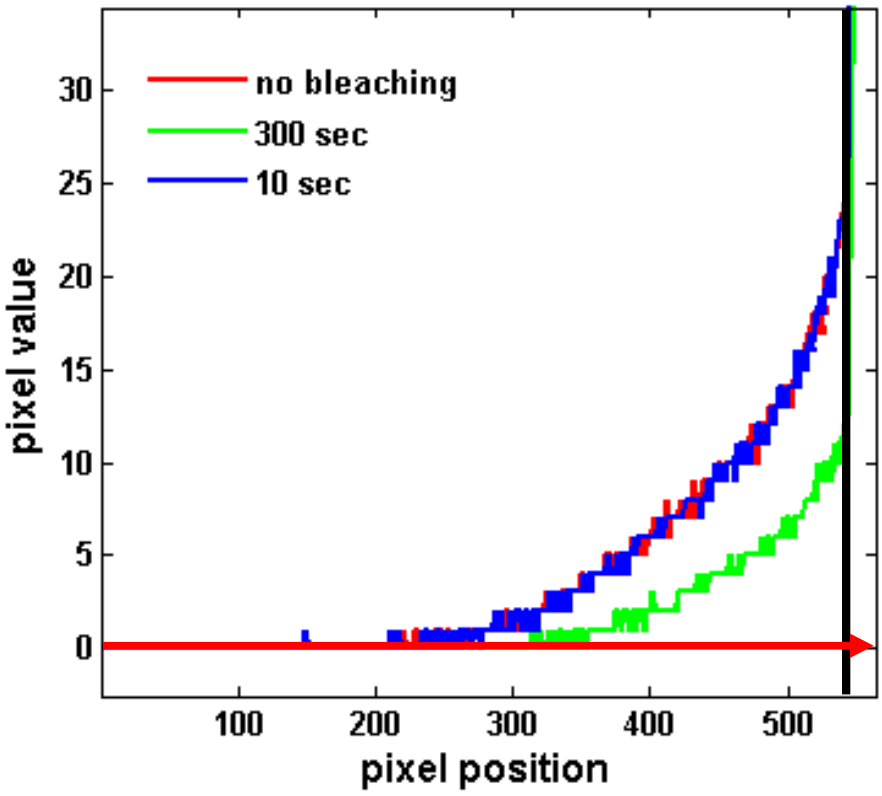


FIG. 5.1.2 | *Bleached and not bleached one-dimentional patterns all coming from the same position on the image. The pixel position axis is represented by the red arrow as in the previous figure.*

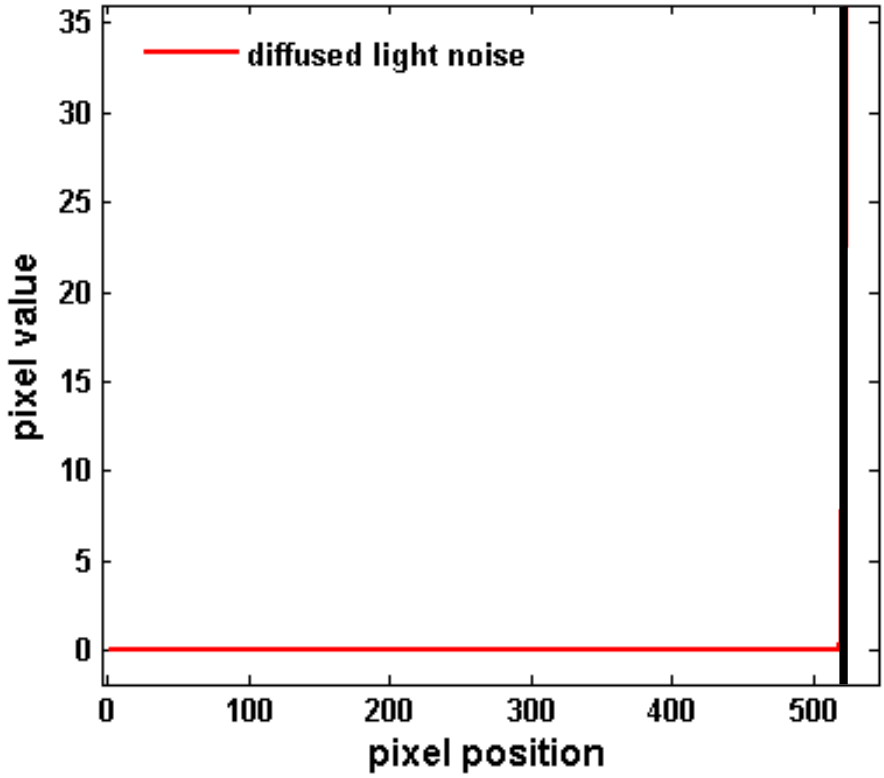


FIG. 5.1.3 | *Diffused light noise one-dimentional pattern. The picture has the same brightness as the quantitative section pictures. It proves there is no diffusion light contribution.*

The choice adopted for the measurement is that of reducing at minimum the exposure of the chamber to the excitation lamp light by manually gating the excitation. This was possible for the presence of the shutter of the lamp light. By manually gating the excitation source an exposure time not higher then one second could be achieved for all the experiments.

As earlier stated the diffused light noise could be neglected. This was further evaluated during the quantitative study. **Figure 5.1.3** depicts the worst case scenario among all different type of tested dextrans i.e. the brightest picture corresponding to 40 kDa dextrans. That picture was taken within the same conditions as for all pictures of the quantitative analysis section. It was captured

very carefully in the beginning of the perfusion in order to not having any leaked dextrans that would create a brightness pattern and so capturing only the diffused light signal of the membrane. As for the bleaching graphics the pixel brightness values are relative to just one vector of the whole picture. The cut was done in correspondence of the brightest zone of the MD probe. The figure shows there is no diffused light noise that otherwise would be present on the graphic as a curve with an exponential damping toward lower pixel position values. The brightness of the membrane is the same as for image taken after the time by protocol, therefore the contribution of the diffused light is also the same.

For what concerns autofluorescence, it was not expected to affect the leakage pattern images. However, the background picture was taken before each perfusion and the subtraction was performed before to process the leakage pattern image. Such operation cuts off also background noise.

The last challenge regards shading. This term is used for image inhomogeneities caused by non uniform illumination or sensor non uniform sensibility. No correction for the shading was performed. It was estimated to be 5 % (the brightness near the edges of the image frame was 5 % less than the brightness at the image center of a silicon tube filled with FICT dextran solution). Ten pictures were used for the shading estimation. The mean value was then considered. Shading is equally present in all pictures. Since the position of the membrane with respect to the image frame was the same and all the patterns had qualitatively the same shape the relative measurement was considered to be not affected by this effect.

5.2 Protocol of the experiments

The experiments were performed as follows. Three 100 kDa cut off brain MD probes (CMA71) were used for the comparison experiments. The probes were purchased from the CMA Microdialysis (Solna, Sweden). Each MD probe was perfused consecutively by 40 kDa, 500 kDa, 250 kDa, 70 kDa and again 40 kDa fluorescent FICT(fluoresceinyl isothiocyanate)-dextrans perfusate. The flow rate used in the experiment was 0.5 µL/min which is a common flow rate when microdialysis is run in vivo [**Schutte et al., 2004**]. This sample sequence was chosen in order to minimize possible probe clogging. The last perfusion was for the clogging test. The concentration of the dextrans in the perfusion solutions was 30 g/L which is the same concentration as suggested by the probe manufacturer in order to prevent ultrafiltration i.e. for the 60 kDa dextrans (CMA instructions). The mass and molar concentration of the FICT-Dextrans is summarized in the **Table 5.2.1**. High precision pipettes and ultrasound bath were used in the preparation process.

Dextran weight	Mass concentration	Molar concentration
40kDa	38,4 g/L	0,96 mmol/L
70kDa	30 g/L	0,48 mmol/L
250kDa	30 g/L	0,12 mmol/L
500kDa	30 g/L	0,06 mmol/L

TAB. 5.2.1 | *Table of the perfusion solutions concentration. The solutions were prepared with use of high precision pipettes, ultrasound bath.*

Before each perfusion the MD probes underwent a cleansing process with distilled water followed by a perfusion with the Ringer's solution. The cleansing procedure was first 20 µL/min perfusion for 6 minutes followed by a 10 µL/min perfusion for 2 hours with distilled water. Then, the probe was perfused for 6 minutes by 10 µL/min with Ringer solution.

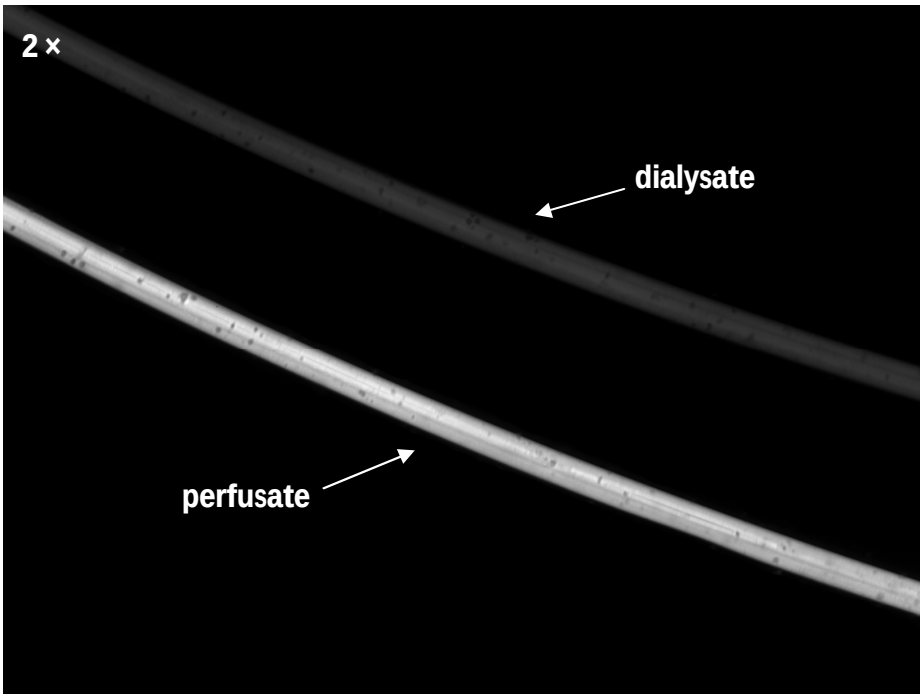


FIG. 5.2.2 | *Perfusate and dialysate picture reIative to the 40 kDa dextrans perfusion. Inflow (lower) and outflow (higher) tubings.*

Before each perfusion the probe holder device was cleaned with the distilled water and filled with Ringer's solution. With the chamber being installed, a normal microscopy mode (not epi-fluorescence mode) picture of the probe was taken for the focusing, central and horizontal positioning of the probe and for

detection of the edges of the probe in the image processing phase. The dialysate collector was placed at the same level of the probe.

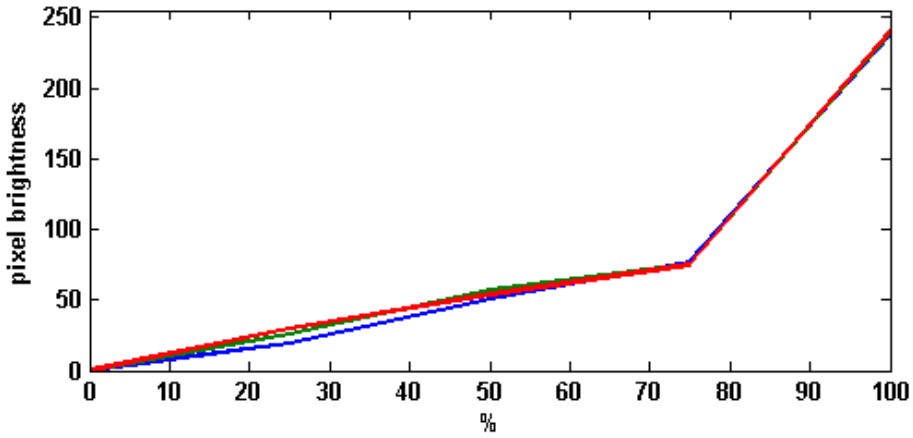
Before activating the pump a background picture was taken. After the activation of the pump the arrival of the fluorescent perfusate was monitored from the live image of the probe. The exposure time of 150 ms was chosen to give a quantitative signal for the weakest FICT dextrans (500 kDa) but providing a non overexposed probe picture in order to be able to have also qualitative information from all the pictures in case of necessity. A picture was taken at 5 and 10 minutes of perfusion after the arrival of the perfusate with the same camera settings but only the 5 minutes pictures were then considered, being the most suitable for the comparison of interest. Those pictures presented large leakage pattern with the whole pattern being present inside the picture frame. This optimum shooting time as well as the other protocol parameters were assessed with test experiments performed before to do this comparison. After the perfusion a picture of the inflow tube with the fluorescent perfusate was taken for the normalization purpose of pictures in the image processing phase (**Fig. 5.2.2**). **Figure 5.2.2** shows also the MD-outflow tube. Indeed, it was decided that a useful help for a better understanding of the leakage behavior could be provided by comparing the brightness of two tubes. Such a comparison could provide information regarding the concentration lost during the microdialysis process.

5.3 System response linearity

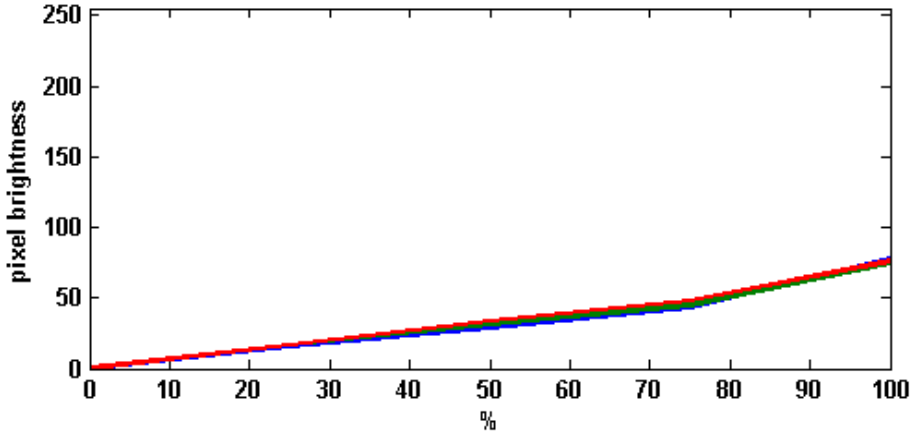
In order to be able to process the images, the linearity of the relationship between the image brightness and the dextran concentration needed to be assessed. This was done by taking pictures of the different concentrated perfusates using same tubings and same set-up used for the perfusate-dialysate pictures. The results for each dextran types are shown in **Figure 5.3.1.** Each type of the dextran perfusion fluid was diluted with Ringer's solution to have 0% (pure Ringer's solution), 25%, 50%, 75%, 100% of the original perfusion fluid (see the **Table 5.2.1**). The sequence of the pictures was taken by increasing the concentration and was performed three times. Each sequence in the figure is marked by a color. The brightness values were extracted from the same radial position from the center of the image. Four values were taken and the mean value of that data was performed.

Figure 5.3.1 show that the linear zone exists for small concentration values. The non-linearity is present for 40 kDa dextrans and slightly for 250 kDa dextrans at high concentrations. This behavior is due to the non-linear relationship between the fluorescence intensity and concentration and not to the sensor response. Indeed, the pixel brighness is linearly proportional to the number of photo-generated electrons or to the number of incoming photons. **Figure 5.3.2** shows the maximum value of a MD-probe picture as a function of exposure time. Excellent linearity exists over the entire range of brightness values.

A | 40 kDa dextrans



B | 70 kDa dextrans



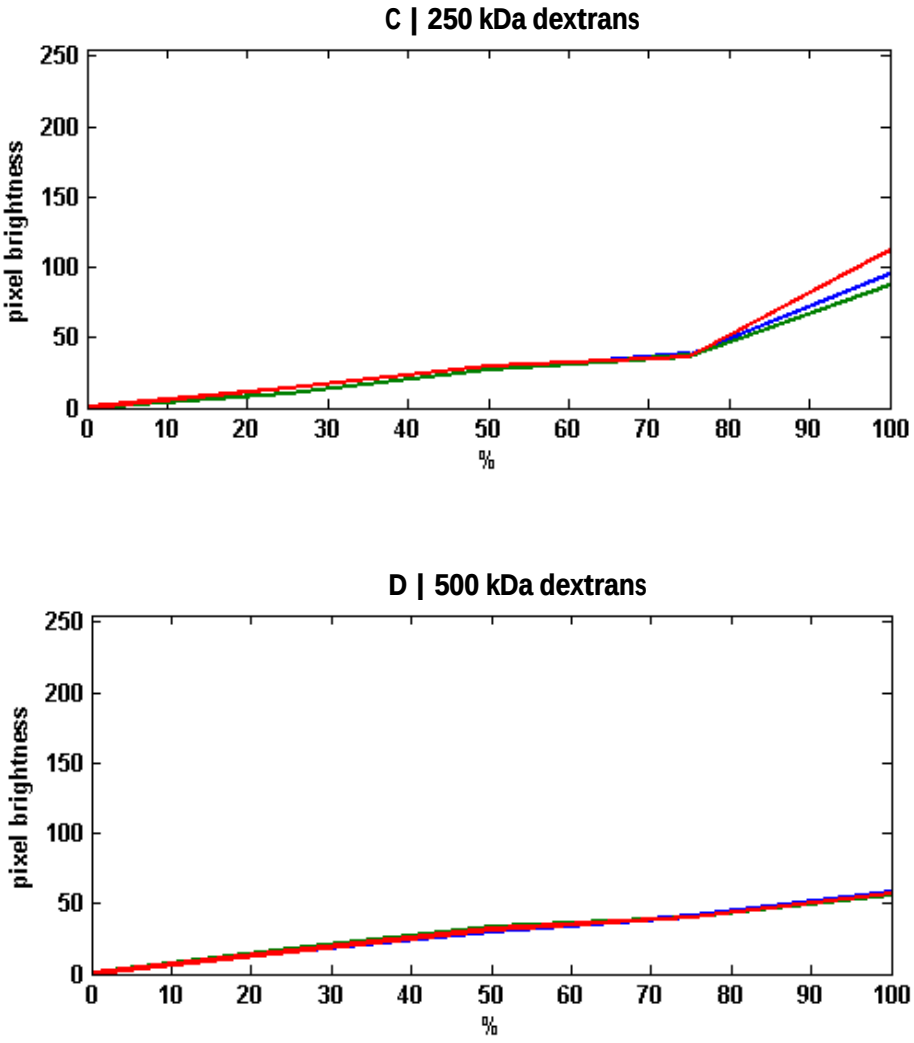


FIG. 5.3.1 | *Dextran concentration – image brightness relationship, for different weight dextrans. (**A**) 40 kDa dextrans, (**B**) 70 kDa dextrans, (**C**) 250 kDa dextrans, (**D**) 500 kDa dextrans. Each graphic shows three broken lines relative to three experiments. Each broken line is defined by 5 values connected by line. The concentration values are 0%, 25%, 50%, 75%, 100% with respect to the original concentration showed in Table 5.2.1. The linear zone exists for small concentrations.*

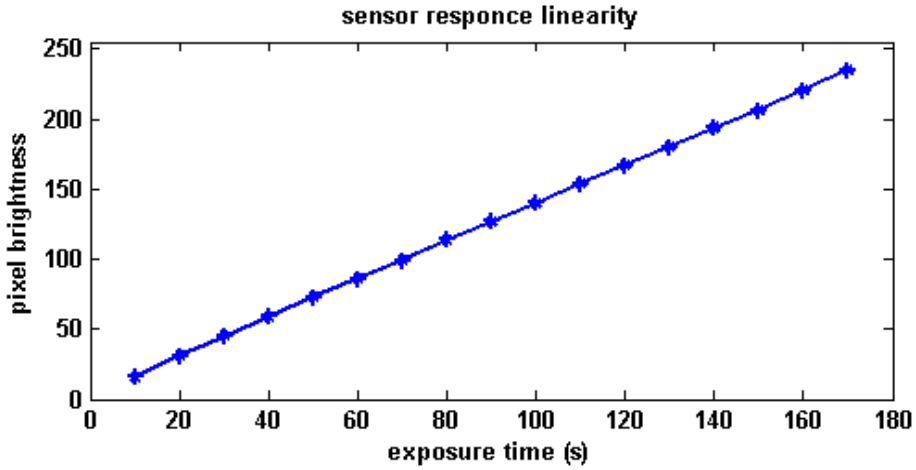


FIG. 5.3.2 | *Image brightness versus sensor exposure time. The figure shows excellent linearity.*

5.4 Image processing

In order to perform the sum over the leakage pattern the MD probe edges should be identified. All MD probes used for the comparison had highly linear edges. The edge evaluation was done simply by looking at the normal microscopy picture and calculating the equation of the edge line. The parameters of the equation were then used as inputs for the Matlab sum code. With respect to an evaluation by code this way to identify the edges seemed to be the faster and easier, and still accurate. The normalization factor relative to each leakage pattern was extracted from the MD-inflow picture taken after each perfusion. The extraction was performed in the same way as for the linearity test of **Figure 5.3.1**. The leakage patterns were observed to have pixel values lying within the linear zone of brightness-concentration relationship. The 40 kDa inflow tube pictures were affected by non linearity instead. Therefore, the extracted values were corrected in order to shift them to values they would have in case of totally linear brightness-concentration relationship. The linearity correction factor was calculated from the **Figure 5.3.1**. The linear value for 100% concentration was extrapolated from the line fitting the mean values relative to 0% - 75% concentration interval. The correction factor was then the ratio between the linear value for 100% concentration and the actual mean value for 100% appearing in **Figure 5.3.1**. The so-found factor was then used for all 40 kDa dextrans inflow tubes pictures in order to find the linearized normalization factor. Such a procedure was adopted also for the 250 kDa dextrans case.

The sum over the leakage pattern is performed by a Matlab function, called in the Matlab browser. The code is shown and discussed in **Appendix 1**. Before using the code, the background was removed from the image by subtracting the background image to the image of interest.

As mentioned earlier the MD-outflow tubes could provide useful information

about concentration loss during the microdialysis process. Since for 40 kDa dextrans there is a non-linearity due to high concentration, the ratio Cd/Cp between the dialysate tube and the perfusate tube concentrations is not simply the ratio between the respective image brightness. The concentration in dialysate tube for the 40 kDa case was evaluated by using the curves of **Figure 5.3.1** as standard curves. Since the experimental environment conditions (discussed in **Section 5.1**) were varying, the perfusate tubes' brightness were varying too. Therefore, in order to read the concentration value in the standard curve, the dialysate tube pictures were scaled so that the brightness of the perfusate tube matched the mean value relative to 100% concentration in the standard curve of **Figure 5.3.1**. Since the standard curve has only five points the concentration loss data was regarded only as supplementary qualitative information to the dextran loss data. For other type dextrans (including 250 kDa) the concentration ratio was evaluated simply by the brightness values ratio.

5.5 Results and discussion

The results of normalized sums over the leakage dextrans patterns are shown in **Figure 5.4.1** Three membranes are grouped by the dextran type.

The normalization makes the data to contain only the information of the capacity of a certain dextran type to leak through the membrane of the MD probe. Therefore, the difference in leakage sum data is due only to the type of dextran and the probe itself. It is crucial to notice that the room temperature was approximately constant during the experiments (± 1K). Therefore, the variation of the diffusion coefficient due to the temperature is negligible.

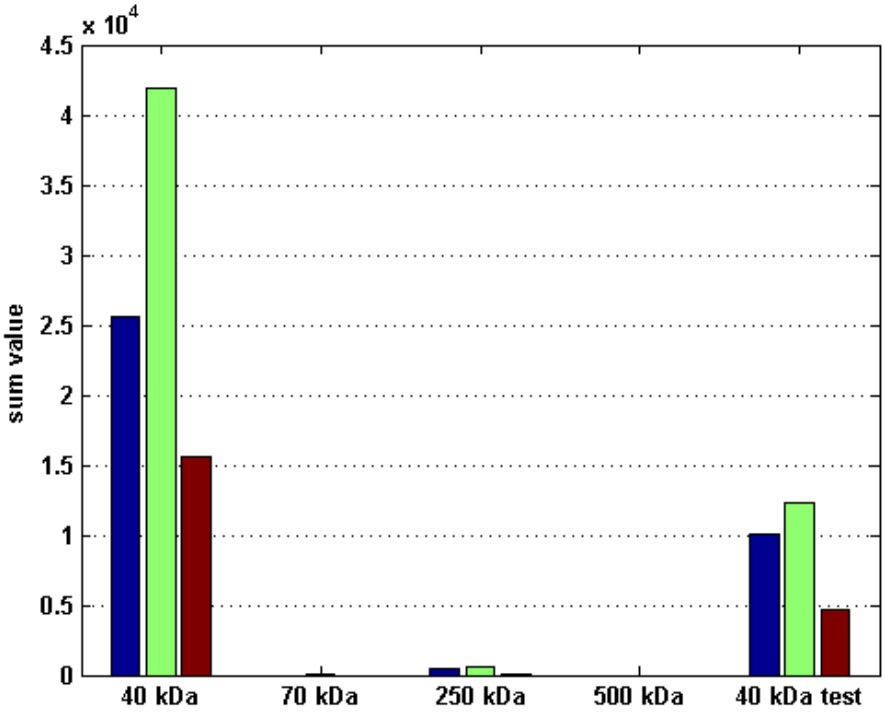


FIG 5.4.1 | *Normalized sums of leaked dextrans. Five sets relative to different weight dextrans are present including the clogging test performed at the end of the measurements.*

First of all we can see, for each dextran type, the same behavior of the three probes. The MD-probe 2 results to be the most leaking, followed by the probe 1 and then by the probe 3, for each dextran type. If we assume in first approximation the clogging during the different perfusions of a probe to be the same for each probe then an assessment of repeatability of the measurement could be done by comparing the three 40 kDa experiments and the three 40 kDa clogging test experiments. The **Figure 5.4.2** shows the comparison between the two 40 kDa dextrans experiments were the data relative to the clogging test is normalized. We can see the same shape so the measure is not affected by random errors. *The measurement is repeatable*, so the variation of the sum within the same dextran type is mostly due to the morphological differences of the membranes of the three probes and not to the random errors. *Therefore, we can conclude that the leaking and sampling properties of probes of the same type are not the same.*

Other important issue to point out is that *although the molecule weight cut off of the membrane is 100 kDa some of 250 kDa dextrans perfusate leaked out anyway*. This could be due to a larger than 100 kDa effective cut off of the membrane or to a high molecular weight variance of the FICT-Dextrans by Sigma Aldrich. The 70 kDa dextrans seems to be less leaking than the 250 kDa dextrans. This behavior could be due to a smaller variance of molecular weight dispersion for 70 kDa dextrans sample than that of the 250 kDa dextrans. A reasonable explanation could be also that of the clogging of the probe since the 70 kDa perfusion was performed as the last perfusion before the 40 kDa dextrans clogging test. Only probe 2 presented the sum different from zero and so it results to be the most leaking one also for the 70 kDa dextrans experiment. Sigma Aldrich reported that no experiments were performed to assess the MW despersivity of the different dextrans.

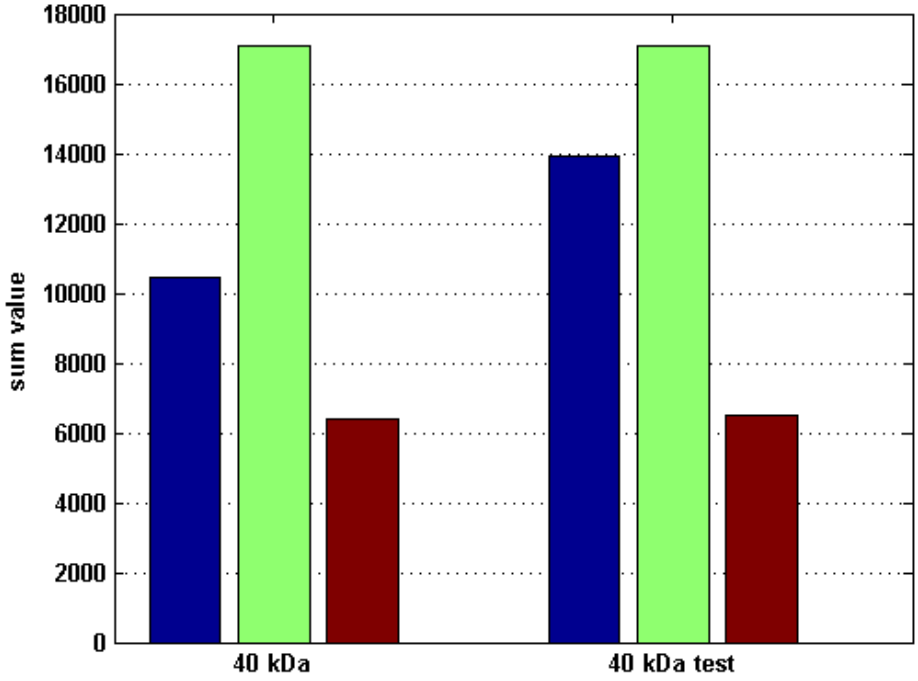


Fig 5.4.2 | *Comparison between the first 40 kDa dextrans set of experiments and 40 kDa clogging test set. The shape of two sets is similar leading to a conclusion the measurement is repeatable.*

Concerning the 500 kDa dextrans, the sum resulted to be zero for all the three perfusions. Therefore, *for what concerns the method deployed here there is no leakage of the 500 kDa type dextrans.* It is worth to point out that for 500 kDa dextrans the method has the lowest sensibility. The choice to use the same exposure time for the CCD sensor for all types of dextrans lead the 500 kDa dextrans perfusate to have less intense image. A higher concentration of 500 kDa dextrans in the perfusate fluid that would lead to a higher fluorescent signal would also lead to a too viscous perfusate with respect to what suggested by the MD probes constructer. As mentioned above, the MD manufacturer suggests

adding 30 g/L of 60 kDa dextrans to the perfusate in order to avoid the ultrafiltration. The method uses a near 30 g/L mass concentration for all the different dextrans perfusate fluids, as discussed above. The viscosity increases with the molecular weight provided the mass concentration to be the same. The 500 kDa dextrans perfusate was already more viscous than the other dextran perfusion fluids with molecular weight nearer to the suggested condition and the difference was easily notable with the naked eye. The viscosity magnitude controls the pressure gradient inside the probe and hence controls all the process of the dextrans leakage. Therefore, raising the concentration of the 500 kDa perfusate seemed not to be convenient. On the other hand, lowering the concentration of 40, 70 and 250 kDa dextrans perfusate would also lead to be further from the suggested condition and seemed to be inconvenient too.

Let us consider now the data regarding the dialysate-perfusate dextrans concentration ratio showed in the **Figure 5.4.3** in the attempt to provide a useful help to understand the dextran loss data. The data was extracted from the inflow and outflow tubes as described in **Section 5.3**. **Figure 5.4.3** shows the mean value relative to the three probes for each dextran type. It seems to be more convenient to focus the discussion directly on the mean value instead of considering each probe separately since, as discussed in **Section 5.2**, the Cd/Cp data serves just as a help to the main data.

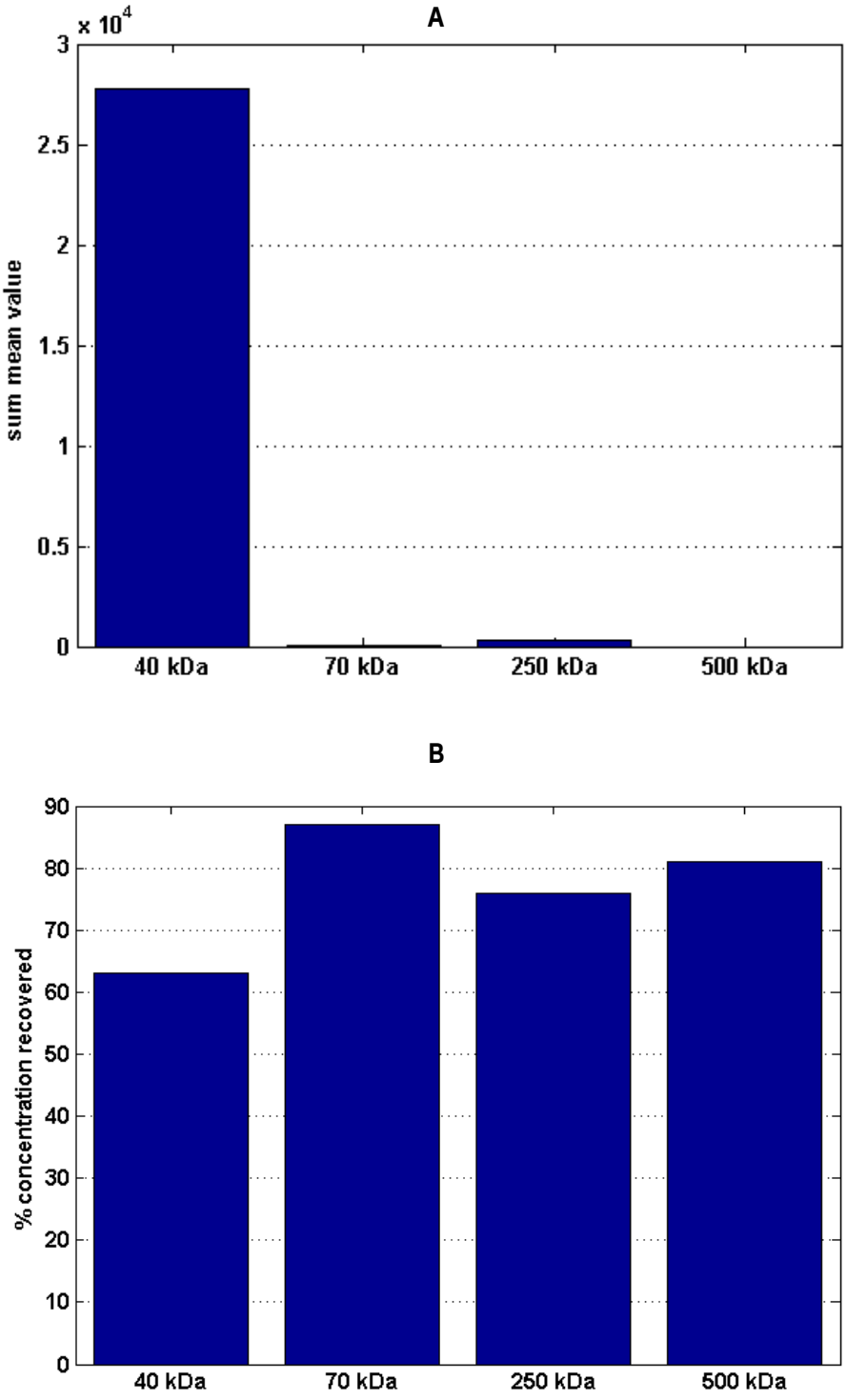


Fig 5.4.3 | *Comparison between the sum over the leakage pattern and the concentration recovered by dialysate. Mean values over three experiments are considered.* ***(A)*** *Normalized sum over the leakage pattern.* ***(B)*** *Concentration recovered by dialysate values. The percentage is calculated with respect to perfusate concentration.*

The comparison between the two figures shows the existence of the highest leakage the lowest Cd/Cp ratio relationship. This behavior shows that for a not leaking dextran (500 kDa) the flow regime is so that there is only a weak inward osmotic ultrafiltration of the Ringer's solution filling the chamber. It dilutes the perfusate making the dialysate less fluorescent than the perfusate. This is due to the presence inside the probe of the dextrans as osmotic agents creating the transmembrane gradient of osmotic pressure inwardly directed. Other type dextrans are more leaking leading to lower Cd/Cp values. The Cd/Cp value of 40 kDa dextrans is however not so low as it is expected to be from the leakage data of the **Figure 5.4.4**. The reason could be that for 40 kDa dextrans case (with the relative concentration and flow rate) the presence of outward ultrafiltration is more important than in case of other leaking dextrans. Indeed, the presence of only an outward UF over the whole length of the MD probe would lead the flow recovery to be less than 100% but the Cd/Cp value would be 1 (or more that 1 if MW>>MWCO of the membrane), whereas in case of pure diffusion the flow recovery would be 100% and Cd/Cp value less than 1.

The flow recovery data was also obtained by measuring the weight of the dialysate but this data is not shown here since it seems to be not reliable due to a short perfusion time used and therefore unhelpful for the actual discussion.

The understandable behavior of the Cd/Cp data relatively to the leakage data could be a validation of the latter one being the aim of the study.

To sum up all, we say that this study gives a validation of the use of fluorescence microscopy as tool for quantitative and qualitative studies of microdialysis, relatively to specific purposes. Moreover, it finds out the relative behavior of different type dextrans, used in microdialysis as osmotic agents, relatively to the capacity to leak through the membrane of the large-sized pores microdialysis probe.

6. Conclusions

A MD probe holding device for fluorescent microscopy (FM) imaging studies was designed, manufactured. The main materials were PDMS (polydimethylsiloxane) and glass. A qualitative FM analysis showed the FM could provide useful information for specific studies. A method for quantitative imaging was developed and applied for comparison of leakage behavior of four different weight dextrans, used in MD to regulate osmotic pressure of the perfusion fluid. The method consisted in the numerical sum performed by C code of pixel brightness values over the dextrans leakage pattern image. The images were normalized before the processing in order to make the leakage sum data to be sensitive only on the capacity of dextrans to leak through the MD membrane and not on other variables varying among the experiments (e.g dextran concentration, experimental environment parameters). The measurement resulted to be repeatable. Following conclusions were made. Probes of the same type resulted to have different leaking properties. Although the MWCO of the membrane was 100 kDa some of 250 kDa dextrans perfusate leaked out anyway. There was no leakage of 500 kDa dextrans instead. Concentration loss was also assessed from pictures of inflow and outflow tubes. The concentration loss data was consistent with leakage sum data.

Appendix 1

The image - object of the sum- is treated by Matlab as a 1200×1600 single valued matrix since the image is a 1200×1600 black-and-white picture and is used as the input **im** for the code. The probe's edges that bound the upper and the lower zones over which the sum is performed are the functions of the type $y=\mathbf{a}_{i,s} \cdot x + \mathbf{c}_{i,s}$ where **i** is for the upper and **s** is for the lower edge. The $\mathbf{x}_i$ input is the starting x coordinate of the sum process that is done over M=1440 pixels. The $\mathbf{x}_i$ was chosen together with **a** and **c** parameters directly from the normal microscopy picture of the MD probe. The variable M, inside the function S was chosen to maximize the length of the sum zone.

---

function S=somma(**im**,$\mathbf{a}_i$,$\mathbf{c}_i$,$\mathbf{a}_s$,$\mathbf{c}_s$,$\mathbf{x}_i$)

```
M=1440;
x=xi-70;xf=x-M;
S=0;
S=uint32(S);
qv=1:1200;

for x=xi:-1:xf          // scan over the horizontal coordinate x

  y=ai*x+ci;
  y=uint32(y);
  yi=y+3;
  qv=im(:,x)            // identification of the lower vertical vector

    for y=yi:1:1200  // scan over the y coordinates of the lower vertical vector
        q=qv(y);
        q=uint32(q);
        S=S+q;

    end

  y=as*x+cs;
  y=uint32(y);
  yi=y-3;
  qv=im(:,x);          // identification of the upper vertical vector
```

```
    for y=yi:-1:0      // scan over the y coordinates of the upper vertical vector
        q=qv(y);
        q=uint32(q);
        S=S+q;

    end

end
```

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