Wall permeability of isolated small arteries. Role of the endothelial surface layer

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6 INCORPORATION OF THE LIPOHILIC TRACER DII IN ENDOTHELIAL CELLS OF ISOLATED ARETERIS OCCURS INDEPENDENT OF VESSEL PERFUSATE COMPOSITION

6.1 ABSTRACT

The fluorescent carbocyanine dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate (DiI) was used in several permeability studies on isolated cannulated arteries to label the endothelial cells of the arterial wall. In these permeability studies we determined the dependence of FITC-dextran transport through the endothelial surface layer (ESL) on molecular size, solvent ionic strength and the presence of albumin in the perfusate. Fluorescence kinetics of DiI, measured at the endothelium, may be influenced by transport of DiI through the ESL, by the dynamics of the incorporation process into the membrane or by spreading of DiI over the endothelial membranes by lateral diffusion. We aimed to evaluate by which of these processes the DiI fluorescence kinetics were dominated. DiI was infused into the cannulated arteries simultaneously with FITC-dextrans (FITC-Δs) by means of a double-barreled θ-pipet. Fluorescence kinetics of DiI, due to incorporation of DiI in the endothelial cell membranes, were recorded with confocal laser scanning microscopy. Accumulation of DiI in the endothelial cells of the arterial wall appeared observable in the recorded arterial fluorescence distributions as peaks, increasing in intensity during the dye perfusion period. Location of the DiI peaks (i.e. the endothelial position) was constant over time. Maximal peak intensity increased at approximately constant rate. The shape over the DiI peaks, as characterized by their full-width-at-half-maximal-value, remained also constant. No differences in DiI fluorescence characteristics were observed between left and right wall per artery. No differences were observed between the different groups of arteries per protocol and no differences were observed between the different protocols. Moreover, no effect of prolonged illumination of the arteries with a bright mercury lamp (light-dye-treatment) on DiI fluorescence characteristics was observed. In conclusion, we demonstrate that fluorescence kinetics due to incorporation of the lipophilic tracer DiI in endothelial cell membranes of isolated arteries were not dependent on experimental conditions such as the presence of different anionic macromolecules, solvent ionic composition, or the presence of proteins in the perfusate. These fluorescence kinetics appear to be mainly determined by the incorporation process itself and not by transport of DiI through the endothelial surface layer (ESL) or by spreading of DiI over the endothelial membranes. Moreover, ESL transport of DiI was found to be very fast (<<1.5 min) in comparison to ESL transport of anionic macromolecules (FITC-Δs, ~30 min). Thus, the initial difference in fluorescence column width (i.e. a measure for the luminal filling) of DiI and FITC-Δs provided a fair estimate of ESL thickness.

6.2 INTRODUCTION

Fluorescent carbocyanine dyes such as DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; see figure 6.1) have been applied to label neurons

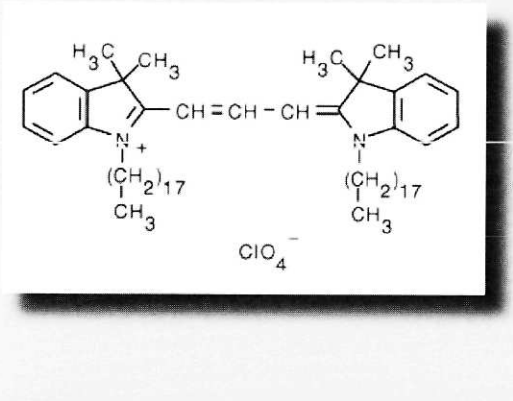

Figure 6.1: Schematic representation of the structure of DiI (copied from http://www.probes.com/servlets/structure?item=282, copyright © Molecular Probes, Inc.).

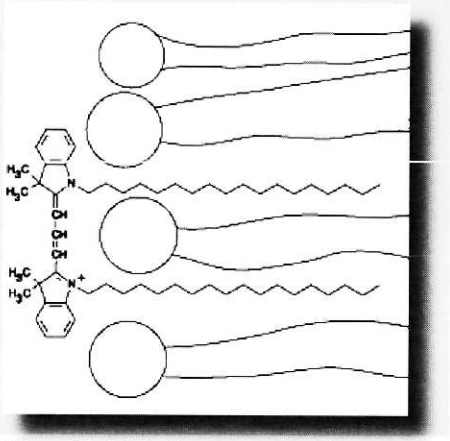

Figure 6.2: Schematic representation of the incorporation of DiI in a membrane (copied from http://www.probes.com/handbook/figures/0912.html, copyright © Molecular Probes, Inc.).

(2;5;8;9;17;18;20;22), tumor cells (7), leukocytes (4), red blood cells (12;19), low-density lipoproteins (11;16), smooth muscle cells (15) and endothelial cells (ECs) (3;13-15). We applied DiI in various permeability experiments to label the endothelial cells in isolated cannulated small arteries (chapter 2, 4 and 5). This way we were able to accurately localize the position of the luminal arterial wall in relation to the location of different fluorescent macromolecules, in order to determine dimension and properties of the barrier for transport of these macromolecules.

The lipophilic tracer DiI has a relatively low fluorescence quantum yield in an aqueous solution and a high fluorescence quantum yield in a membrane-like-environment (6). Thus upon incorporation in the endothelial membrane (see figure 6.2) of isolated perfused arteries, DiI will become brightly visible with an appropriate fluorescence microscope. However, after infusion into the lumen of the artery, DiI first needs to permeate through the endothelial surface layer (ESL) – a gel-like layer of ~2-3 μm in thickness that covers the luminal surface of the endothelium and that forms a profound barrier for anionic solute transport (chapter 2) – to reach the endothelium and to be able to incorporate in the ECs.

We originally hypothesized that the kinetics of DiI fluorescence due to its incorporation in the endothelium would reflect the transport properties of DiI through the ESL. Altered ESL transport

properties of DiI, for example due to alterations in ionic composition of the perfusate solution (see chapter 4) or due to alterations in protein composition of the perfusate (see chapter 5), might change DiI fluorescence kinetics measured at the endothelium. Nevertheless, many other factors may influence DiI fluorescence kinetics as well, such as the dynamics of the incorporation process or the spreading of DiI over the endothelial membranes by lateral diffusion. In the present study we evaluated DiI fluorescence kinetics as measured in the various permeability studies in this thesis. In particular, we evaluated whether the dynamics of DiI fluorescence were dominated by ESL transport properties or by the incorporation process of DiI in the endothelial membranes. This way one could obtain more insight in transport properties or vessel wall interactions of e.g. vasoactive agents or hormones that have to interact with the endothelium to exert their function, using DiI and its fluorescence dynamics as a model for these agents.

6.3 MATERIALS AND METHODS

6.3.1 Experiments

A full description of the experiments in which all DiI fluorescence data was collected is given elsewhere (chapter 2, 4 and 5). Briefly, small arteries (~170 μm) were isolated from rat mesentery, cannulated and perfused with MOPS-buffered saline solutions of various compositions. Table 6.1 summarizes the different perfusion protocols that will respectively be referred to as the "localization-protocol" (chapter 2), the "ionic-strength-protocol" (chapter 4) and the "albumin-protocol" (chapter 5). In the localization-protocol 3 extra experiments had been performed, in which the ESL was destroyed by prolonged illumination of the arteries with a bright mercury lamp (light-dye-treatment, LDT) after 30 min of perfusion with DiI and FITC-Δ148 (for details see chapter 2). As reported previously, no effect of LDT on DiI fluorescence characteristics was observed (data not shown). Kinetics of arterial filling with FITC-dextrans (FITC-Δs) of different sizes and accumulation in the endothelium of the fluorescent membrane probe DiI (see fluorescent probes) were recorded by means of confocal laser scanning microscopy (CLSM). For the localization-protocol a Leica Fluovert CLSM was used; for the ionic-strength-protocol and for the albumin-protocol a newly obtained Leica DM IRBE microscope equipped with a Leica TCS SP2 confocal unit was used (for detailed descriptions of CLSM image acquisition see chapter 2, 4 and 5). Rapid inflow of fluorescent tracers into the arteries was accomplished by using a double-barreled θ-pipet. Radial fluorescence profiles of all confocal recorded images were analyzed with procedures written in Matlab (The Mathworks Inc., USA).

Table 6.1: summary of arterial perfusion protocols

Protocol	***Experimental conditions***						***Results***	
	perfusate	*superfusate*	*fluorescent tracers*			*n*	d_{ESL} *from* $X_s^{t=2}$ *[µm]*	d_{ESL} *from* $X_{DiI-FITC-\Delta}^{t=1.5}$ *[µm]*
Localization	MOPS-BSA	MOPS-PSS	FITC-Δ4	and	DiI	7	2.5 ± 0.7	3.0 ± 0.5
	MOPS-BSA	MOPS-PSS	FITC-Δ50	and	DiI	7	3.2 ± 1.7	3.3 ± 0.5
	MOPS-BSA	MOPS-PSS	FITC-Δ148	and	DiI	7	2.8 ± 0.4	3.2 ± 0.8
Ionic strength	LO-MOPS-BSA	LO-MOPS-PSS	FITC-Δ50	and	DiI	7	6.3 ± 1.4	4.8 ± 0.7
	MOPS-BSA	MOPS-PSS	FITC-Δ50	and	DiI	7	2.7 ± 1.0	2.7 ± 0.2
	HI-MOPS-BSA	HI-MOPS-PSS	FITC-Δ50	and	DiI	7	1.1 ± 1.3	1.7 ± 0.3
Albumin	* *MOPS-BSA*	*MOPS-PSS*	*FITC-Δ50*	*and*	*DiI*	*7*	*2.7 ± 1.0*	*2.7 ± 0.2*
	MOPS-PSS	MOPS-PSS	FITC-Δ50	and	DiI	7	3.1 ± 0.3	2.4 ± 0.5

* First group of albumin-protocol (MOPS-BSA-group) is the same as the second group of the ionic-strength-protocol.

n : number of arteries; d_{ESL}: ESL thickness;

$X_s^{t=2}$: exclusion zone to FITC-Δ after 2 min, as determined from the shift of the arterial fluorescence profile in comparison to profile in a glass tube (ref);

$X_{DiI-FITC-\Delta}^{t=1.5}$: distance between the front of the DiI and FITC-Δ fluorescence profiles after 1.5 min.

6.3.2 DiI fluorescence profiles analyses

Accumulation of DiI in the endothelial cells of the arterial wall appeared observable in the fluorescence profiles as peaks, increasing in intensity over time (see for example fig. 2.2, chapter 2). The location of the maximum of these fluorescence peaks was determined after subtraction of the luminal DiI fluorescence (i.e. the profile after 1.5 min of dye perfusion) and was used as the position of the endothelium, as described previously (chapter 2). The normalized position of the DiI peaks (NP_{DiI}), introduced to allow for comparison of arteries of different diameters, was calculated as follows (see figure 6.3):

$$NP_{DiI}(t) = \frac{X_{DiI-midlumen}(t)}{< X_{DiI-midlumen} >} \tag{6.1}$$

where:

$NP_{DiI}(t)$ = normalized DiI peak position over time [norm]

$X_{DiI\text{-}midlumen}(t)$ = distance from DiI-peak to midluminal position over time [µm]

$< X_{DiI\text{-}midlumen} >$ = average distance $X_{DiI\text{-}midlumen}$ from 2-30 min of dye perfusion [µm].

Normalized DiI positions were calculated for left and right DiI peak (i.e. left and right arterial wall) separately. When DiI peak positions remain constant over time, $NP_{DiI}(t)$ will be equal to unity. Furthermore, maximal peak intensity was measured over time and normalized to midluminal DiI fluorescence intensity. The shape of the DiI peaks was characterized by their full-width-at-half-maximal-value (FWHM).

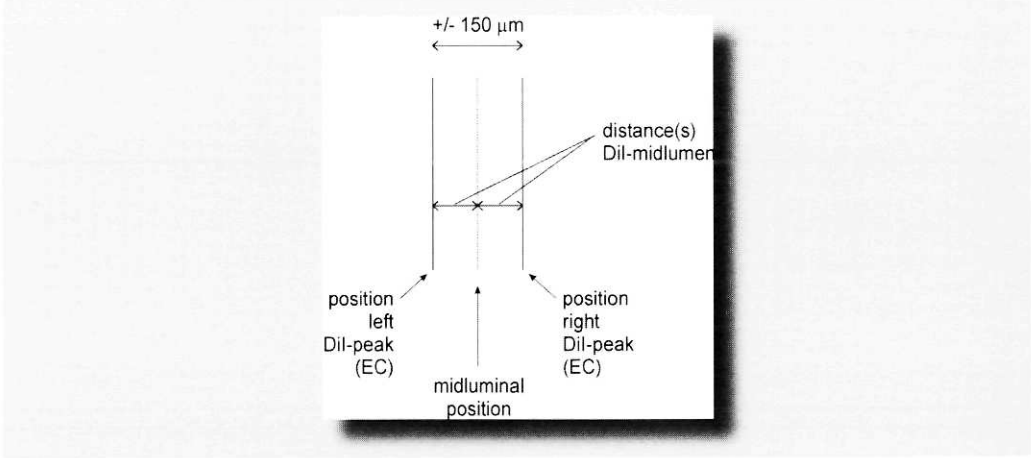

Figure 6.3: Calculation of the normalized DiI peak position (NP_{DiI}). NP_{DiI} was calculated for left and right DiI peak separately according to eq. 6.1.

6.3.3 Fluorescent probes

The lipophilic membrane tracer DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate) was purchased from Molecular Probes (D-282). DiI was applied in a concentration of $1.0 \cdot 10^{-5}$ M in all experiments. Once incorporated in the endothelial membrane (fig. 6.2), DiI will stay there for the remainder of the experiment. DiI is able to spread along the membrane of an endothelial cell, but cannot migrate from one cell to another (6-9;20).

6.3.4 Statistics

Data are presented as means ± SEM. Parameters describing fluorescence characteristics for the different protocols were compared using ANOVA and bonferroni post-hoc tests. Paired t-tests were used for the comparison of parameters at 2 min versus 30 min.

6.4 RESULTS

The average development of DiI fluorescence peaks during the 30 min dye perfusion period for the different protocols is shown in figure 6.4. Left and right DiI peak were averaged per artery. The position of the maximum was taken here as position 0 μm, which presumably represents the location of the endothelium. Positive positions represent luminal of the endothelium, negative positions represent abluminal of the endothelium. Since fluorescence of DiI bound to the endothelium appeared rather heterogeneous and different from one artery to another, the DiI peaks in fig. 6.4 were normalized to maximal peak fluorescence after 30 min of dye perfusion, to allow for comparison of different arteries. In the localization-protocol no differences in the DiI peaks were observed when perfusion with either FITC-Δ4 (4 kD, fig. 6.4A), FITC-Δ50 (50 kD, fig. 6.4B) or with FITC-Δ148 (148 kD, fig. 6.4C) in combination with DiI. In the ionic-strength-protocol no differences in the DiI peaks were observed for the different ionic strength conditions (low ionic strength (LO-MOPS, fig. 6.4D), normal ionic strength (MOPS, fig. 6.4E) or high ionic strength (HI-MOPS, fig. 6.4F)). In the albumin-protocol no differences in the DiI peaks were observed between the different perfusate conditions (MOPS-BSA (fig. 6.4G) or protein-free MOPS-PSS (fig. 6.4H)).

DiI peak position remained constant over time. Average standard deviations of variations in this position were 0.24 ± 0.01 μm in the localization-protocol, 0.34 ± 0.01 μm in the ionic-strength-protocol, and 0.26 ± 0.01 μm in the albumin-protocol, i.e. less than one pixel in all

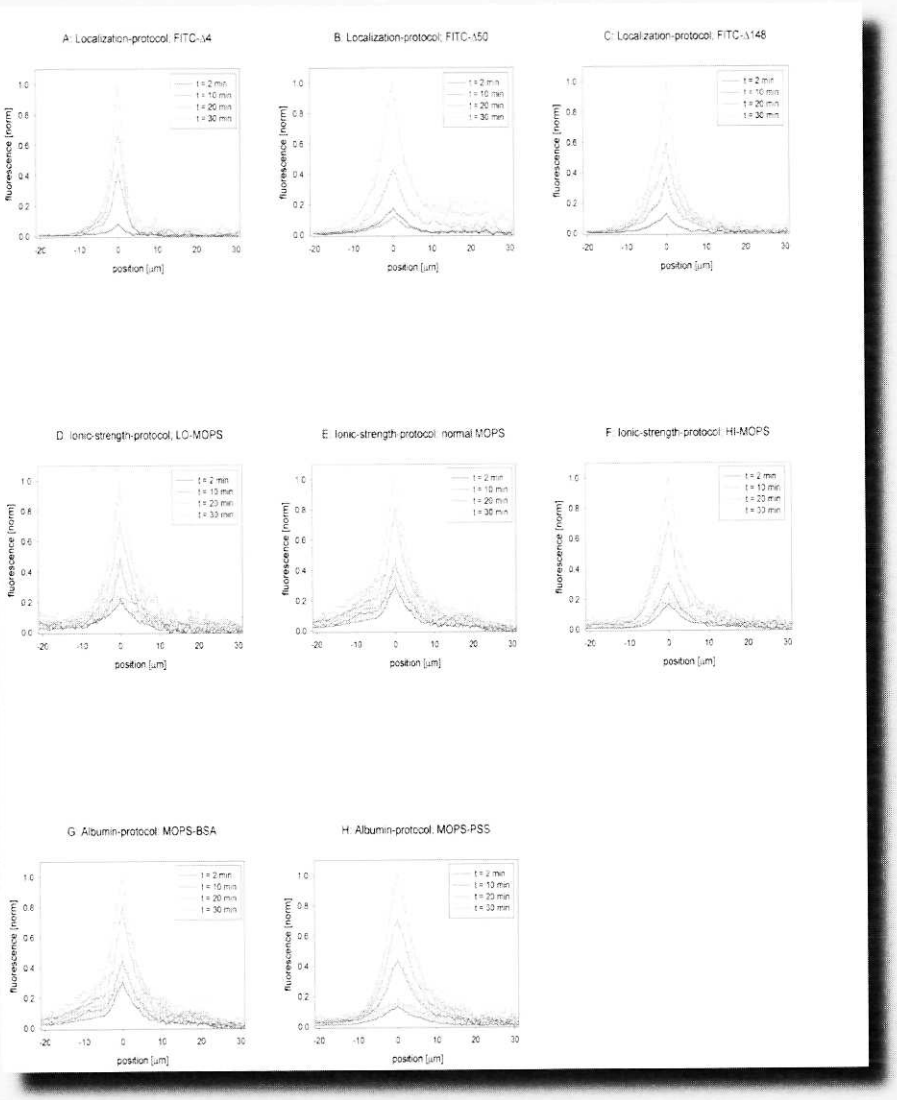

Figure 6.4: Average normalized DiI fluorescence profiles near the arterial wall during 30 min of dye perfusion for the localization-protocol, when perfusing with DiI in combination with FITC-Δ4 (A), with FITC-Δ50 (B) or with FITC-Δ148 (C), for the ionic-strength-protocol when using LO-MOPS (D), normal MOPS (E) or HI-MOPS (F) and for the albumin-protocol when perfusing with MOPS-BSA (G) or with MOPS-PSS (H). Left and right DiI peak were averaged per artery. The position of the maximum was taken as position 0 µm, which represents the location of the endothelium. Positive positions represent luminal of the endothelium, negative positions represent abluminal of the endothelium.

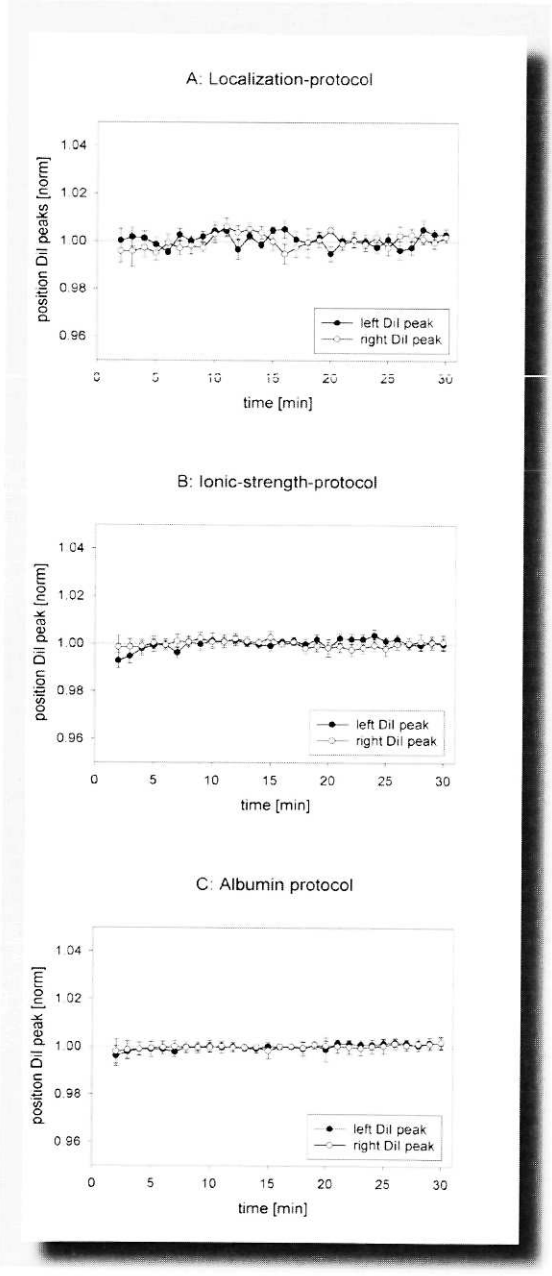

Figure 6.5: Average normalized DiI positions (NP_{DiI}) of the left and right DiI peak for the localization-protocol (A), the ionic-strength-protocol (B) and for the albumin-protocol (C).

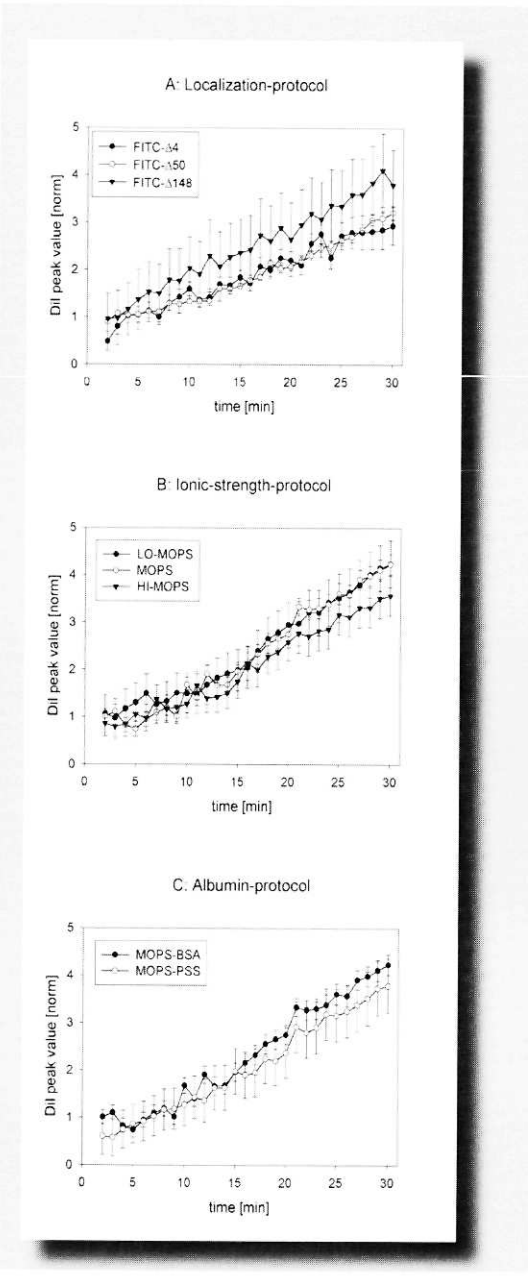

Figure 6.6: Average maximal DiI peak intensity, normalized to mid-luminal DiI fluorescence intensity, for the localization-protocol (A) when perfusing with DiI in combination with resp. FITC-Δ4, FITC-Δ50 or FITC-Δ148 (see also chapter 2), for the ionic-strength-protocol (B) when using resp. LO-MOPS, normal MOPS or HI-MOPS (see also chapter 4), and for the albumin-protocol (C) when perfusing with resp. MOPS-BSA or MOPS-PSS (see also chapter 5).

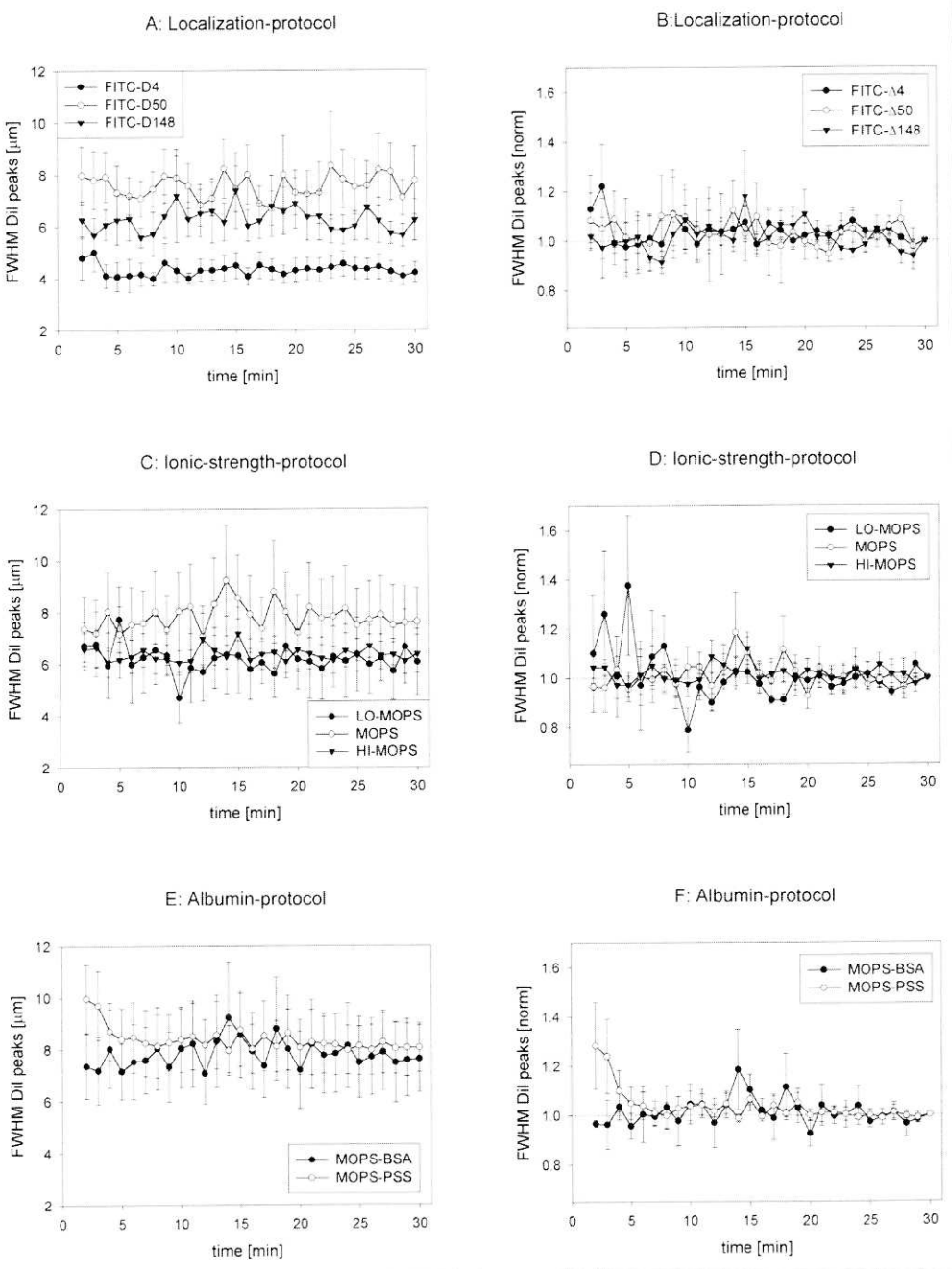

Figure 6.7: Average absolute full-width-at-half-maximal-value (FWHM) of the DiI peaks for the localization-protocol (A), the ionic-strength-protocol (C) and for the albumin-protocol (E). Average normalized FWHM, normalized to the value after 30 min of dye perfusion, for the localization-protocol (B), the ionic-strength-protocol (D) and for the albumin-protocol (F).

protocols. DiI staining was always located luminally of the autofluorescent internal elastic lamina (on average 1.2 ± 0.1 μm), confirming confinement of DiI to the endothelium. Normalized DiI positions, calculated using equation 6.1, for the different protocols are depicted in figure 6.5. This figure confirms that DiI positions remained stable over time. Normalized DiI positions were always close to unity, with deviations less than 0.75%, i.e. in the order of one pixel. No differences were observed between left and right DiI peaks.

Maximal DiI peak intensity over time, normalized to mid-luminal DiI fluorescence intensity, is depicted in figure 6.6. Normalized peak intensity increased at approximately constant rate and did not reach saturation within 30 min of dye perfusion. No differences in normalized peak intensity or in the increase rate of normalized peak intensity between the different groups of arteries or between the different protocols were observed.

The shape of the DiI peaks, as characterized by their full-width-at-half-maximal-value (FWHM), remained constant over time as well (figure 6.7). Although DiI binding is confined to theendothelium, which is ~0.2 μm thick, the DiI fluorescence profile is much broader, with a width (FWHM) of ~6-8 μm. Furthermore, no differences in FWHM between the different groups of arteries or between the different protocols were observed (fig. 6.7A, C and E). We also normalized FWHM to the value after 30 min of dye perfusion. Average results of the normalized FWHM are depicted in figures 6.7B, D and F. These graphs indicate once more that the shape of the DiI peaks did not change during 30 min of dye perfusion. For the MOPS-PSS group in the albumin-protocol (fig. 6.7F) normalized FWHM appeared to decrease over time, but this was not statistically significant.

6.5 DISCUSSION

Analyses of the fluorescence recordings of the lipophilic membrane tracer DiI, whose recordings had been obtained during previously described permeability studies (chapter 2, 4 and 5), revealed that fluorescence kinetics due to incorporation of DiI in the membranes of endothelial cells of isolated arteries were not dependent on experimental conditions such as the presence of different anionic macromolecules, solvent ionic composition, or the presence of proteins in the perfusate.

6.5.1 Application of DiI

Fluorescent carbocyanine dyes such as DiI have been applied to label neurons (2;5;8;9;17;18;20;22), tumor cells (7), leukocytes (4), red blood cells (RBCs) (12;19), low-density lipoproteins (11;16),

smooth muscle cells (15) and endothelial cells (ECs) (3;13-15). Labeling of these cells or particles with DiI has been applied for purposes of cell identification (8-10;17), cell-cell attachment (4;7), cell growth and development (2;5;13;14;18), microvascular measurements such as RBC flux (19), membrane fluidity measurements (3) and permeability studies (11;16). DiI staining of cells or particles can be obtained by amongst others direct application of dye crystals, loading by micro-injection, or labeling in a dye-containing solution (6) and can even be obtained in fixed tissues (5). Once incorporated in a membrane, DiI can spread along the membrane by lateral diffusion (3;5;6;8-10;12). No transfer of DiI from labeled to unlabeled cells occurs, not even when both cell types are attached to each other (6-9;20). Furthermore, DiI does not affect basic physiological cell properties or functions (4;6-8;10).

Due to its properties we considered DiI a suitable tracer to label the endothelial cells in isolated cannulated arteries, in order to localize the arterial wall during confocal fluorescence recordings. We originally hypothesized that the kinetics of DiI fluorescence due to its incorporation in the endothelium would reflect the transport properties of DiI through the endothelial surface layer (ESL). Altered ESL transport properties of DiI, for example due to alterations in ionic composition of the perfusate solution (see chapter 4) or due to alterations in protein composition of the perfusate (see chapter 5), might result in altered DiI fluorescence kinetics measured at the endothelium. Nevertheless, many other factors may influence DiI fluorescence kinetics, such as the dynamics of the incorporation process or the spreading of DiI over the endothelial membrane by lateral diffusion.

6.5.2 Transport of DiI through the endothelial surface layer

Vink and Duling described that transport of small neutral or positively charged molecules is not hindered by the negatively charged endothelial surface layer (ESL) (21). Transport of the small (MW 934), positively charged (valence +1, see fig. 6.1) DiI molecules through the ESL should therefore be relatively fast, especially in comparison to large anionic FITC-Δs. Indeed we observed that, already 1.5 min after simultaneous infusion into the arteries, the fluorescence column of DiI was more wide than the FITC-Δ columns. The difference in the width of these columns should therefore reflect the dimension of the ESL and provides a method to estimate the ESL thickness that is independent of the widening of the FITC-Δ columns over 30 min of dye perfusion. These estimates have been summarized in table 6.1, as well as the estimates of ESL thickness as obtained from a previously developed and verified procedure (see chapter 2). Thus, the estimates of ESL thickness as obtained from the distance of the DiI fluorescence profile to the

FITC-Δ fluorescence profile (i.e. half the difference in fluorescence column width) after 1.5 min of dye perfusion, was essentially equal to the estimates of ESL thickness as obtained from the shift in the arterial FITC-Δ fluorescence profile with respect to the endothelium after 2 min of dye perfusion as compared with the FITC-Δ fluorescence profile in a glass tube with respect to the tube wall Hence, transport of DiI through the ESL is very fast and is – unlike FITC-Δ transport – not influenced by experimental conditions that influence ESL permeability such as solvent ionic composition or the presence of albumin in the perfusate.

6.5.3 Spreading of DiI over the endothelial membranes

Carbocyanine dyes such as DiI spread over membranes in which they are incorporated due to lateral diffusion. Lateral diffusion coefficients for DiI have been reported to be in the order of $10^{-8} - 10^{-7}$ $cm^2 \cdot s^{-1}$ (3;8;10;12). Adamson reported that arterial endothelial cells are approximately ~140 μm long (1). Furthermore, from his data on endothelial cell perimeter and surface area, the width of endothelial cells can be estimated to be approximately ~10 μm (1). Thus, the typical diffusion time for DiI to spread lengthwise over an endothelial cell (EC) is in the order of 6.5 min and to spread over the width of an EC in the order of 8 sec. Since we may assume that the DiI concentration at the luminal surface of the endothelium is uniform and constant, we may also assume that DiI incorporates into the EC membrane along the entire length of the cell and not at only a single position. Thus after incorporation, spreading of DiI over the EC membrane is solely determined by diffusion in circumferential direction (i.e. over the width of the cell), and therefore we feel that spreading of DiI over the membrane should be very fast. Nevertheless, time scales for labeling procedures that make use of lateral dye diffusion have been reported to range from about 10 min to several days, to label cells or neurons over distances ranging from ~100 μm up to several millimeters (2-5;7-20;22).

6.5.4 Incorporation of DiI in the endothelial membranes

As far as we know, no information from literature is available on the dynamics of the incorporation process of DiI into the endothelial membranes. Assuming that continuous incorporation of DiI into the EC membrane takes place and that a single EC membrane can contain a large amount of DiI, the dynamics of the incorporation process are most likely responsible for the relatively slow saturation of the DiI fluorescence signal, as reflected in the maximal peak fluorescence intensity (see fig. 6.6), which did not saturate within 30 min.

6.5.5 Conclusion

In conclusion, we demonstrate that fluorescence kinetics due to incorporation of the lipophilic tracer DiI in endothelial cell membranes of isolated arteries were not dependent on experimental conditions such as the presence of different anionic macromolecules, solvent ionic composition, or the presence of proteins in the perfusate. DiI fluorescence kinetics are mainly determined by the dynamics of the incorporation process, and not by the dynamics of ESL transport of DiI or by spreading over the EC membrane by lateral diffusion.

6.6 REFERENCES

1. **Adamson,R.H.** Microvascular endothelial cell shape and size in situ. *Microvasc Res* 46: 77-88, 1993.
2. **Balice-Gordon,R.J., C.K.Chua, C.C.Nelson, and J.W.Lichtman.** Gradual loss of synaptic cartels precedes axon withdrawal at developing neuromuscular junctions. *Neuron* 11: 801-815, 1993.
3. **Butler,P.J., G.Norwich, S.Weinbaum, and S.Chien.** Shear stress induces a time- and position-dependent increase in endothelial cell membrane fluidity. *Am J Physiol-Cell Ph* 280: C962-C9692001.
4. **De Clerck LS, C.H.Bridts, A.M.Mertens, M.M.Moens, and W.J.Stevens.** Use of fluorescent dyes in the determination of adherence of human leucocytes to endothelial cells and the effect of fluorochromes on cellular function. *Journal of Immunological Methods* 172: 115-124, 1994.
5. **Godement,P., J.Vanselow, S.Thanos, and F.Bonhoeffer.** A study in developing visual systems with a new method of staining neurones and their processes in fixed tissue. *Development* 101: 697-713, 1987.
6. **Haugland,R.P** Handbook of Fluorescent Probes and Research Chemicals. Eugene, *OR: Molecular Probes, Inc.*, 1996.
7. **Hiscox,S. and W.G.Jiang.** Quantification of tumour cell-endothelial cell attachment by 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI). *Cancer Lett.* 112: 209-217, 1997.
8. **Honig,M.G. and R.I.Hume.** Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures. *J Cell Biol* 103: 171-187, 1986.
9. **Honig,M.G. and R.I.Hume.** Carbocyanine dyes. Novel markers for labelling neurons. *Trends.Neurosci.* 12: 336-338, 1989.
10. **Honig,M.G. and R.I.Hume.** DiI and diO: versatile fluorescent dyes for neuronal labelling and pathway tracing. *Trends in Neurosciences* 12: 333-335, 1989.
11. **Kao,C.H., J.K.Chen, J.S.Kuo, and V.C.Yang.** Visualization of the transport pathways of low density lipoproteins across the endothelial cells in the branched regions of rat arteries. *Atherosclerosis* 116: 27-41, 1995.
12. **Kapitza,H.G. and E.Sackmann.** Local measurement of lateral motion in erythrocyte membranes by photobleaching technique. *Biochim Biophys Acta* 595: 56-64, 1980.
13. **Nehls,V., R.Herrmann, and M.Huhnken.** Guided migration as a novel mechanism of capillary network remodeling is regulated by basic fibroblast growth factor. *Histochem Cell Biol* 109: 319-329, 1998.
14. **Nehls,V., R.Herrmann, M.Huhnken, and A.Palmetshofer.** Contact-dependent inhibition of angiogenesis by cardiac fibroblasts in three-dimensional fibrin gels in vitro: implications for microvascular network remodeling and coronary collateral formation. Cell & Tissue Research 293: 479-488, 1998.
15. **Ragnarson,B., L.Bengtsson, and A.Haegerstrand.** Labeling with fluorescent carbocyanine dyes of cultured endothelial and smooth muscle cells by growth in dye-containing medium. *Histochemistry* 97: 329-333, 1992.
16. **Rutledge,J.C., F.R.Curry, J.F.Lenz, and P.A.Davis.** Low density lipoprotein transport across a microvascular endothelial barrier after permeability is increased. *Circ Res* 66: 486-495, 1990.
17. **Thanos,S.** Specific transcellular carbocyanine-labelling of rat retinal microglia during injury-induced neuronal degeneration. *Neurosci.Lett.* 127: 108-112, 1991.
18. **Thanos,S. and F.Bonhoeffer.** Axonal arborization in the developing chick retinotectal system. *J Comp Neurol* 261: 155-164, 1987.
19. **Unthank,J.L., J.M.Lash, J.C.Nixon, R.A.Sidner, and H.G.Bohlen.** Evaluation of carbocyanine-labeled erythrocytes for microvascular measurements. *Microvasc Res* 45: 193-210, 1993.
20. **Vidal-Sanz,M., M.P.Villegas-Perez, G.M.Bray, and A.J.Aguayo.** Persistent retrograde labeling of adult rat retinal ganglion cells with the carbocyanine dye diI. *Exp Neurol* 102: 92-101, 1988.
21. **Vink,H. and B.R.Duling.** Capillary endothelial surface layer selectively reduces plasma solute distribution volume. *Am J Physiol-Heart C* 278: H285-H2892000.
22. **von Bartheld,C.S., D.E.Cunningham, and E.W.Rubel.** Neuronal tracing with DiI: decalcification, cryosectioning, and photoconversion for light and electron microscopic analysis. *J Histochem Cytochem* 38: 725-733, 1990.