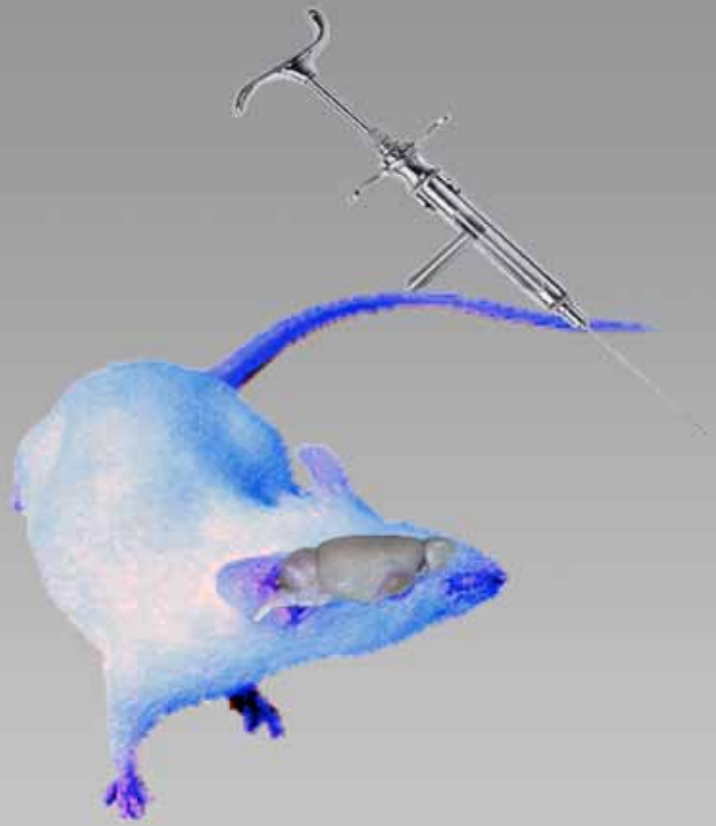
« TESTING BARRIERS AND TRANSPORTS: NEW BASIS »

7th annual *ecopa* WORKSHOP

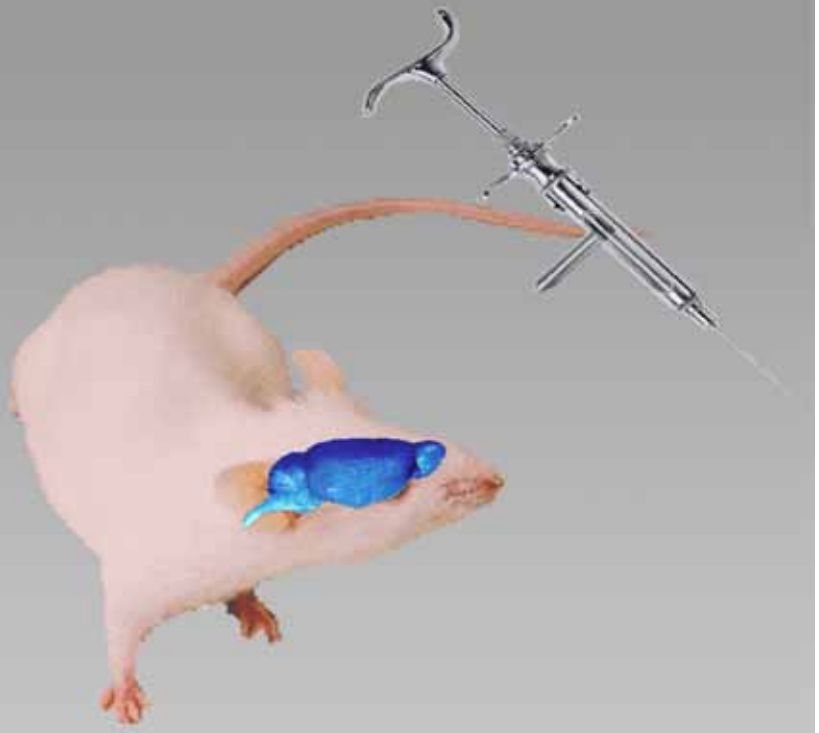
25-26 November 2006, Brussels

REACH for help: Science back-up?

Pr Roméo Cecchelli



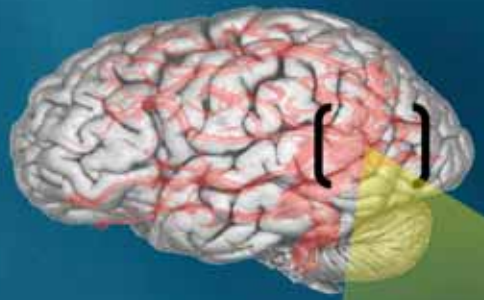
Ehrlich 1885



Goldmann 1913

The BBB was located in brain capillaries

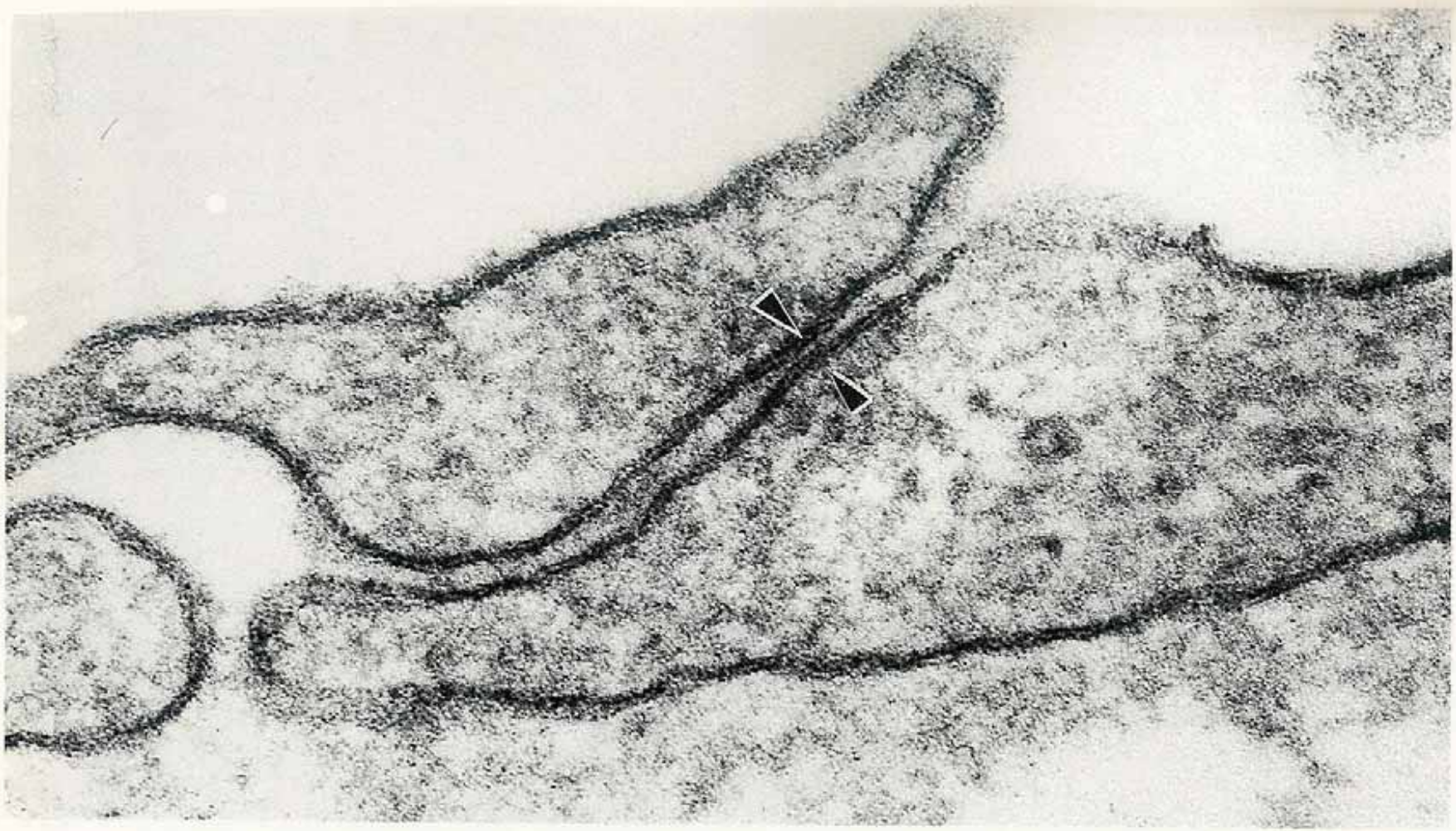
The lenght in Human is around 650 km
Its surface area is around 12 m^2



Brain capillaries

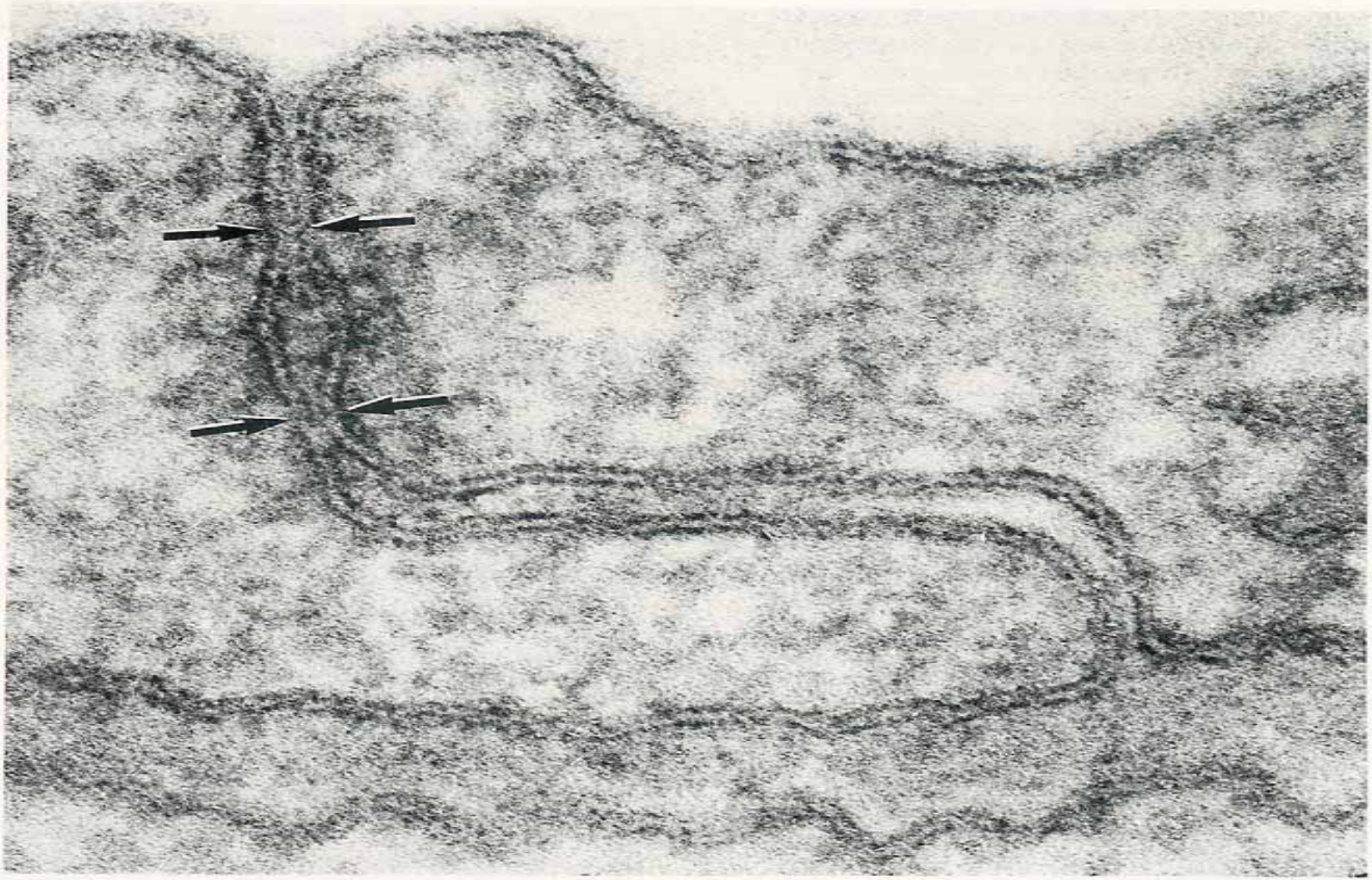
Endothelial cells

Astrocytes



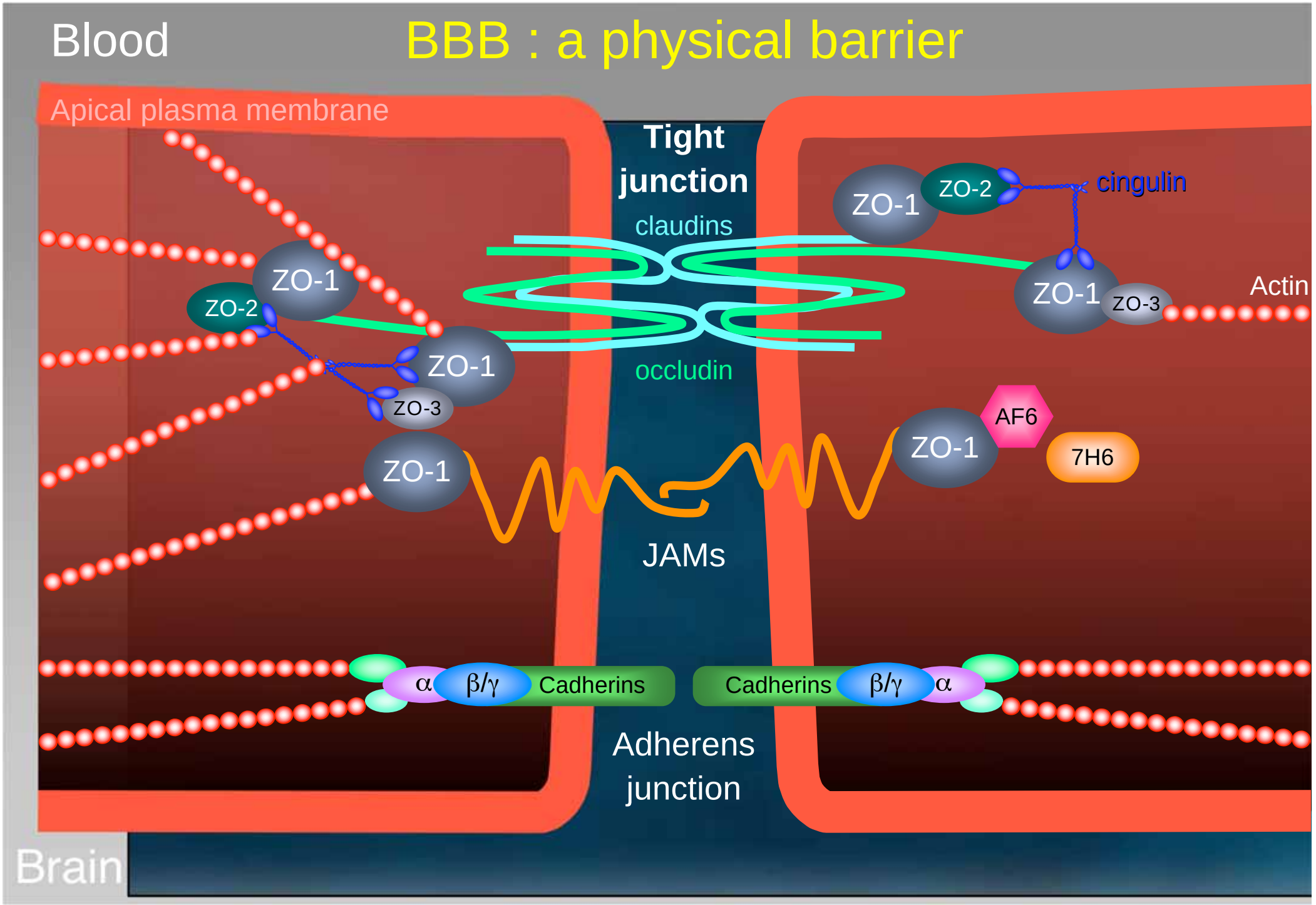
The intercellular cleft at a junction between two endothelial cells of a permeable capillary supplying a choroid plexus papilloma, is narrowed but not occluded. Unlike the tight junction shown in Fig. 1, this junction is patent (*arrowheads*) and is recognized as a junction by cytoplasmic densities situated symmetrically on either side of the cell membrane beneath the arrowheads. The vessel lumen is at top. ×250,000.

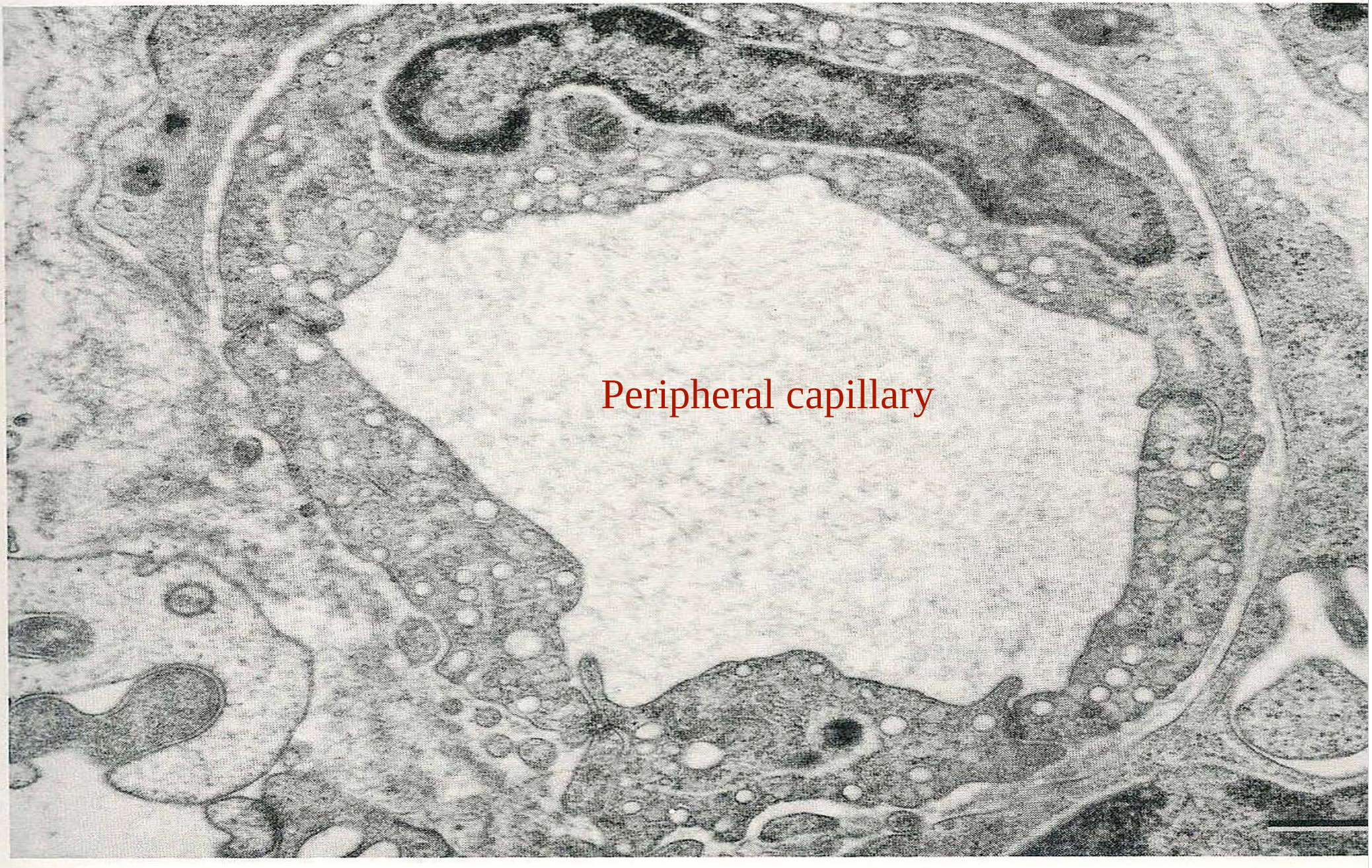
The Blood-Brain Barrier Cellular and Molecular Biology
William M. Pardridge 1993



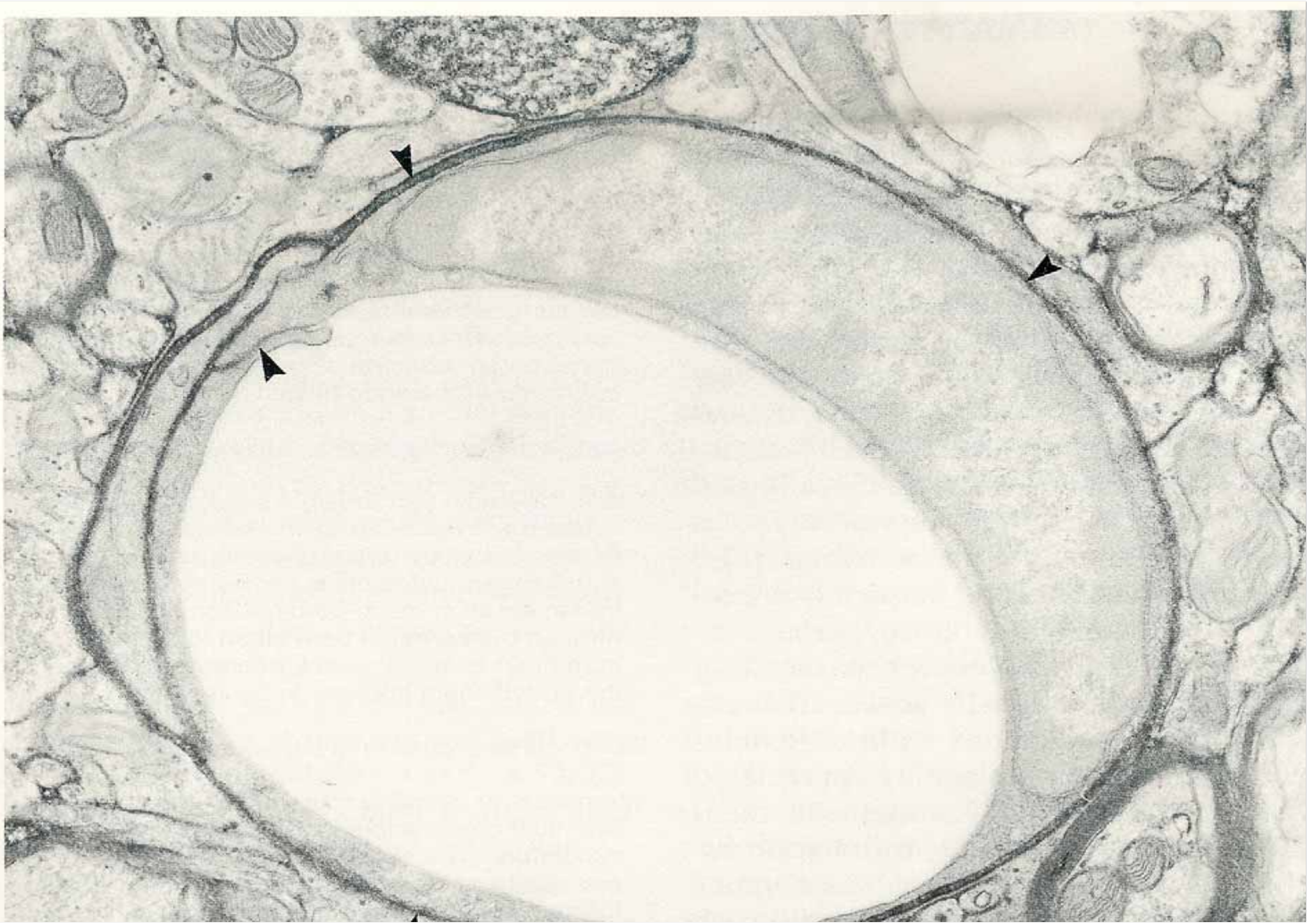
The intercellular cleft between two endothelial cells of a capillary within the brain of a mouse is occluded at two tight junctions (*arrows*) that have been sectioned transversely. In the transverse plane of this thin, plastic-embedded section, the junctions appear as short contacts (1). The vessel lumen is at top. ×380,000.

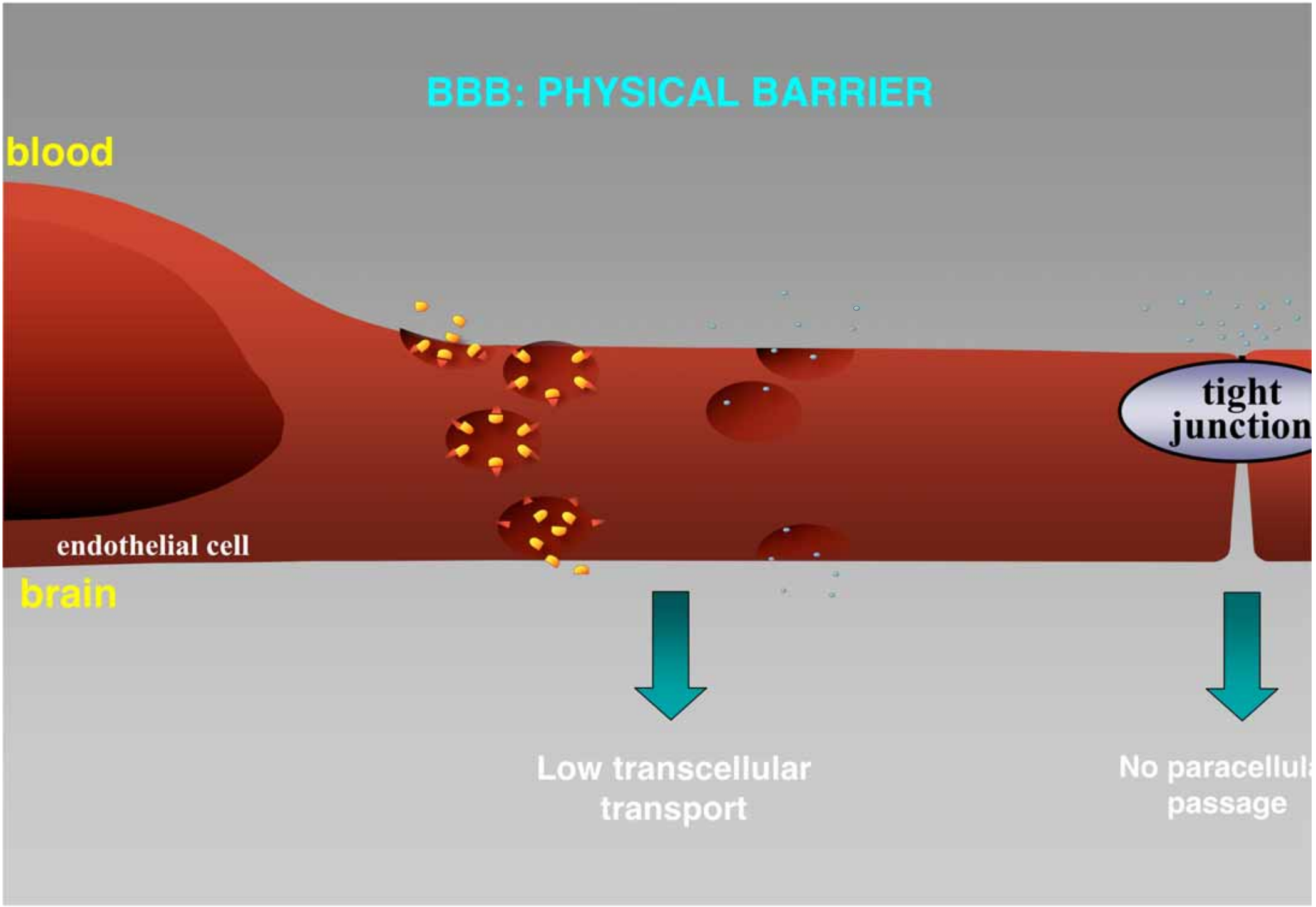
The Blood-Brain Barrier Cellular and Molecular Biology
William M. Pardridge 1993

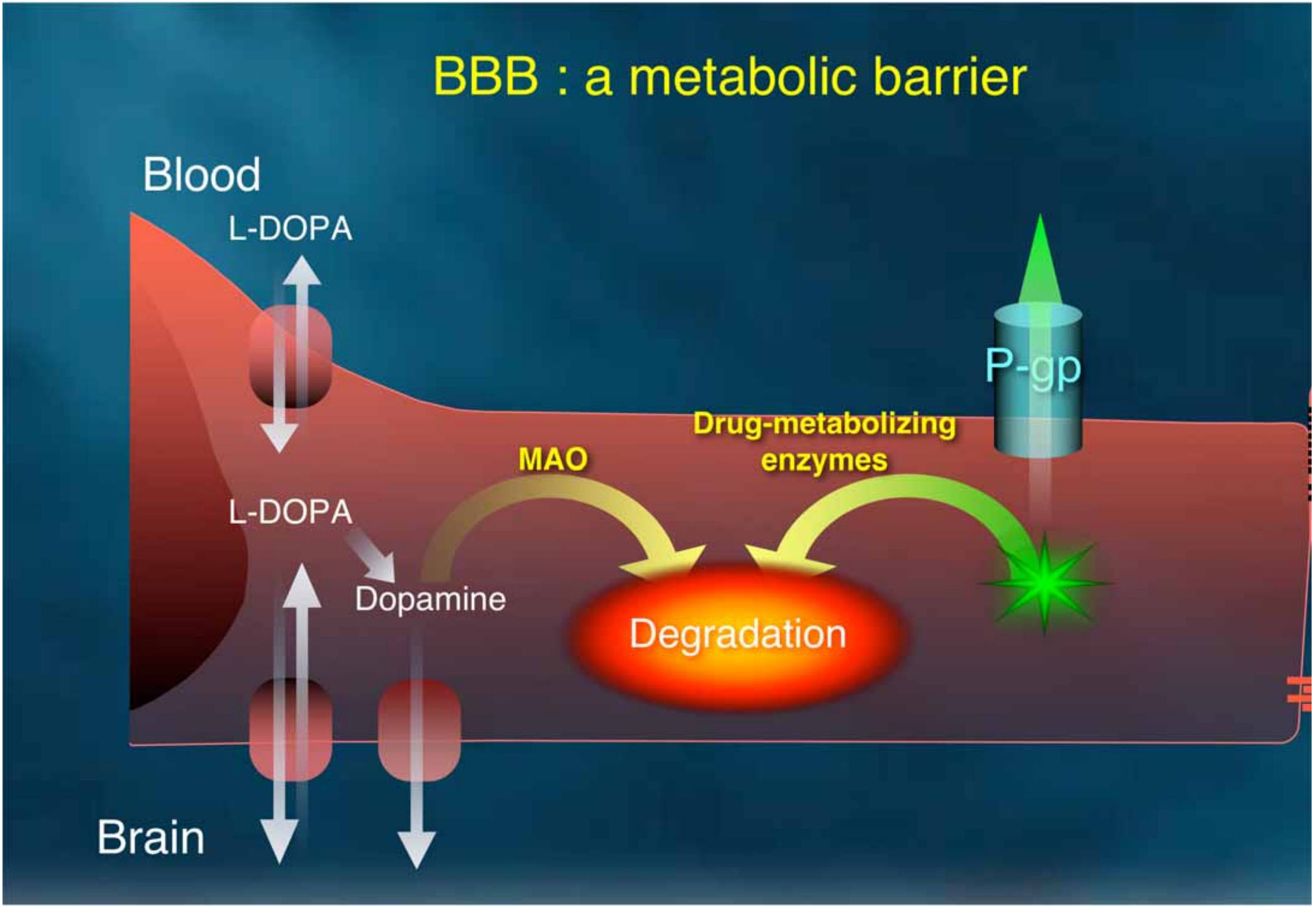

Blood
BBB : a physical barrier
Apical plasma membrane
Tight junction
claudins
occludin
ZO-1
ZO-2
ZO-3
cingulin
Actin
AF6
7H6
JAMs
α
β/γ
Cadherins
Adherens junction
Brain

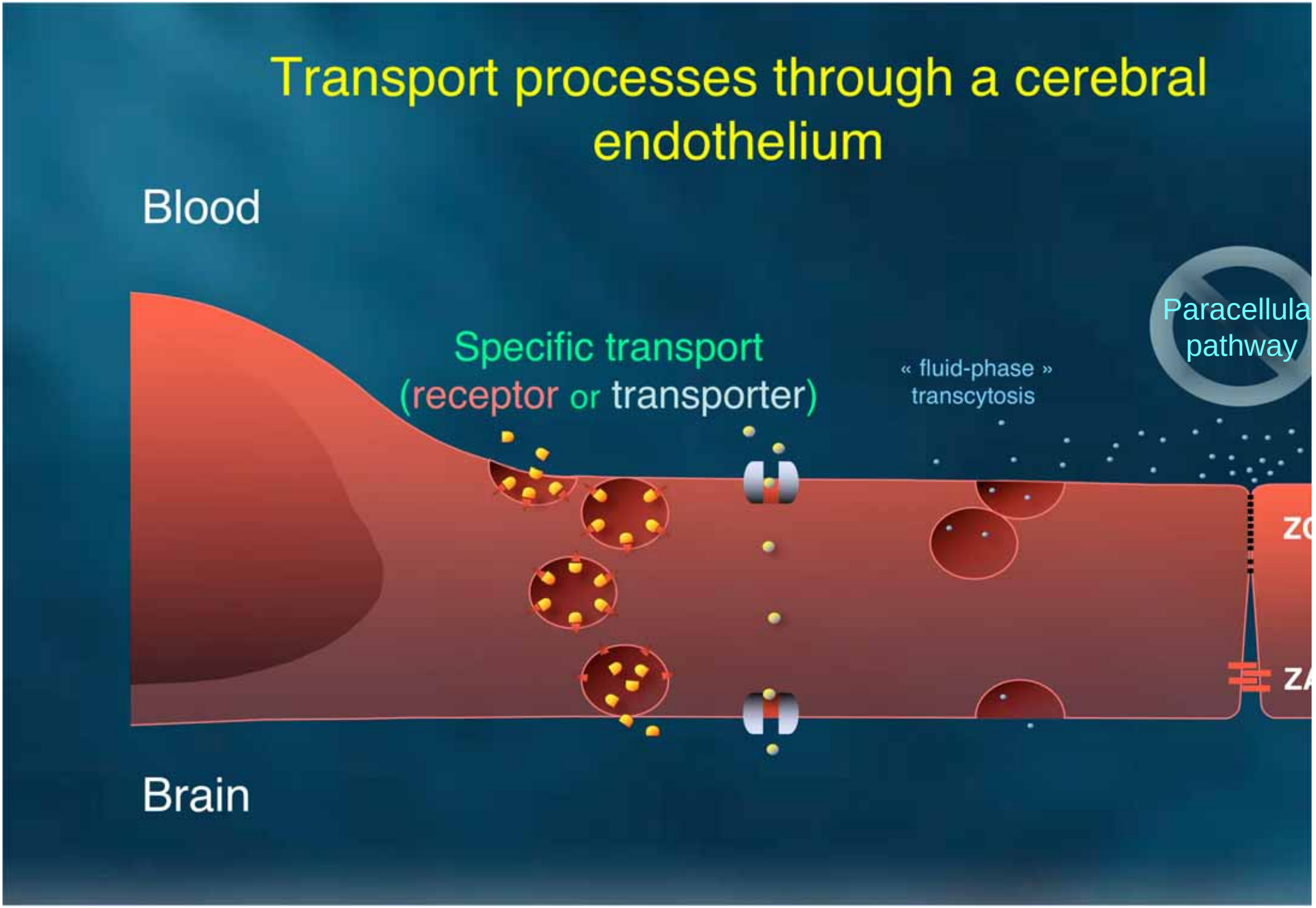


Endothelial Cell Biology
N. Simionescu and M. Simionescu 1988




BBB: PHYSICAL BARRIER
blood
endothelial cell
brain
tight junction
Low transcellular transport
No paracellul
passage


BBB : a metabolic barrier
Blood
L-DOPA
P-gp
Drug-metabolizing
enzymes
MAO
L-DOPA
Dopamine
Degradation
Brain

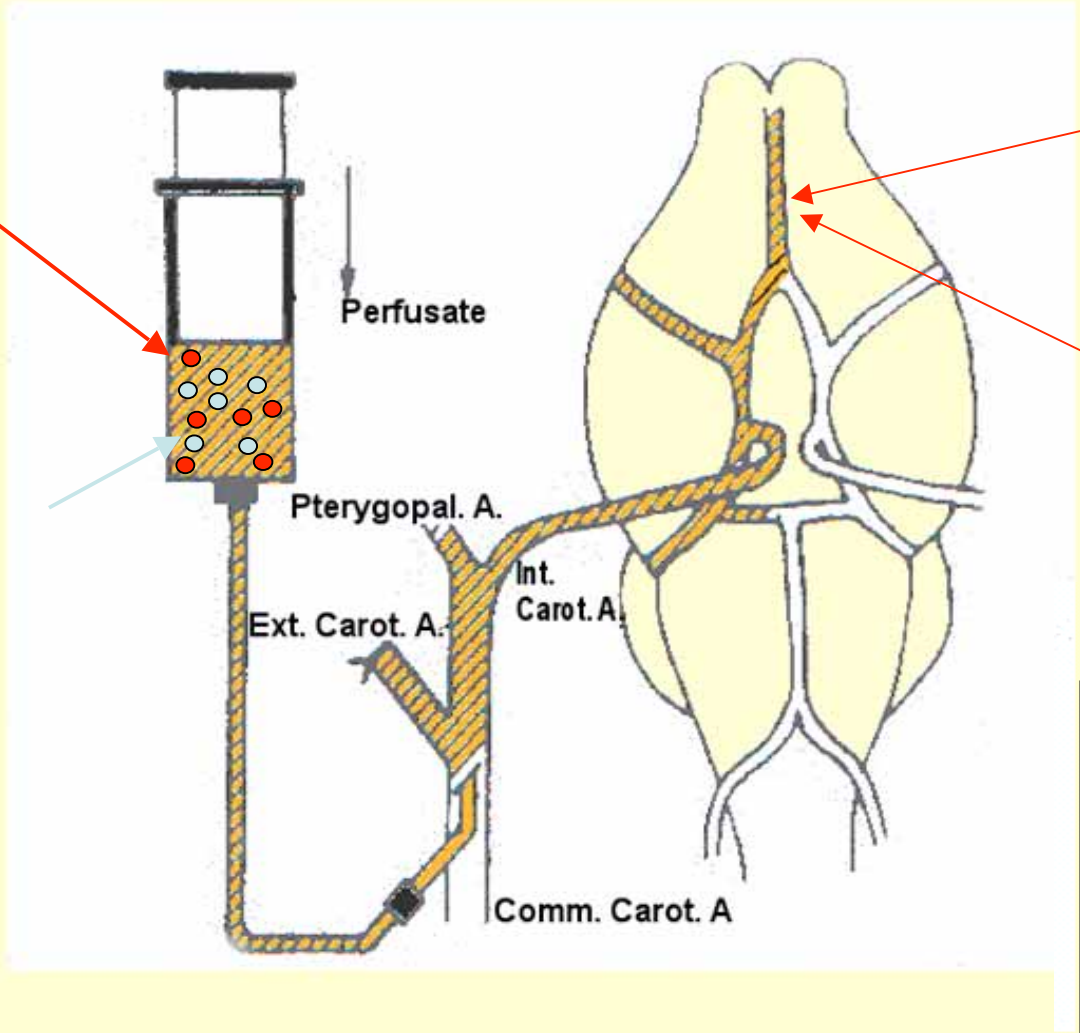

Transport processes through a cerebral endothelium
Blood
Specific transport
(receptor or transporter)
« fluid-phase »
transcytosis
Paracellula
pathway
ZO
ZA
Brain

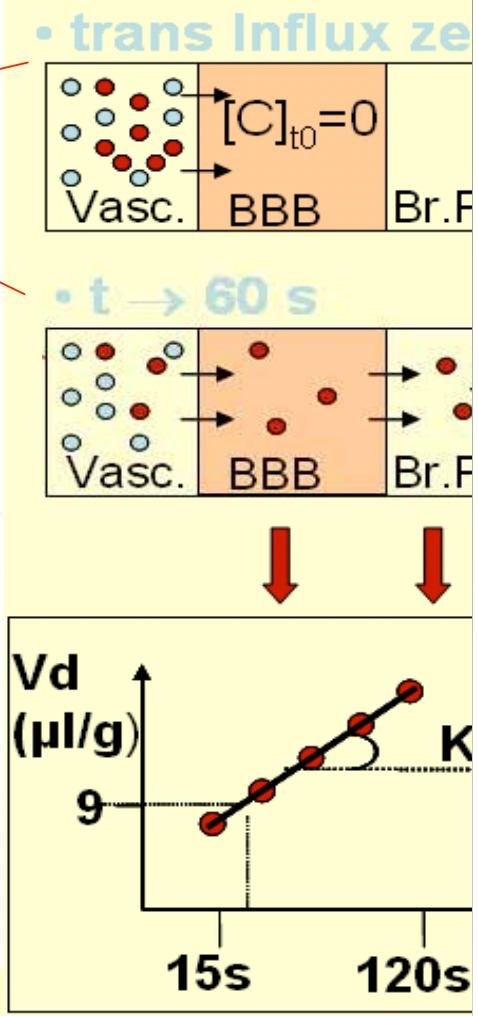
In vivo methods

IN SITU BRAIN PERFUSION TECHNIQUE

$^{3}H/^{14}C$-labelled drug
(to measure BBB permeability)

$^{14}C/^{3}H$ –sucrose/inulin
(to measure cerebro-vascular volume





RAT (Takasato et *al.*, 1984; Smith, 1996)

MOUSE (Dagenais et *al.*, *J Cereb Blood Flow Metab*, 2000)

ZER 2005

- 60 rats are necessery to determine the permeability of a molecule through the BBB

In vitro methods

ECVAM

- Seeks to promote the scientific and regulatory acceptance of alternative methods. Contribution to 3Rs concept (Russel and Burch, 1959) by:

  - ✓ *Reduction*
  - ✓ *Refinement*
  - ✓ *Replacement*

In May 2003: workshop on BBB *in vitro* methods and their application in toxicology

Blood-Brain Barrier *in vitro* models and their application in toxicology

The Report and Recommendations of ECVAM Workshop 49

Altern Lab Anim. 2004 Mar;32(1):37-50.

AIM OF THE WORKSHOP

Discuss the current status of BBB *in vitro* models, focusing on their application in toxicology and their possible use in integrated testing strategies to assess neurotoxicity

TOPICS

A) Minimal criteria needed for an *in vitro* BBB model:

- availability, transferability and ease of culture of the model
- restrictive paracellular permeability and high TEER
- physiological realistic cell architecture
- expression of functional transporters present *in vivo*
- possibility of studying the transport polarity

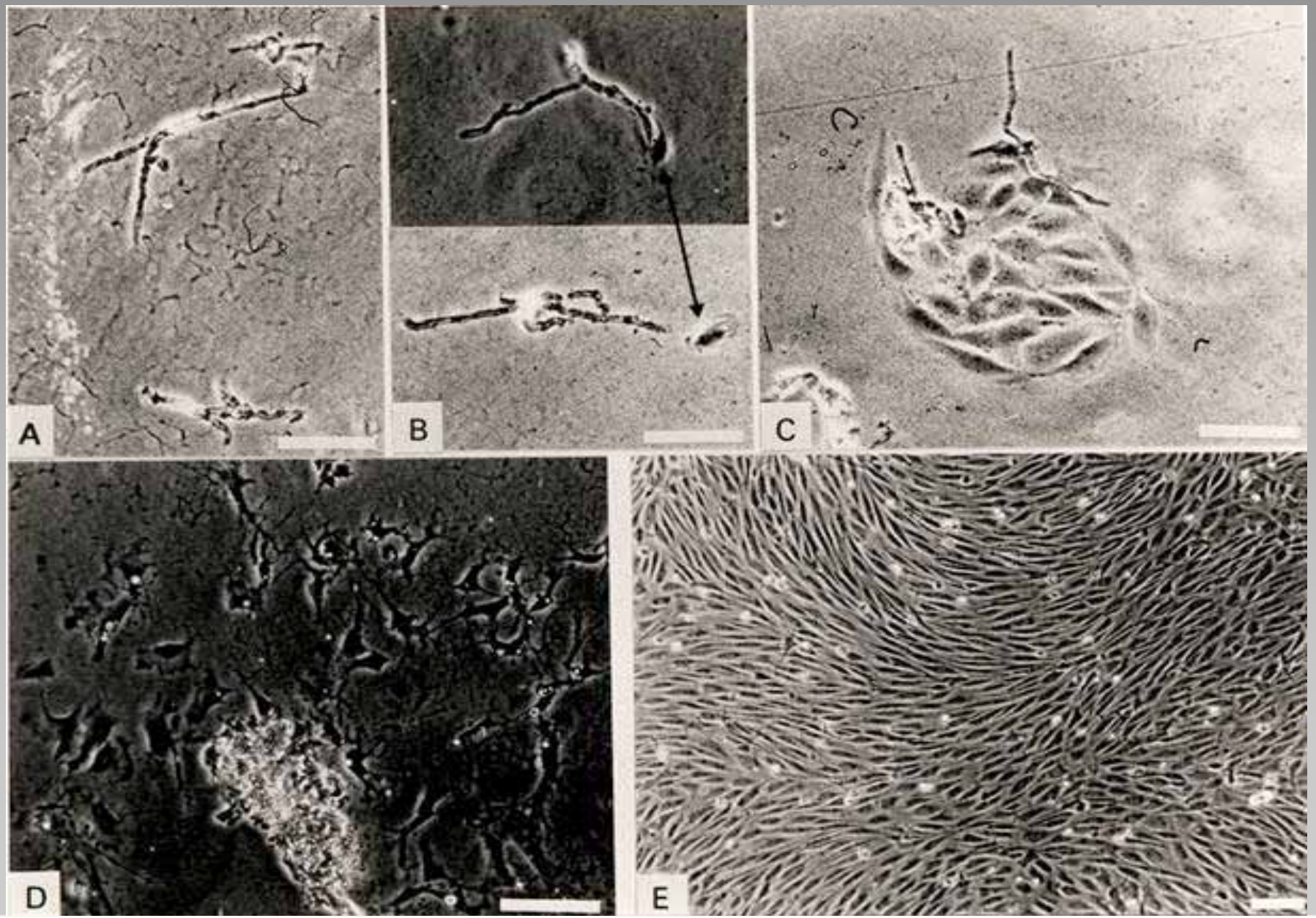
B) Discussion of available BBB *in vitro* models:

- Primary brain endothelial cells (co-cultured with astrocytes)
- Immortalised brain endothelial cells
- Cell lines of non-cerebral origin

Cell lines

- All immortalized brain capillary endothelial cell lines (RBE4, MBEC line, TR-BBB) do not expressed most of the tight junction proteins and are very leaky.
- They cannot be used to study transcellular transport.

⇨I just want to focus my presentation on primary and long term culture of brain capillary endothelial cells.


A
B
C
D
E

Conclusions

Sure to obtain brain capillary endothelial cell culture non contaminated by pericytes.

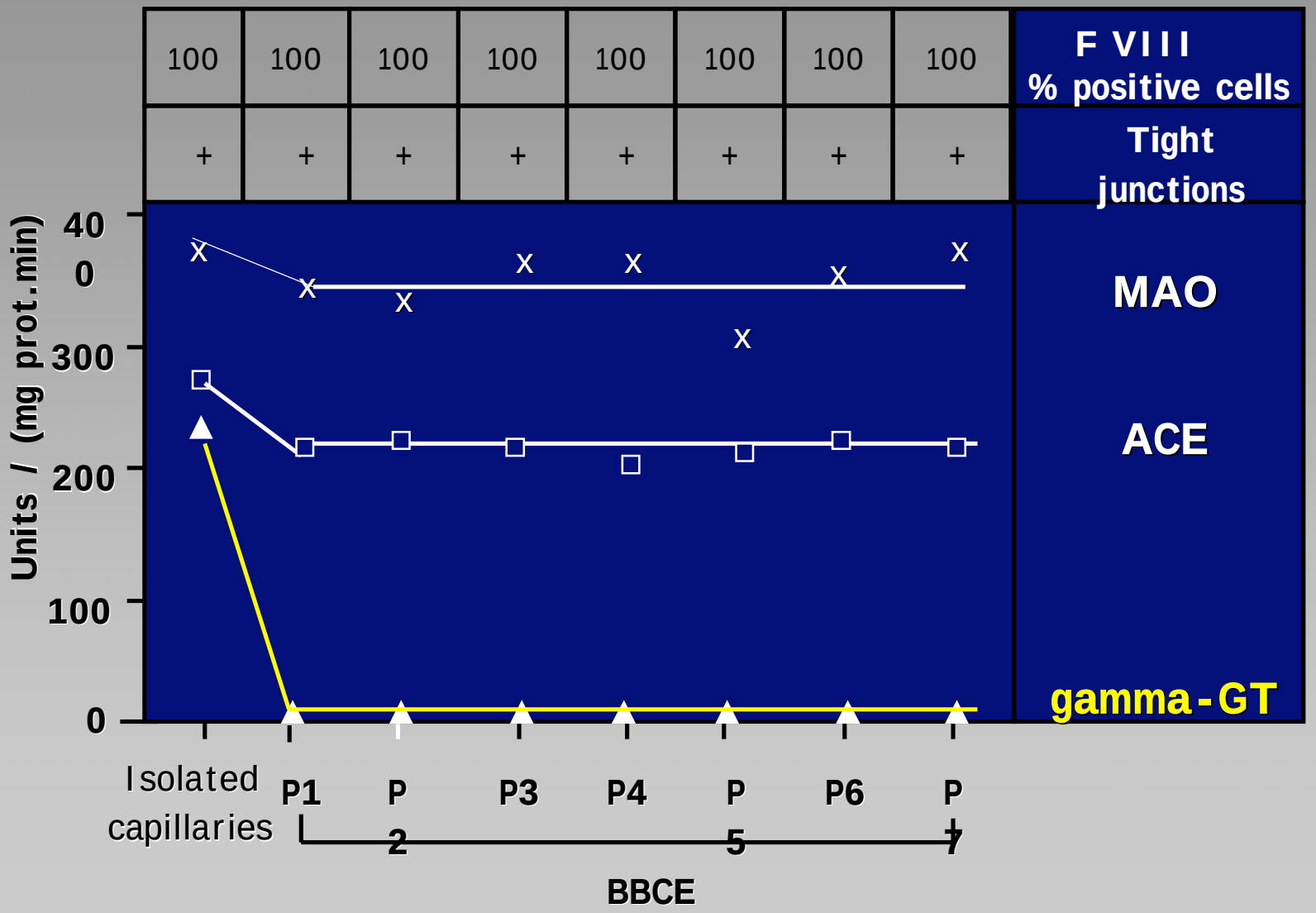
Provide a large quantity of endothelial cells.

What about the Blood Brain Barrier marker ?

Freeze fracture examination of endothelial cells

Transendothelial electrical resistance = 400 Ohms.cm^2

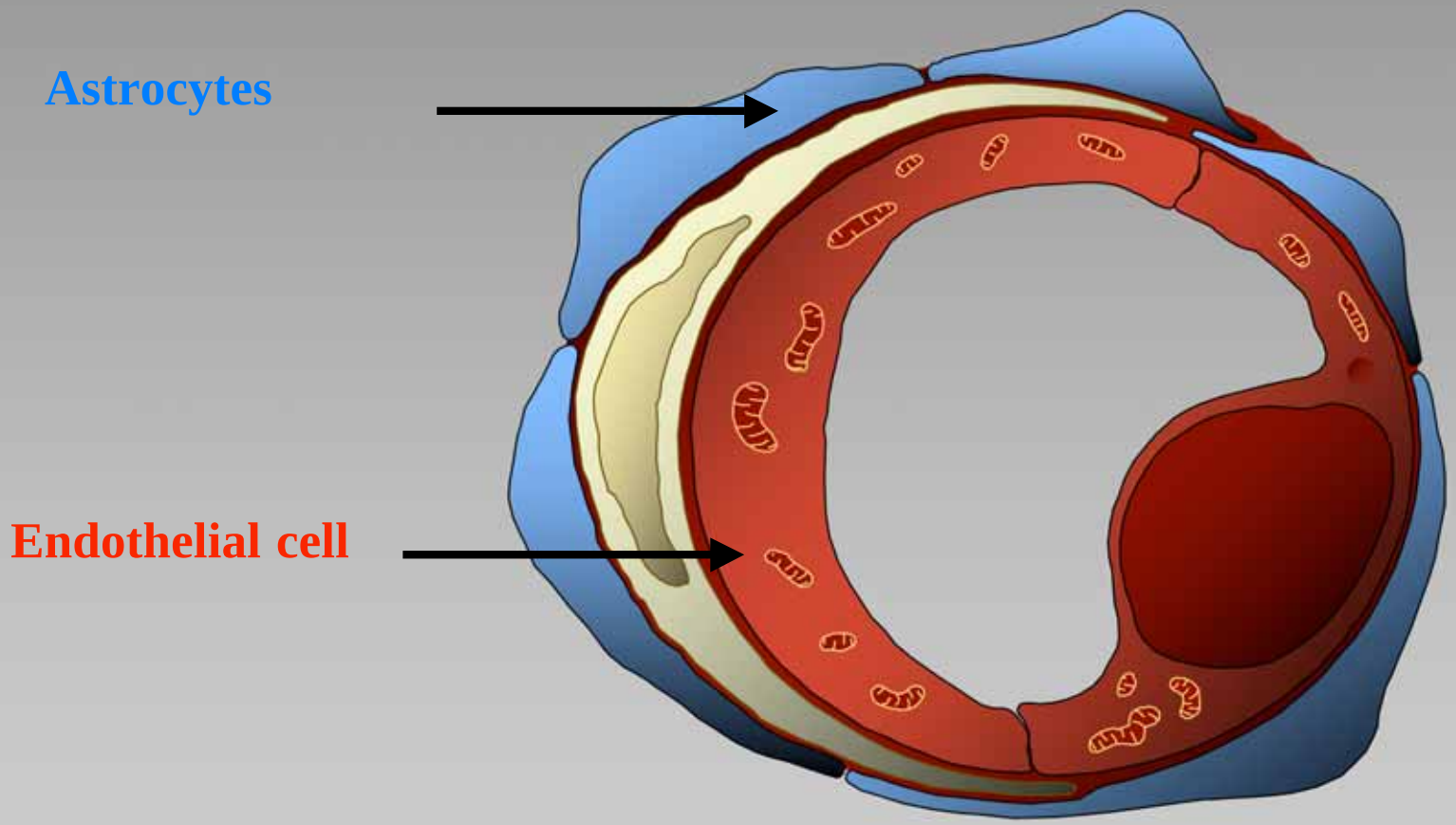
Gamma-GT expression



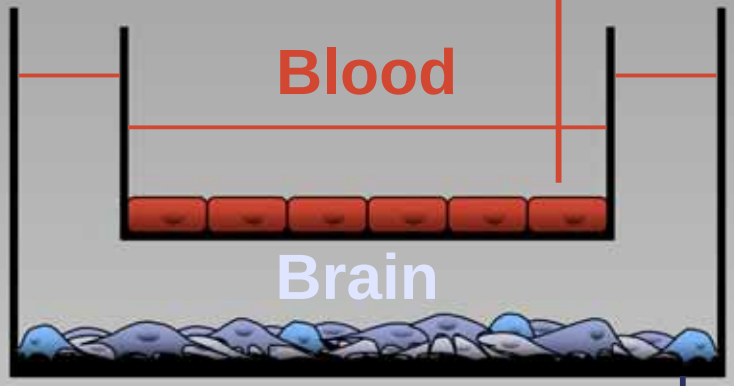
Conclusion

Consequently, endothelial cells in culture alone can not be considered as a relevant blood-brain barrier model

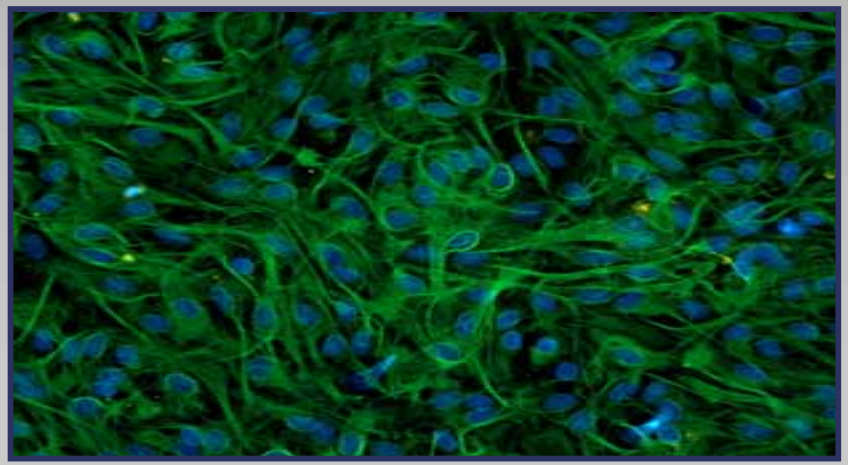
The BBB localisation



The *in vitro* BBB model

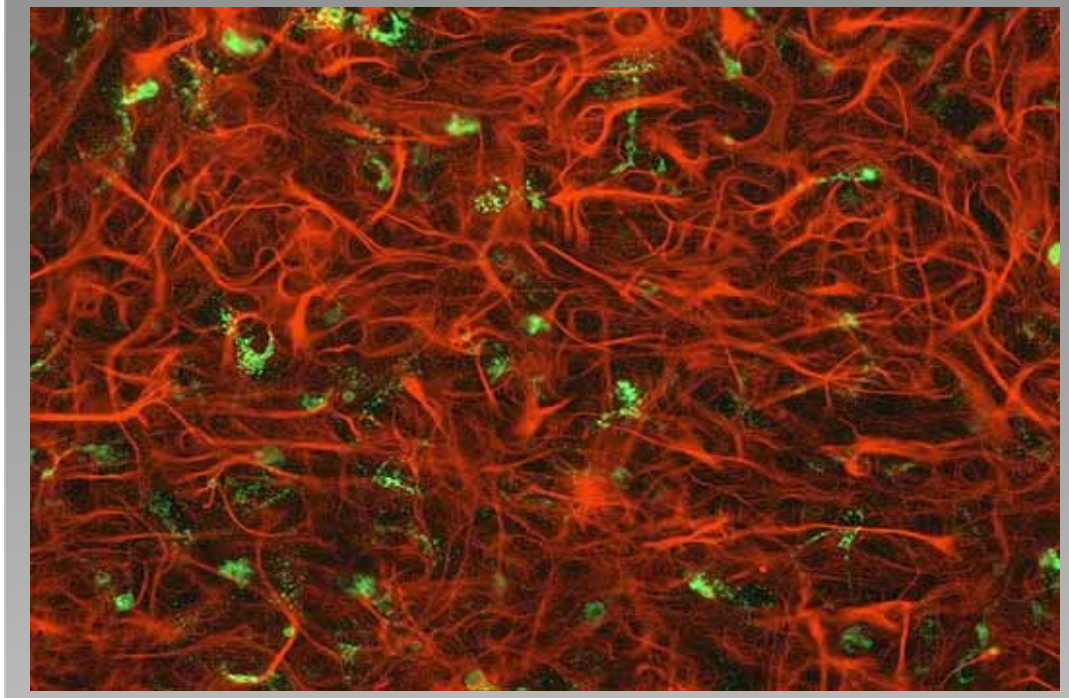


Endothelial cells

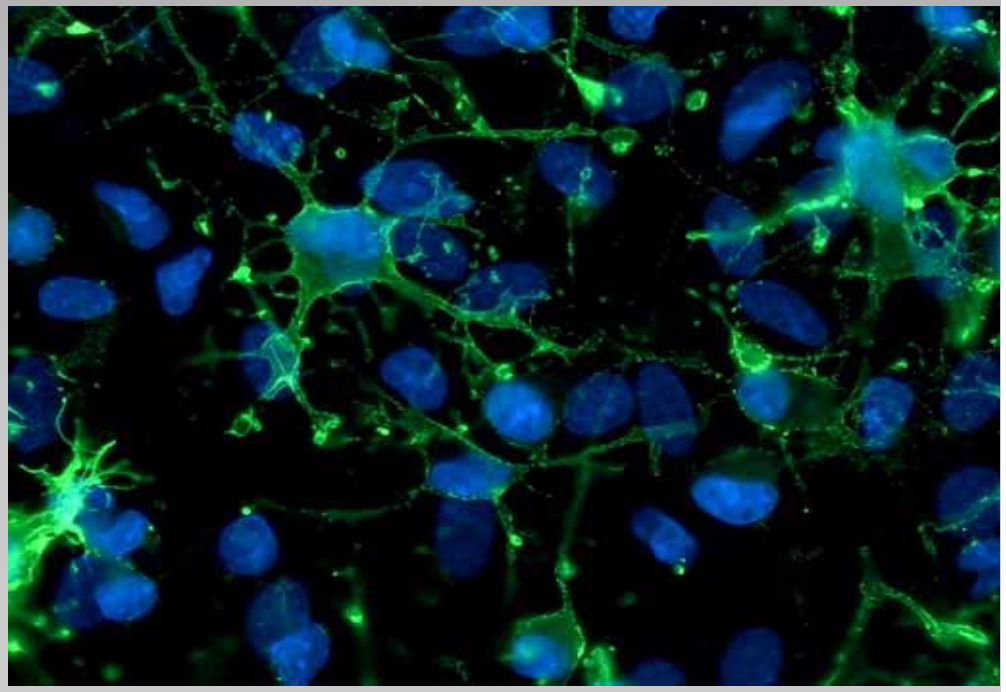


Glial cells

Rat glial cell characterization



GFAP-positive astrocytes
ED1-positive microglial cells



O4-positive oligodendrocytes

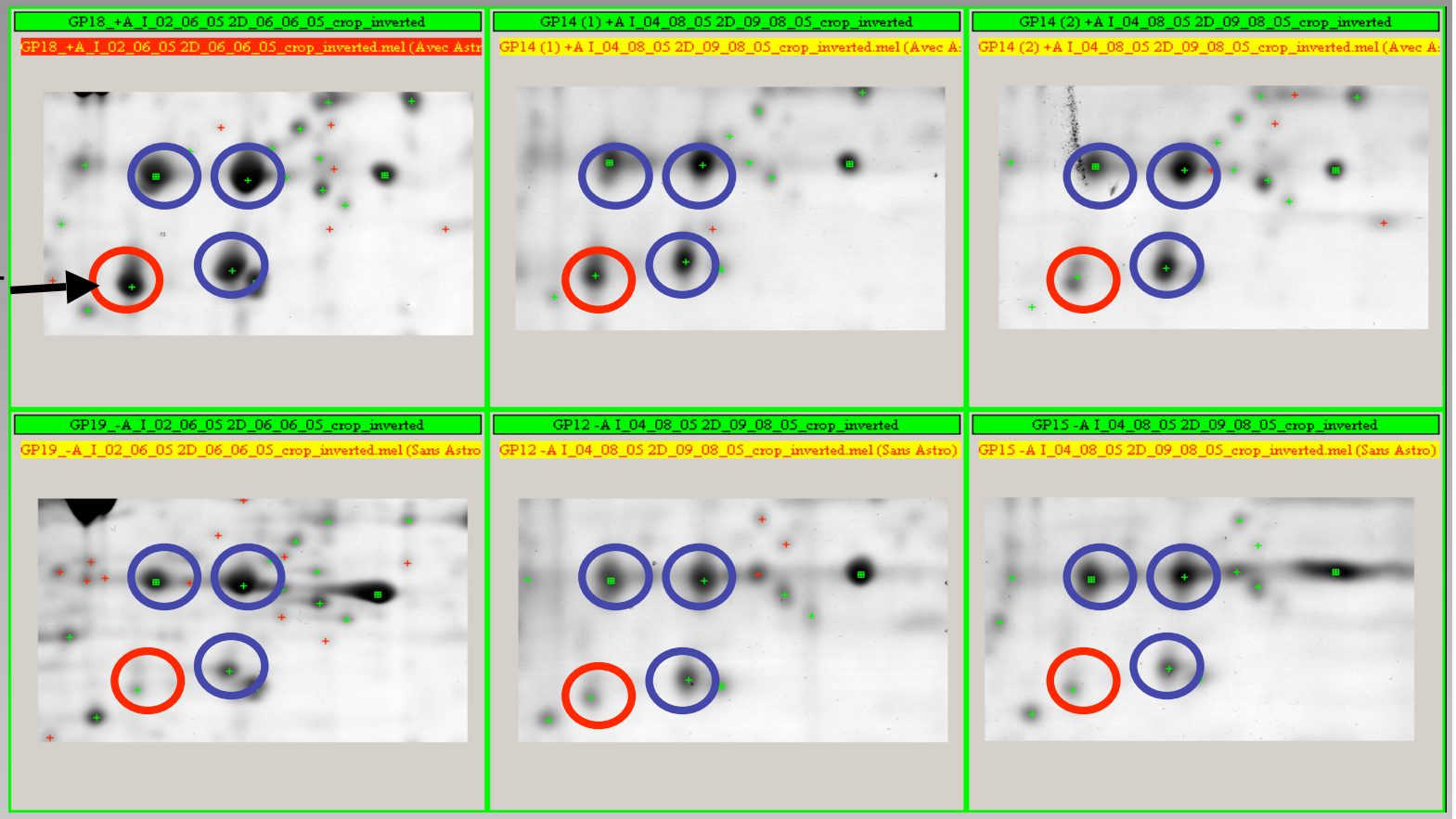
What about the g–GT activity ?

Statistical study of 2D-PAGE area from BCECs

Co-culture

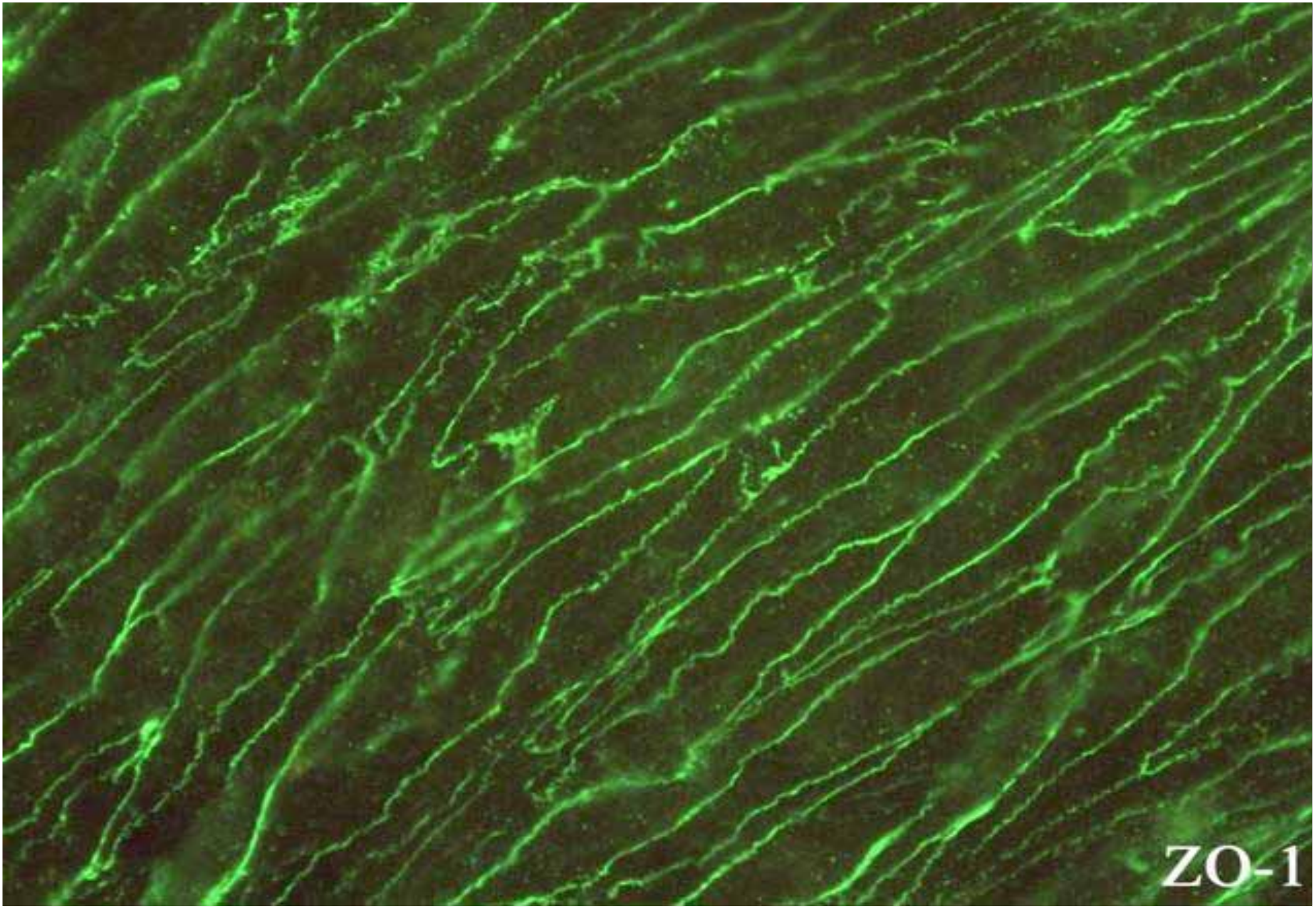
Gamma-GT

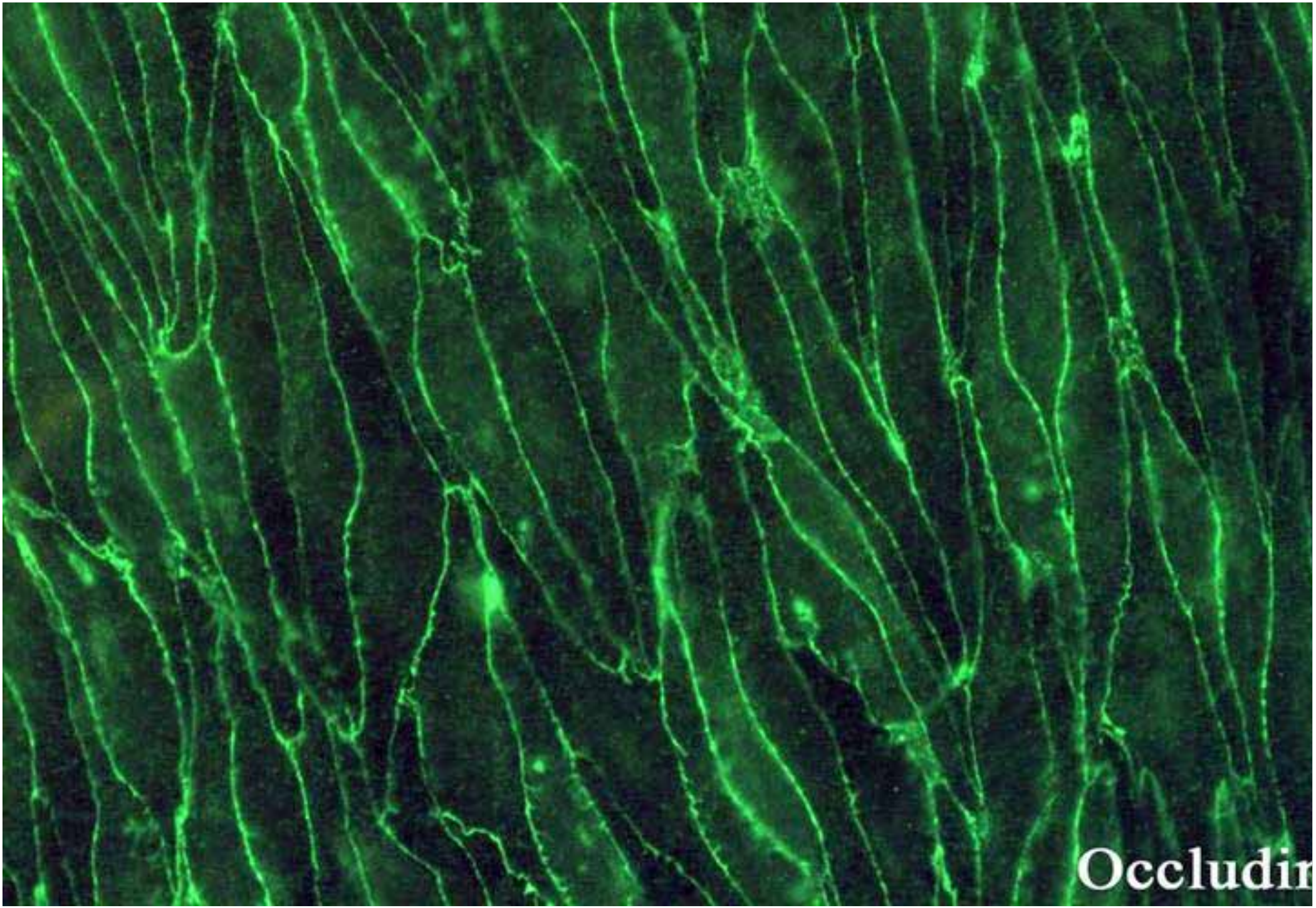
Solo-culture



Important variation : overexpressed in co-culture

No statistical significant variation


ZO-1


Occludin


Claudin-1

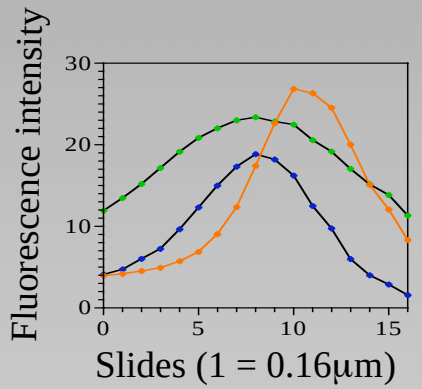
The transendothelial electrical resistance raises

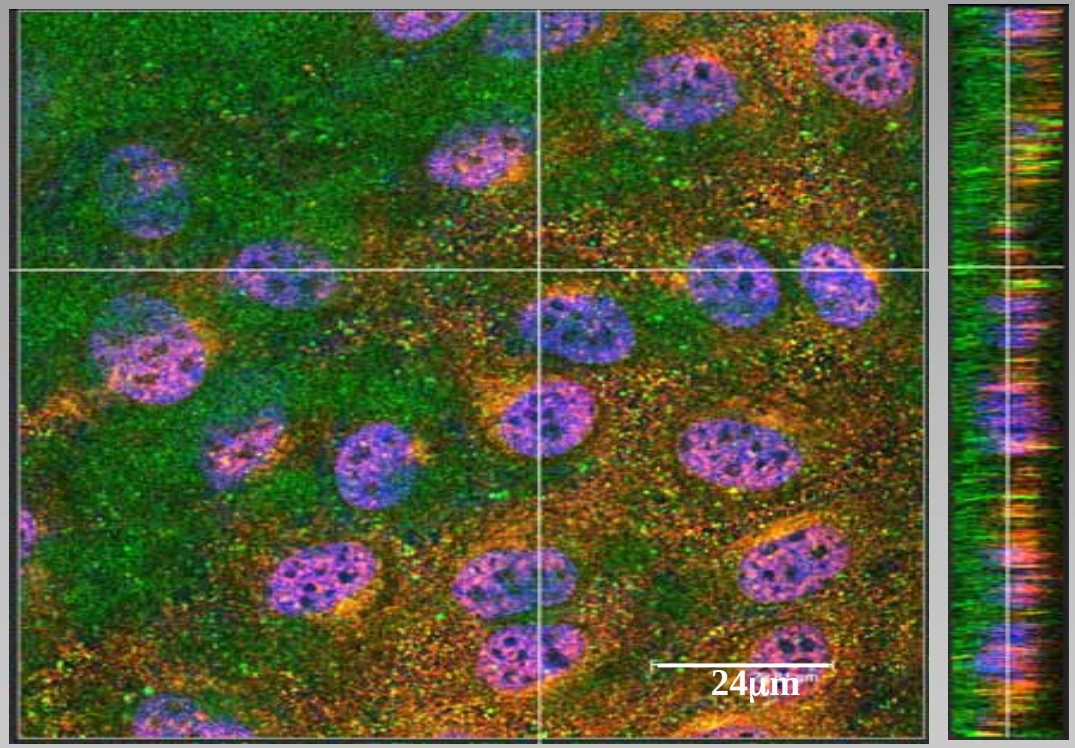
from 400 Ohms.cm^2 in soloculture

to 800 Ohms.cm^2 in coculture

Analysis of OCTN2 and P-gp immunofluorescent staining in apical and basolateral membrane of endothelial cells

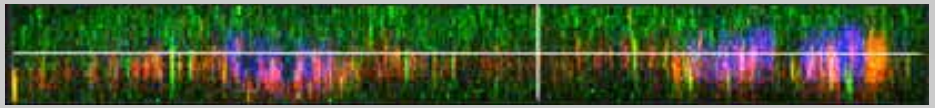
OCTN2
P-gp
nuclei



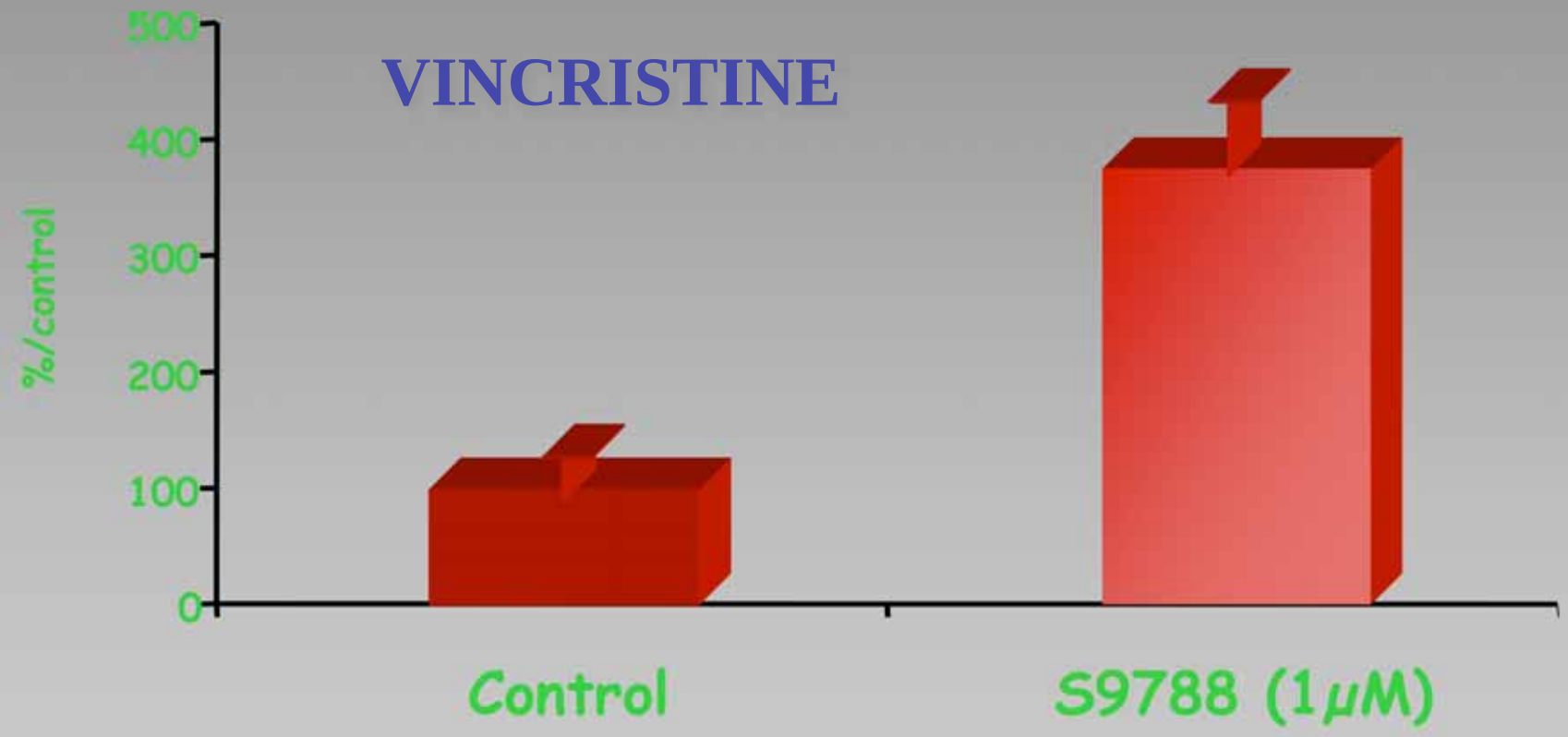


Apical membrane →

Basolateral membrane →



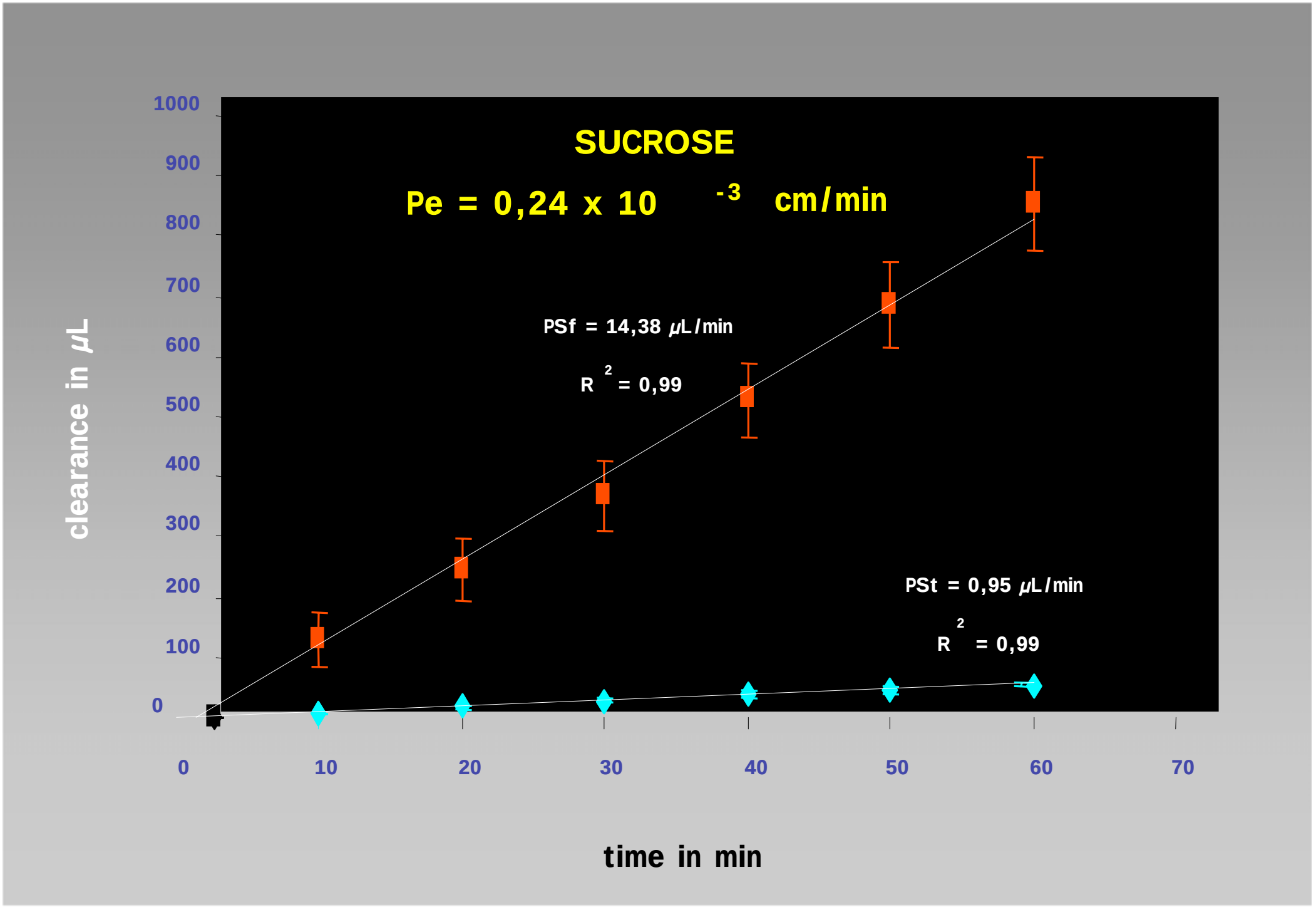
Inhibition study (Uptake)

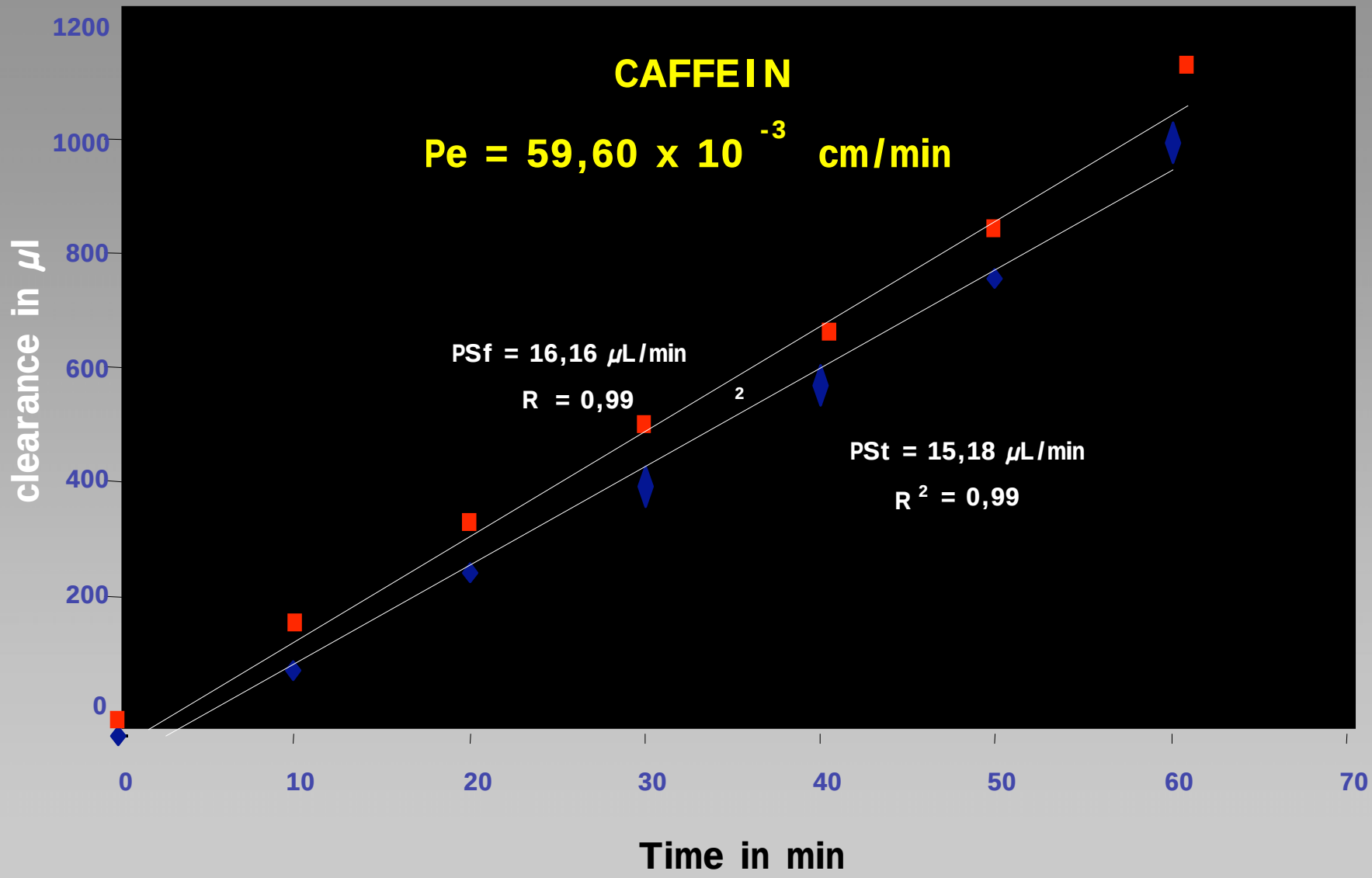


Conclusion

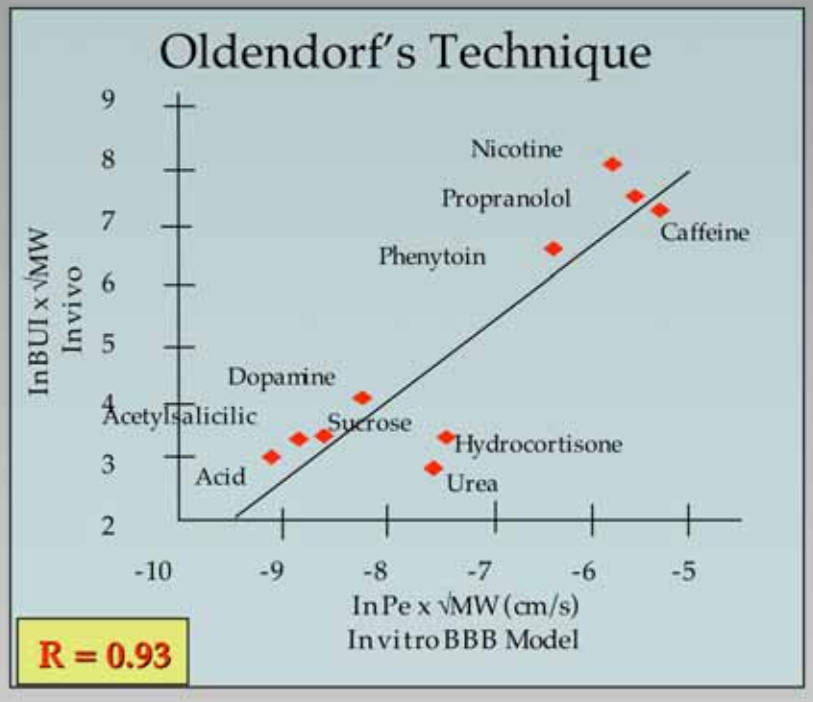
In coculture with astrocytes, brain capillary endothelial cells present most of the characteristics that are known to have important functions *in vivo*

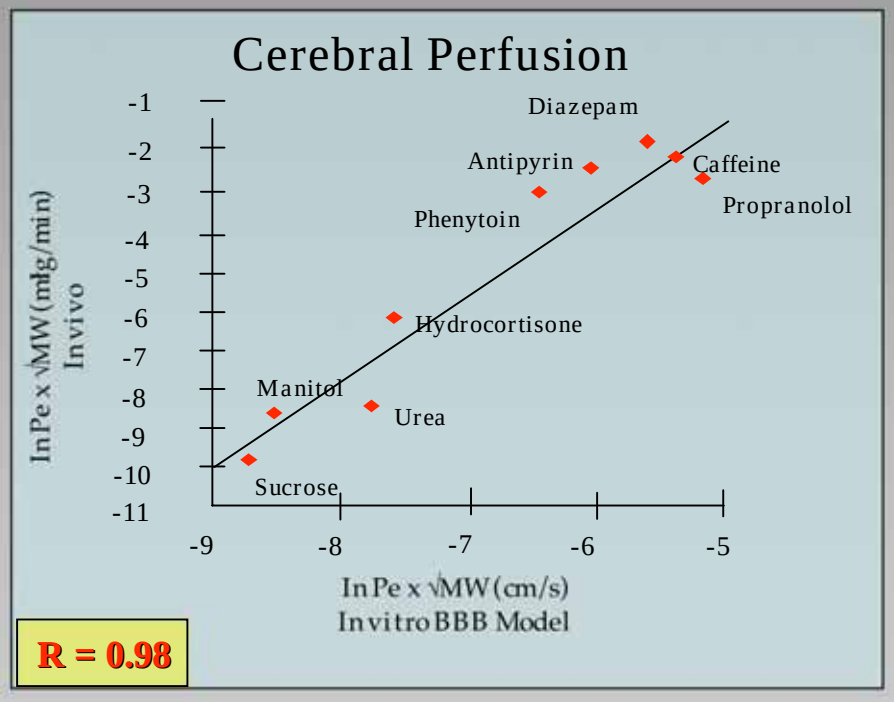
How can we use this model to predict the drug brain penetration ?


SUCROSE
Pe = 0,24 x 10 -3 cm/min
PSf = 14,38 µL/min
R 2 = 0,99
PSt = 0,95 µL/min
R 2 = 0,99
clearance in µL
time in min
1000
900
800
700
600
500
400
300
200
100
0
0
10
20
30
40
50
60
70


CAFFEIN
Pe = 59,60 x 10 -3 cm/min
PSf = 16,16 µL/min
R 2 = 0,99
PSt = 15,18 µL/min
R 2 = 0,99
clearance in µl
Time in min
0
200
400
600
800
1000
1200
0
10
20
30
40
50
60
70

Correlation between permeability values obtained *in vivo* with the in vivo techniques (Brain perfusion and Oldendorf) and *in vitro* with the BBB model





Ranking

Example of product	Pe (x 10^{-3} cm/min)	Brain penetration
Caffeine	59.90	VERY GOOD
Nicotine	58.76	
Diazepam	19.86	GOOD
Antipyrine	18.74	
DiPhenylHydantoine	7.37	
Urea	2.22	
Morphine	1.90	LOW
Verapamil	1.74	
Warfarin	1.72	
L Dopa	1.29	
Vinblastine	1.19	
Alanine	1.07	
Leucine	0.91	VERY LOW
Lactic acid	0.63	
Sucrose	0.58	
Glycerol	0.44	
Cyclosporin	0.42	
AZT	0.39	
Cimetidine	0.26	
Digoxin	0.21	
Vincristine	0.15	
Inulin	0.04	

30.10^{-3} cm/min

2.10^{-3} cm/min

1.10^{-3} cm/min

This model is already used in different pharmaceutical companies (Europe, Canada Japon) and academic groups

Reproducibility

- 6 last months

- 3 different clones (B1, PK, Lau1) between P4 et P7

- 4 different technicians

$$Pe\ (sucrose) = 0{,}28 \pm 0{,}12\ (n=81)$$

Astrazeneca (Sweden)

$$Pe\ (sucrose) = 0{,}32 \pm 0{,}18\ (n=52)$$

This *in vitro* model constitutes a RELEVANT model to the BBB

Blood-Brain Barrier Penetration and Drug Development from an Industria Point of View

Katharina Mertsch* and Jochen Maas

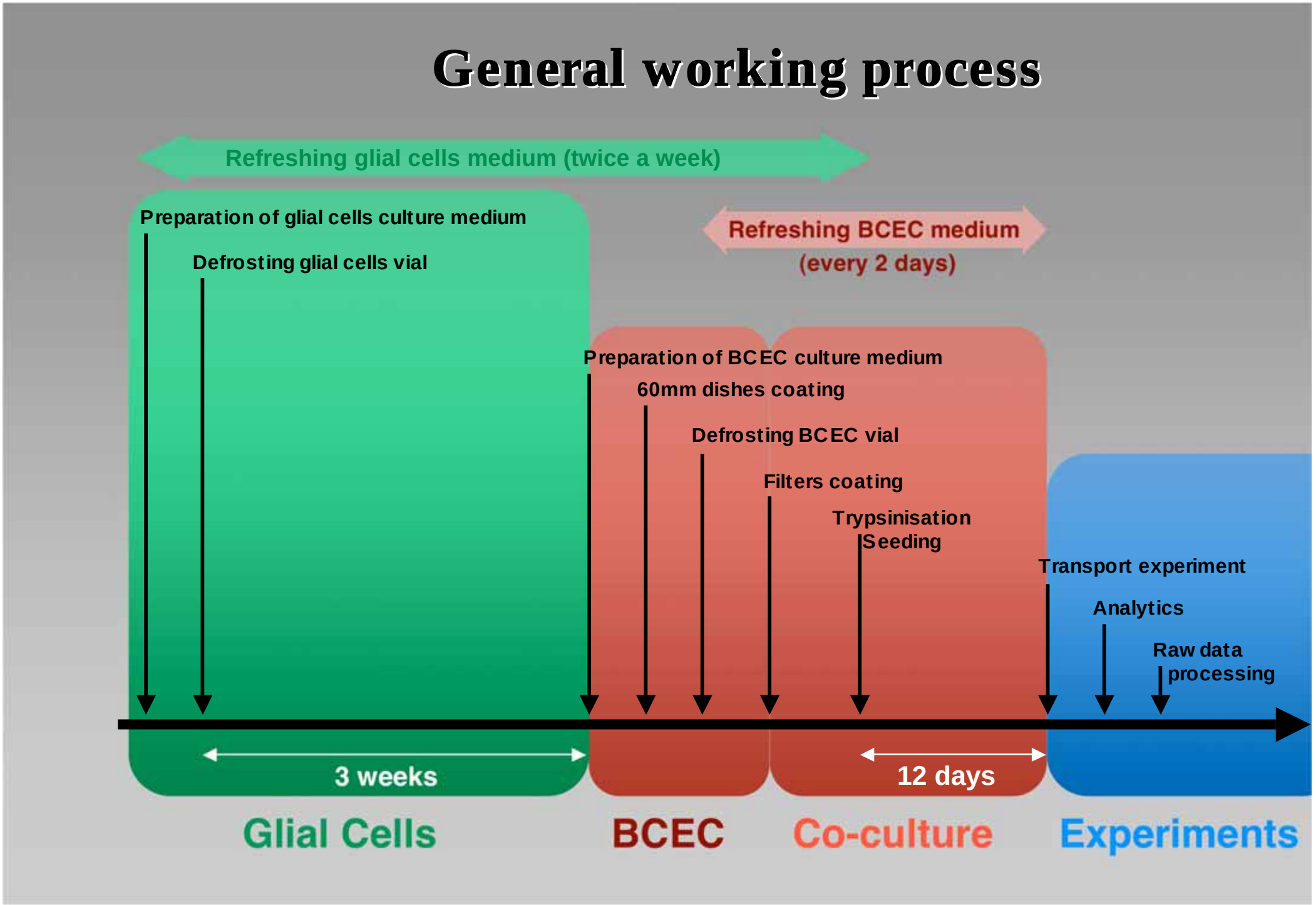
DI&A, Lead Optimization Drug Metabolism/Pharmacokinetics, Aventis Pharma Deutschland GmbH, Building G877, Industriepark Höchst, D-65926 Frankfurt am Main, Germany

Abstract: To be effective as therapeutic agents, centrally acting drugs must cross the blood-brain barrier (BBB). Conversely, to be devoid of unwanted CNS effects, peripherally acting drugs must show limited brain accessibility. This demonstrates clearly the need for different methods to assess the blood-brain penetration at different levels of project development in industry. Since the experimental determination of blood-brain partitioning is difficult, time consuming and expensive, also other methods like in-silico approaches mainly based on physicochemical properties like solubility, lipophilicity, molecular size, hydrogen-bonding capacity and charge are used. Approaches for drug delivery and drug modification are also reviewed in the present article.

In vitro cellular models based on cell cultures growing in two-chamber systems for transport studies or isolated microvessels play an important role for compound screening. To achieve and use the full potential of these models a characterization of anatomical, physiological and biochemical properties is needed. The more strict the criteria for BBB models the better prediction of penetration and cellular mechanisms. Unfortunately, the throughput decreases often in parallel. Therefore, though high-throughput assays as the MDCK/CACO assay or artificial membrane assays are used but they still suffer from low predictability for specifically transported substances (transporters, P-glycoprotein, brain specific receptors) due to differences between peripheral epithelial cells and brain endothelial cells.The animal experiment with radiolabelled and non-radiolabelled compound not only have the highest level and the highest predictability but the highest cost and lowest throughput as well. There is no "golden rule" for approaching brain penetration in industry but models available are used often in parallel (*in silico, in vitro, in vivo*).

The coculture model of BCEC and astrocytes is time and labor intensive and cannot be used as high throughput assay. However, it is the *in vitro* BBB model next to *in vivo* at the moment and the models characterized show good correlation to *in vivo* data

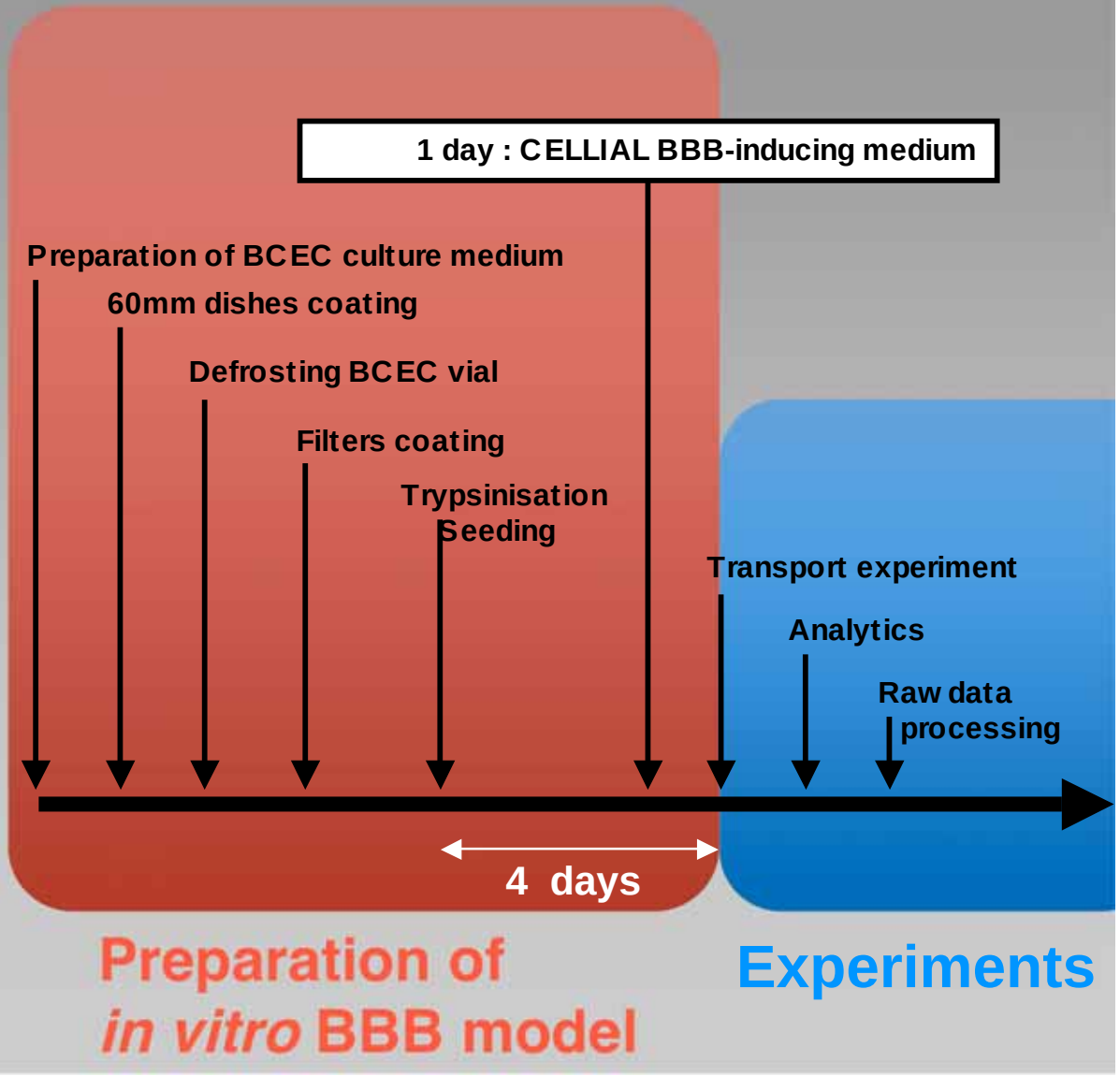
General working process



In order to recreate the interactions between BBCE and glial cells,

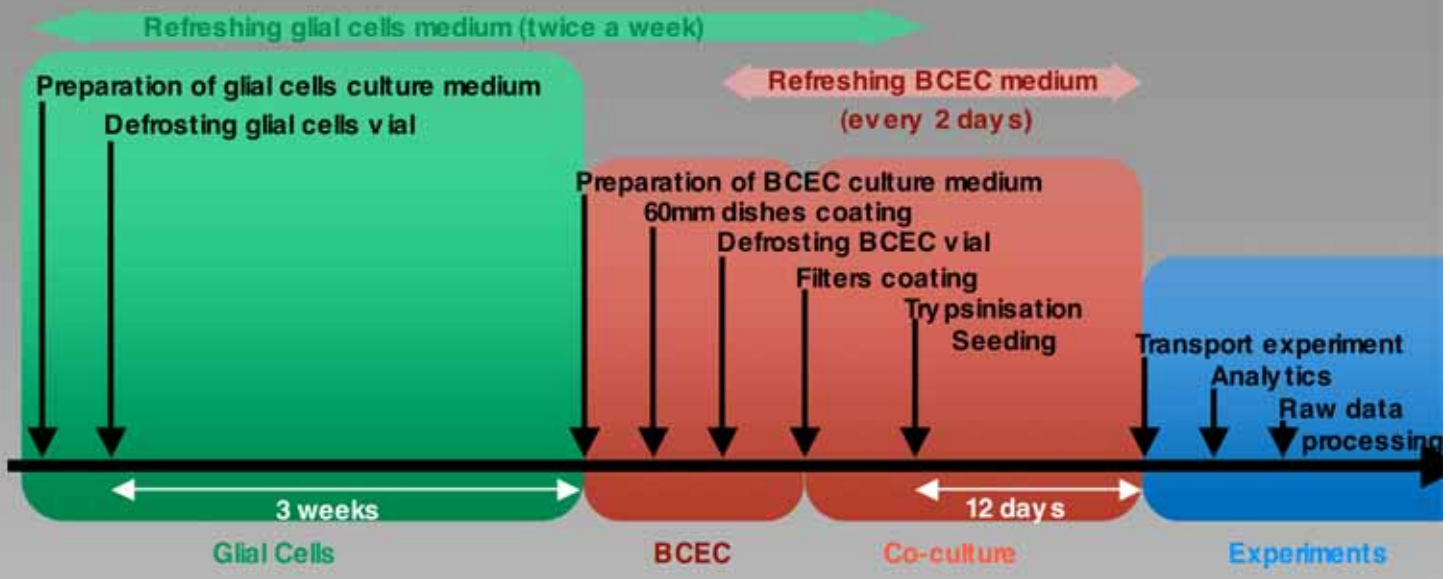
we worked on the preparation of a medium , which will be able to indu
all the properties of the BBB without the presence of glial cells

General working process

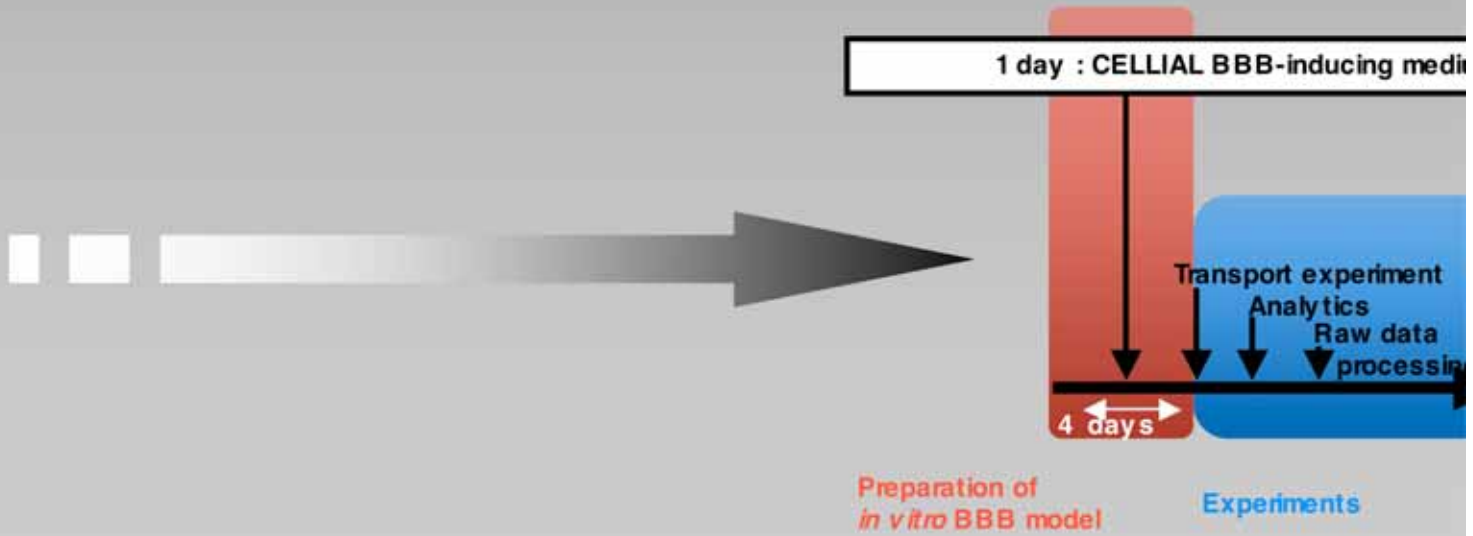


General working process

CT Bovial@Screen Pack

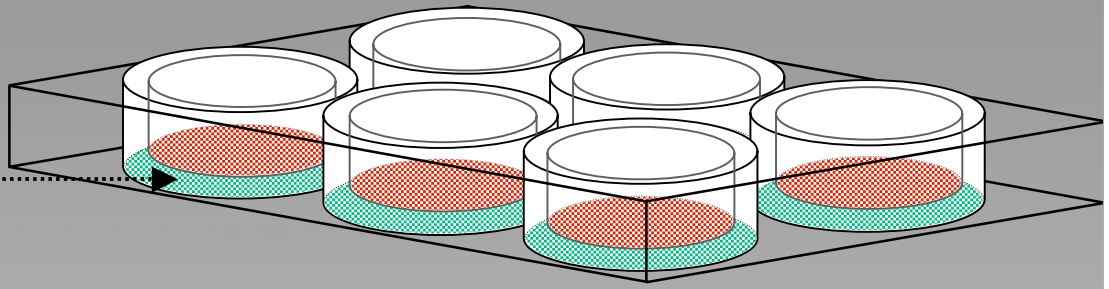


4D@Screen Pack



Culture Time

CT Bovial@Screen Pack / 4D@Screen Pack



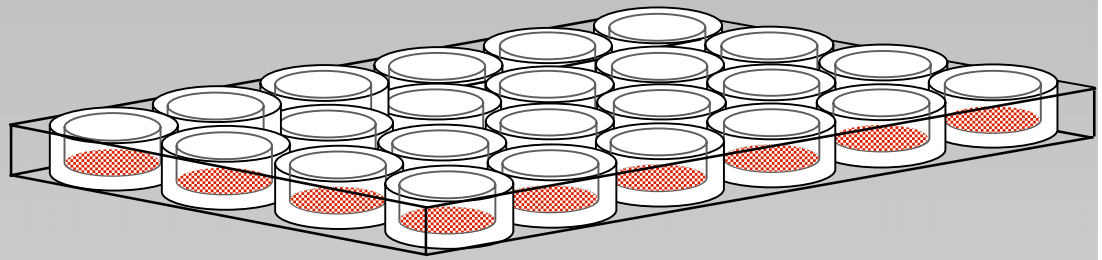
3 weeks (glial cell culture)

Co-culture

+ 7 days (confluence)+ 5 days : BBB ready

60mm-culture dish

4 day-culture

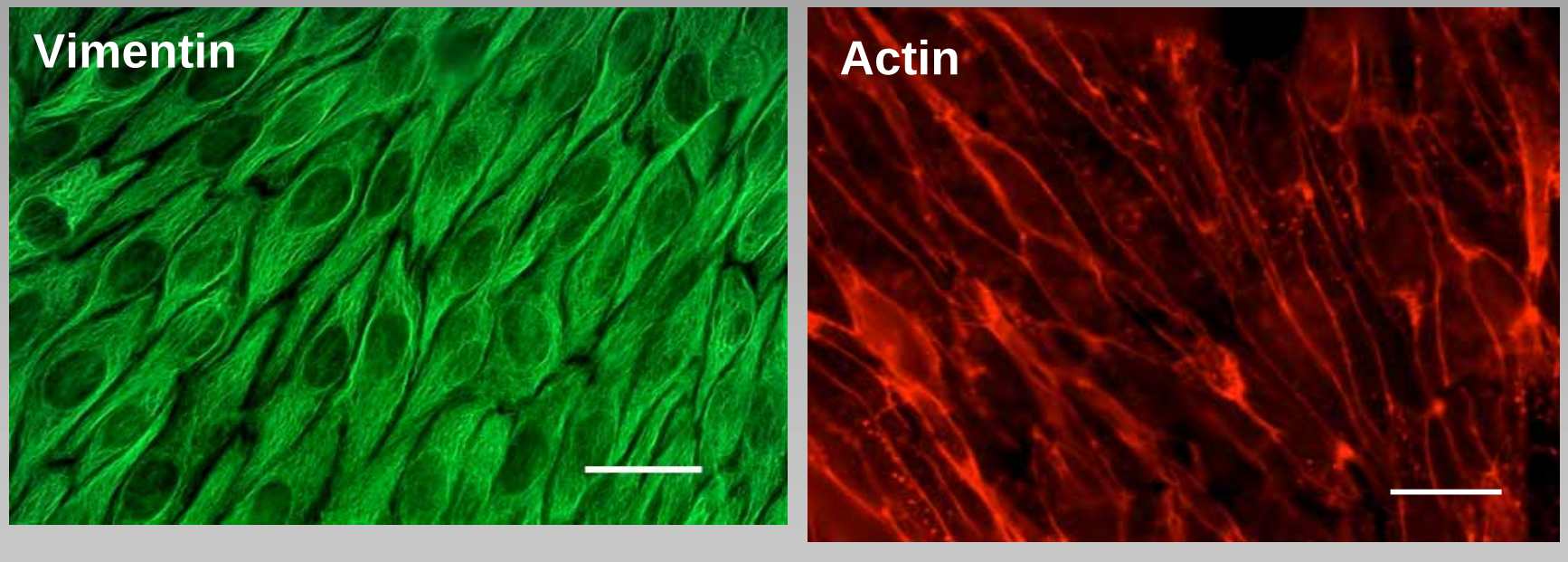


4 days : BBB ready

BBB characteristics

4D@Screen Pack

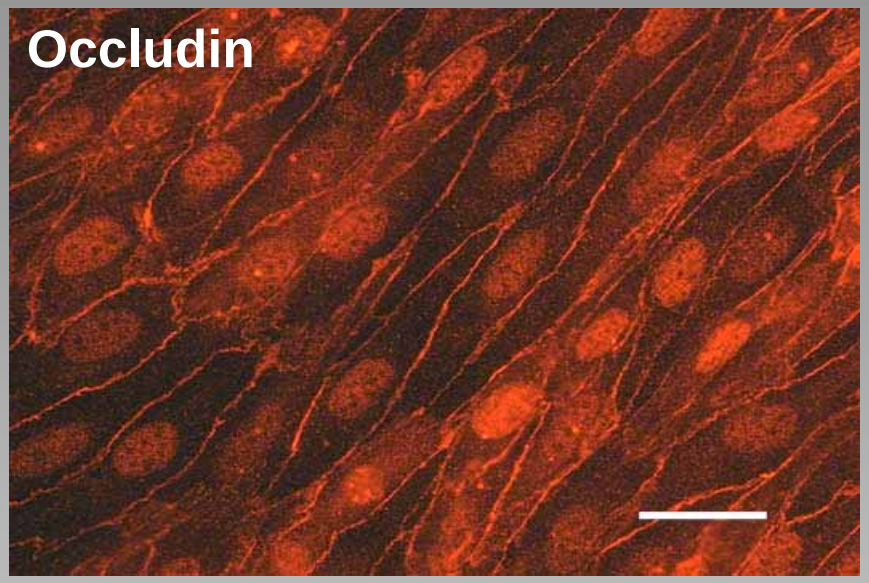
Cell monolayer morphology:

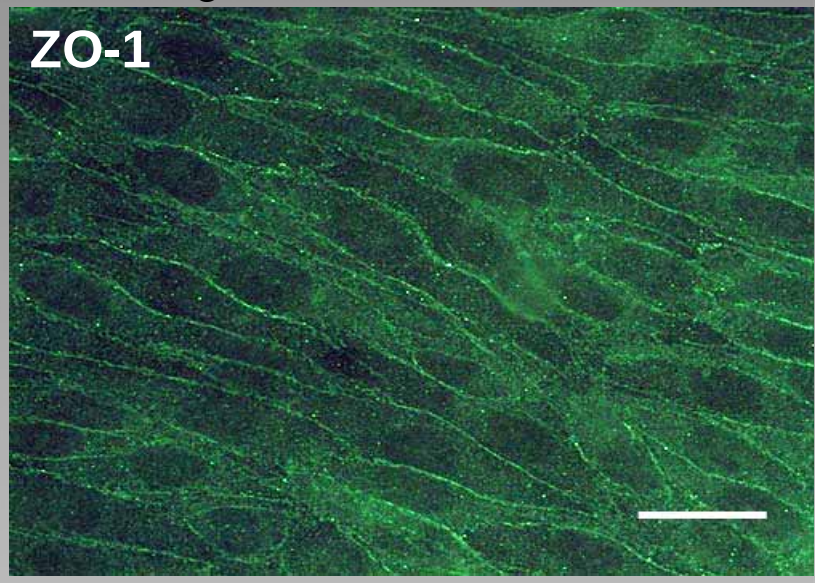


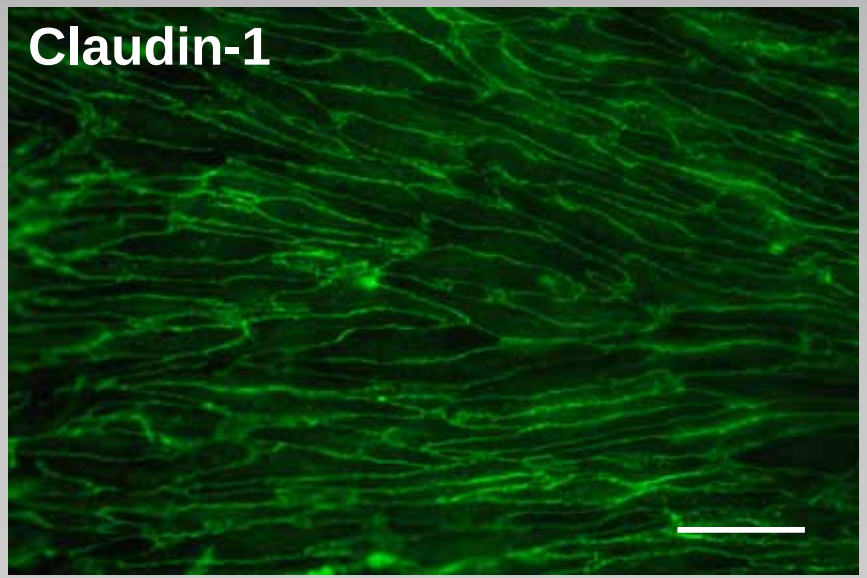
Bar = 25 μm

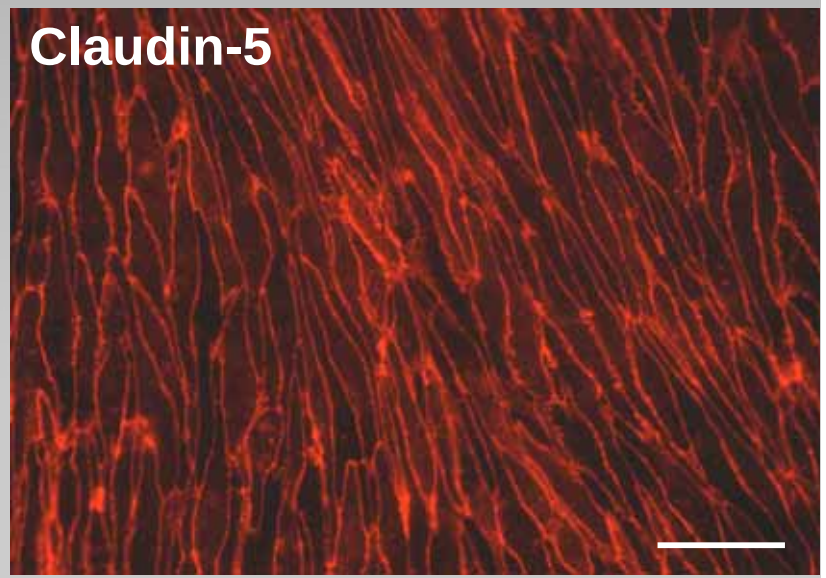
BBB characteristics

Tight junctions:







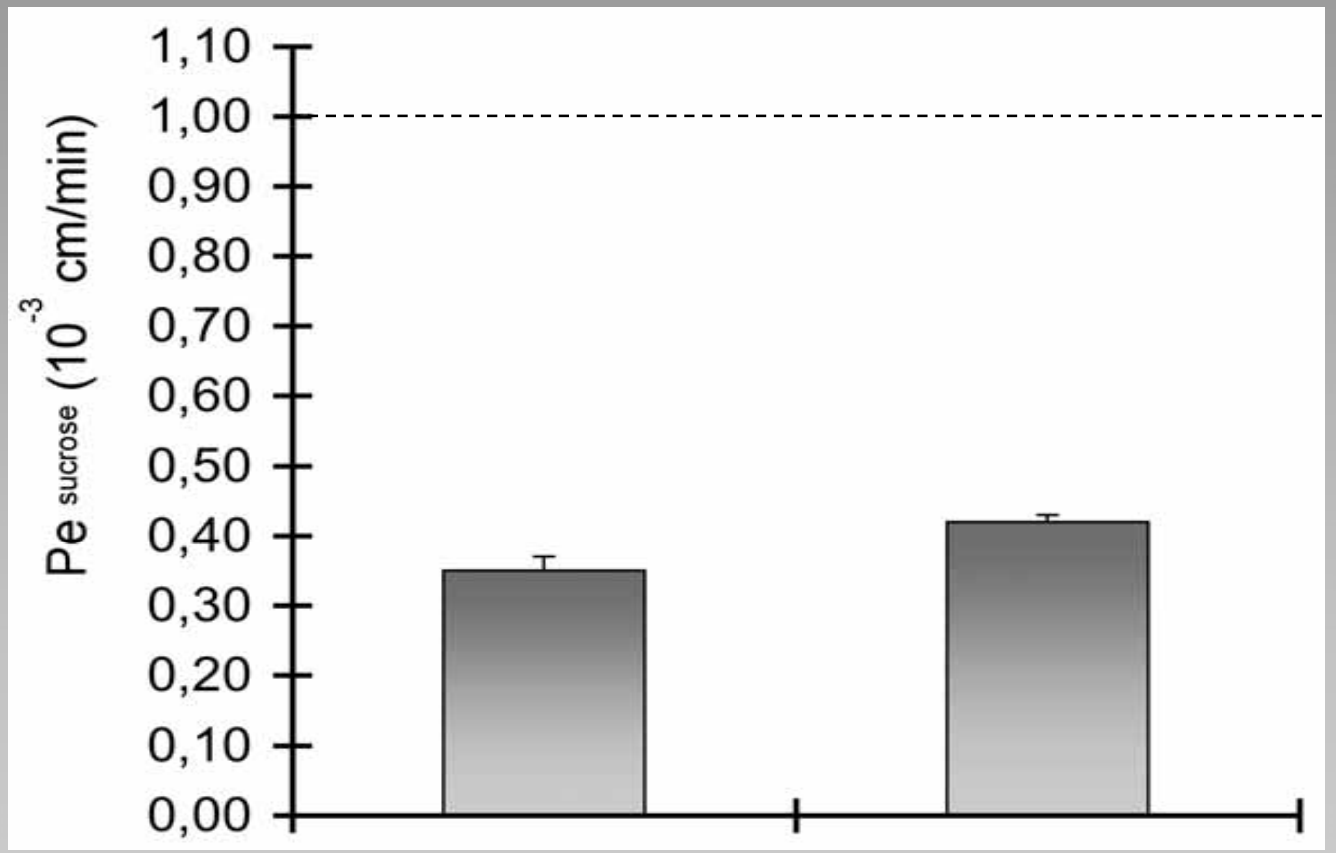


Bar = 25 μm

Control of BBB integrity

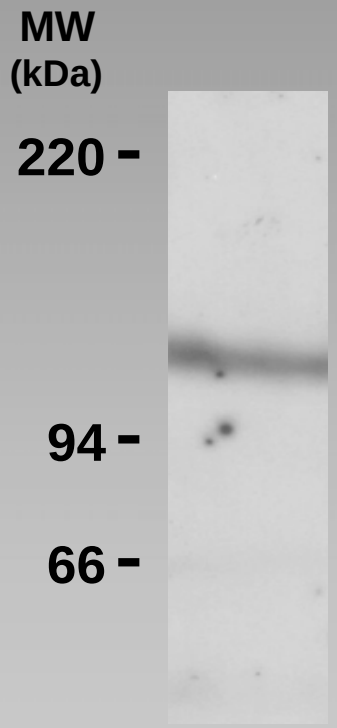
4D@Screen Pack

Sucrose permeability



P-glycoprotein

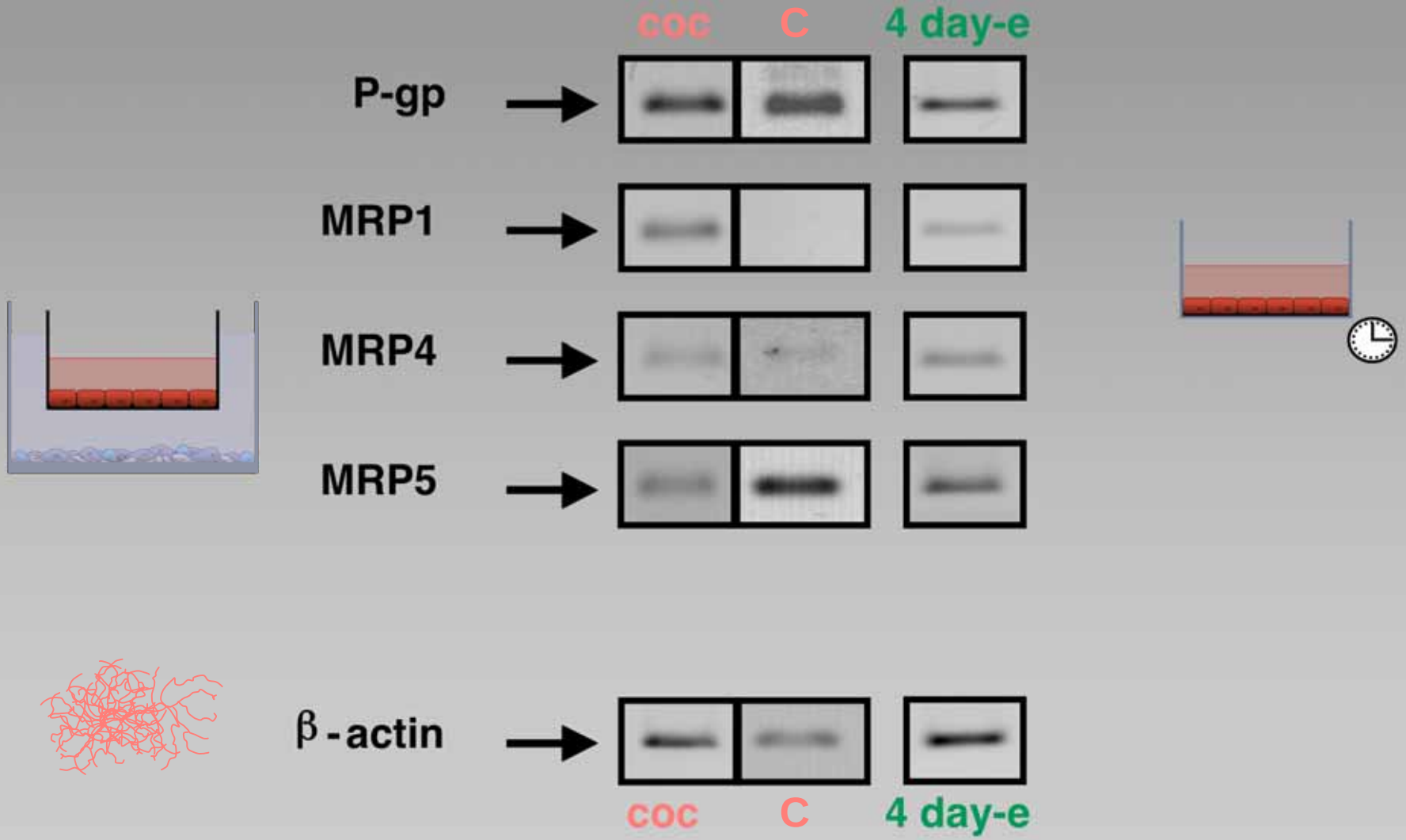
Presence of the protein (Western Blot)



Functionality of P-gp (inhibition assay)

Transporters (mRNA)

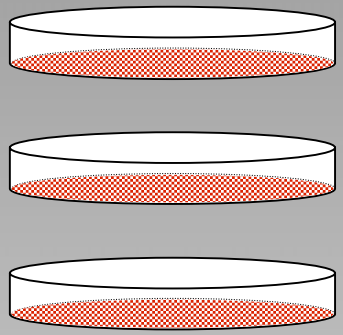
RT-PCR from co-cultures, capillaries and 4 day-endothelial cells



Seeding

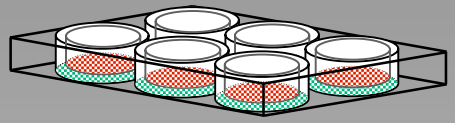
CT Bovial@Screen Pack / 4D@Screen Pack

1 vial of
endothelial cells



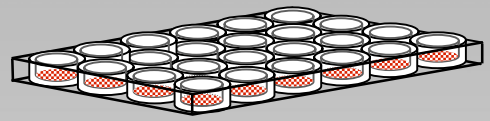
3 x 60mm-culture dishes

90 filters



12 day-system
6 well-filters

226 filters

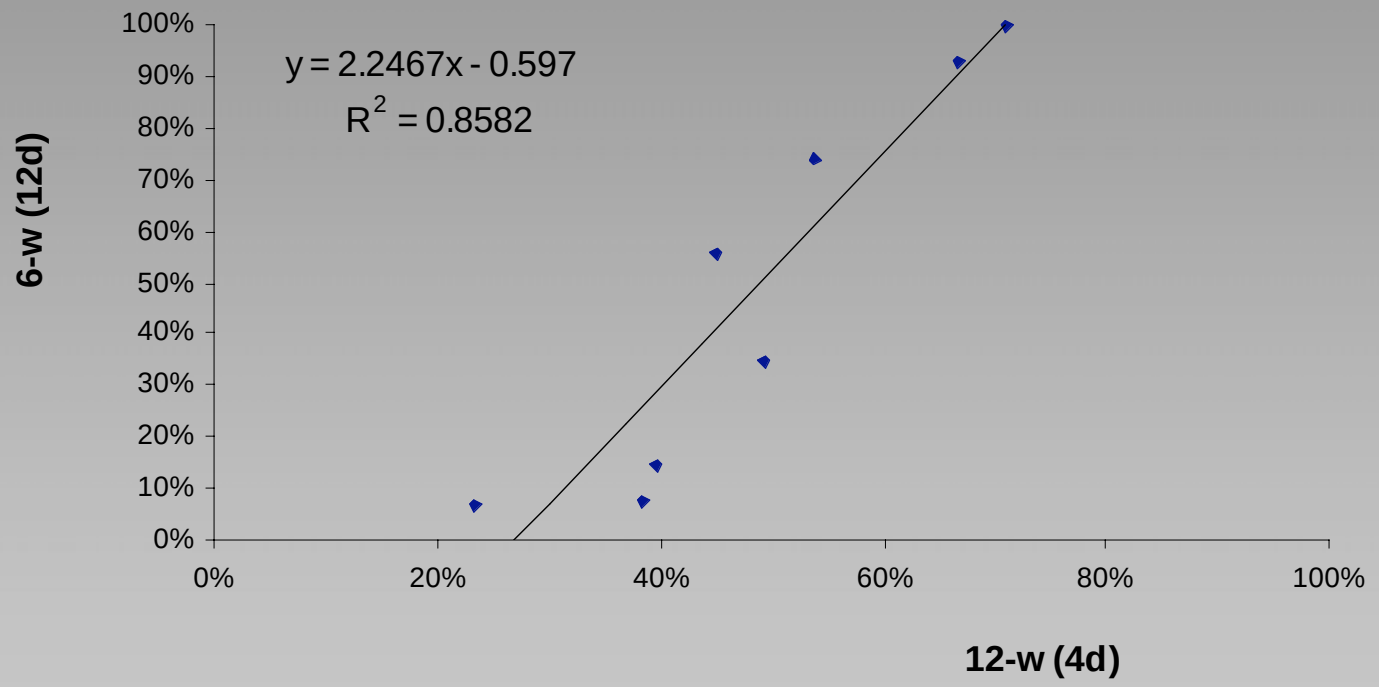


4 day-system
24 well-filters

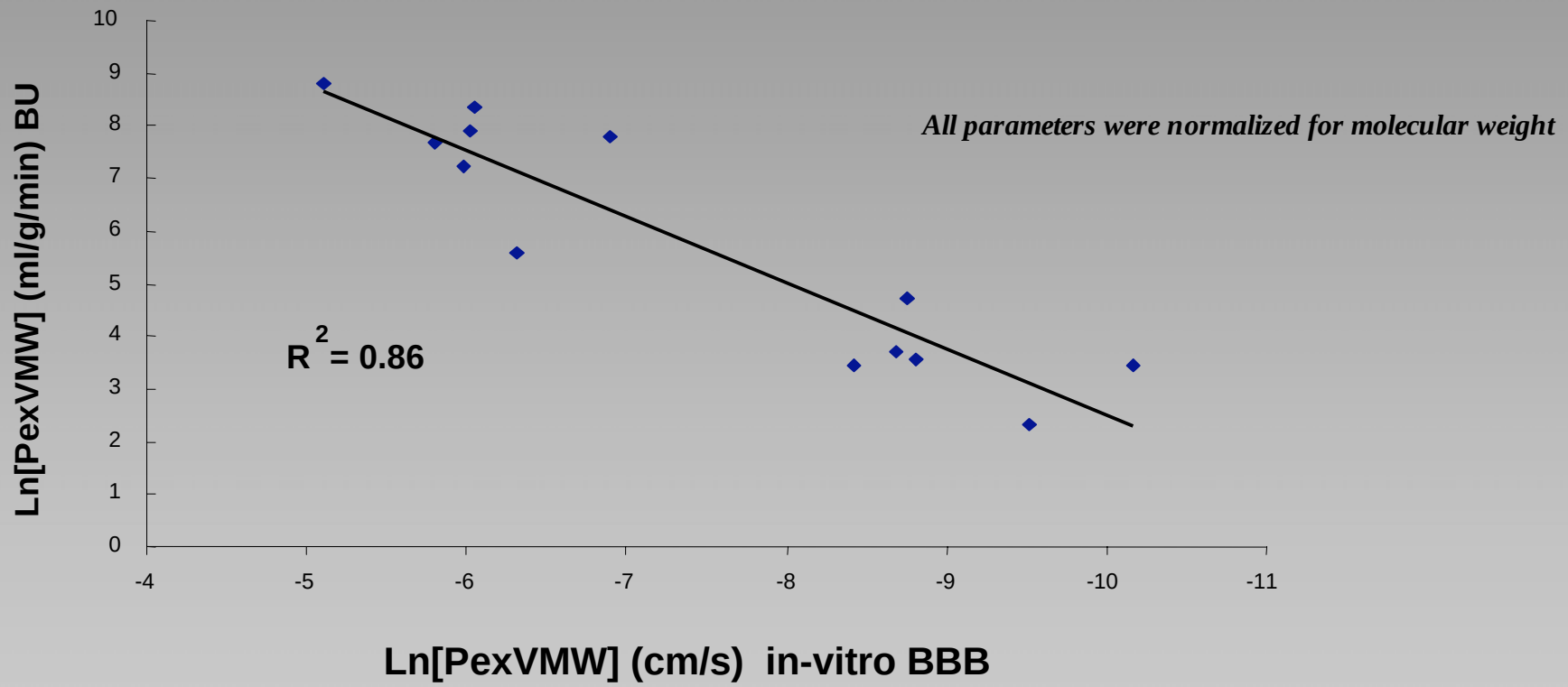
Correlation between 12-w (4d) and 6-w (12d) (% passage versus filter)

Tested compounds:

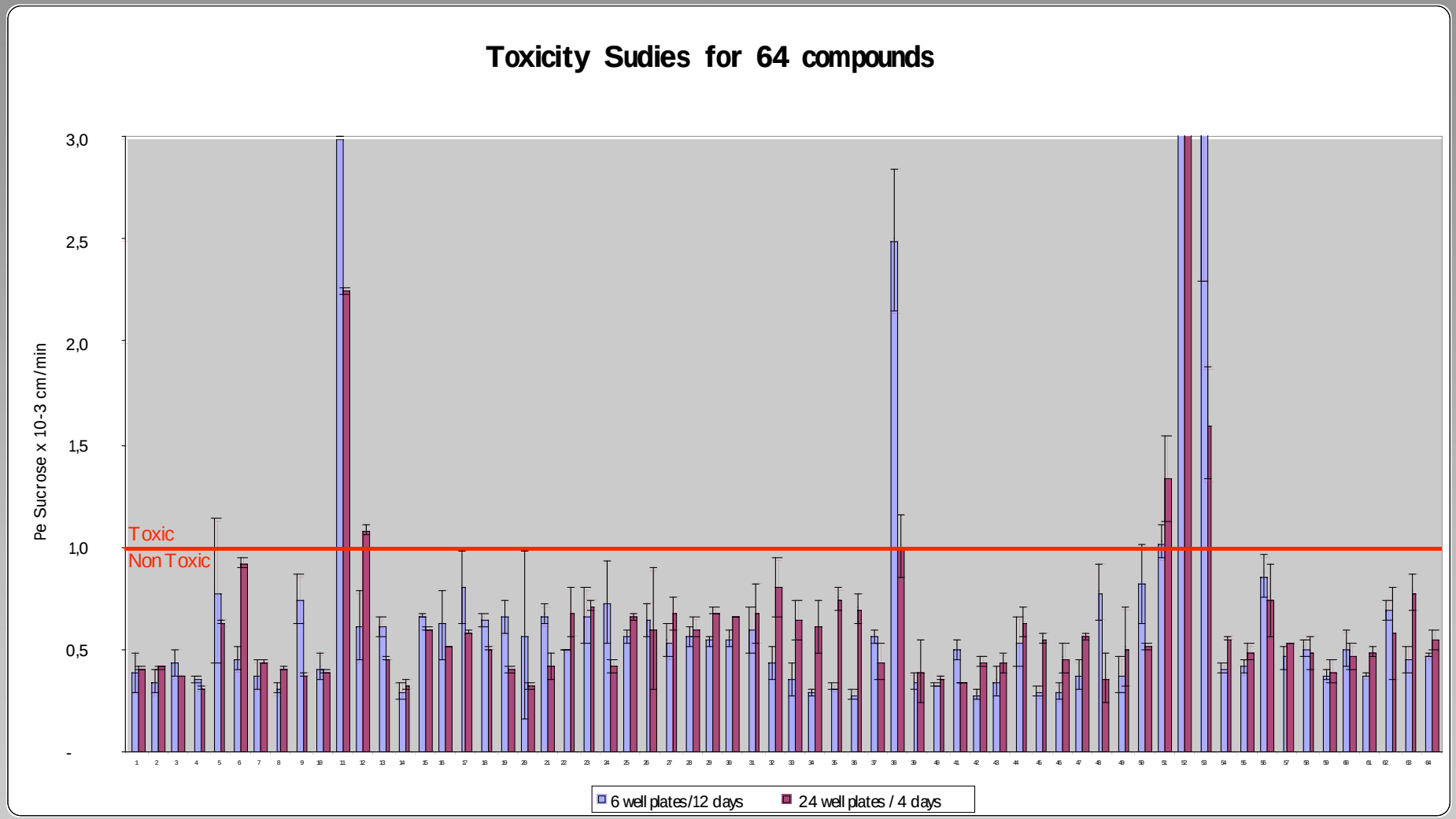
Lucifer Yellow
Acetaminophen
Acetylsalicylic acid
Atropine
Caffeine
Colchicine
Cycloheximide
Phenobarbital

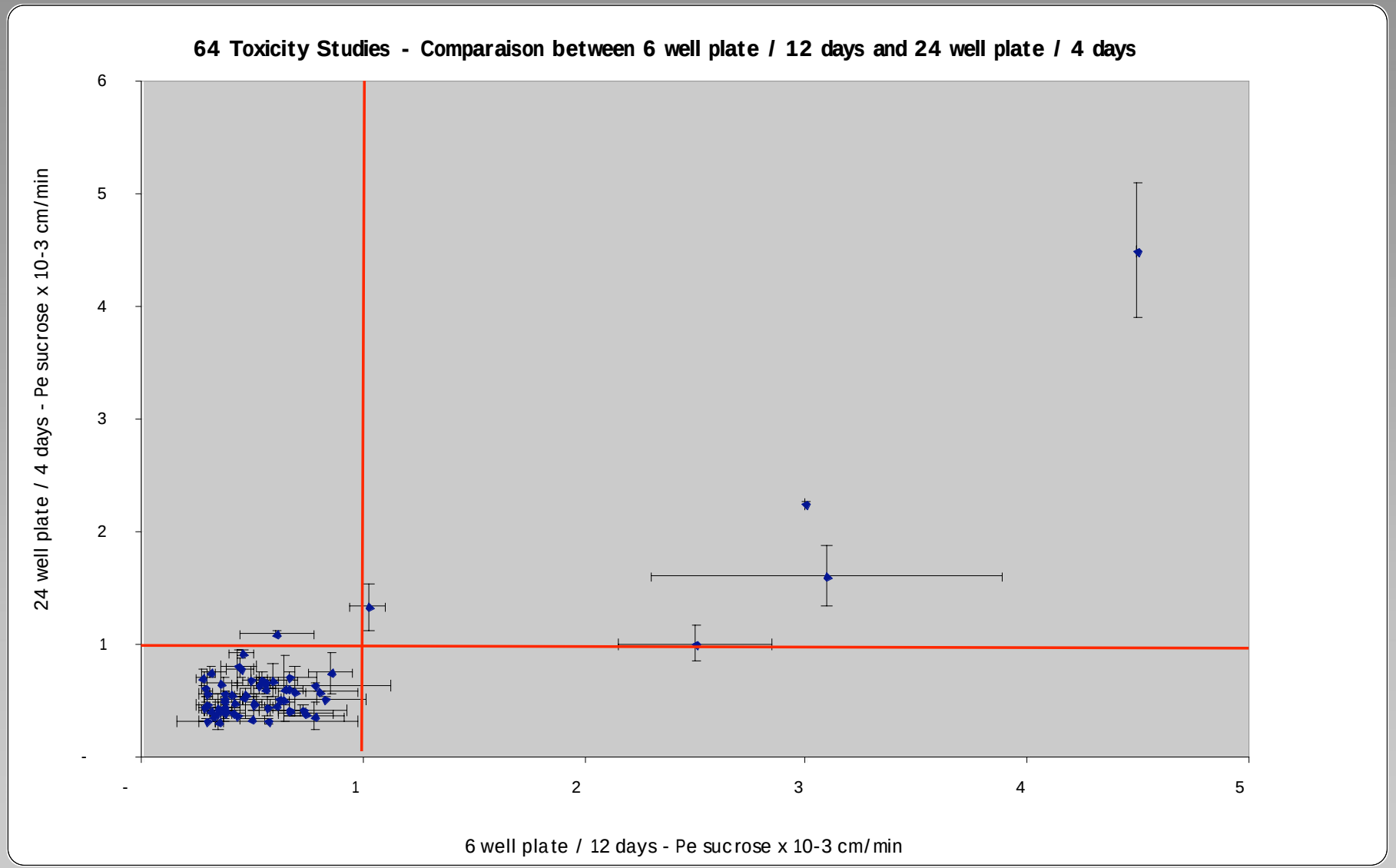


Correlation between *in vitro* BBB (24w-4d permeability) and in vivo BU (brain perfusion)



A-cute-Tox IP


Toxicity Sudies for 64 compounds
Pe Sucrose x 10-3 cm/min
3,0
2,5
2,0
1,5
1,0
0,5
-
Toxic
Non Toxic
6 well plates/12 days
24 well plates / 4 days


64 Toxicity Studies - Comparaison between 6 well plate / 12 days and 24 well plate / 4 days
24 well plate / 4 days - Pe sucrose x 10-3 cm/min
6 well plate / 12 days - Pe sucrose x 10-3 cm/min
6
5
4
3
2
1
-
-
1
2
3
4
5

Conclusions

The 4 days model is a simple ,robust and relevant model of the BBB

Two important endpoints in toxicology could be achieved with this model:

The toxicity of the compound for the barrier
« alerts » : high permeability can give high concentration in the brain
possible neurotoxicity