Effects of mobile phone signals (GSM and UMTS) on the blood-brain barrier *in vitro*

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In vitro-Experiments on exposure to RF-fields of mobile telecommunication
C. Blood brain barrier

- BBB in vitro (rat brain endothelial cells)
- GSM 1800 exposure
- UMTS exposure

- differential gene expression (genechip arrays)
- selection of BBB related candidates
- verification of diff. gene expression (rt-PCR)

overview: project parts

- establishment and characterization of RBEC cultures as BBB in-vitro model
- design of exposure unit
- exposure of RBEC and isolation of RNA
- gene expression analysis
- RT-PCR analysis of regulated genes

goals

- Influence of RF-EMF on endothelial cells of the BBB ?
- reduction of BBB towards an in vitro model
- investigations on cellular level
- identification of potential EMF-targets on molecular level
- no hypotheses on pathophysiological issues

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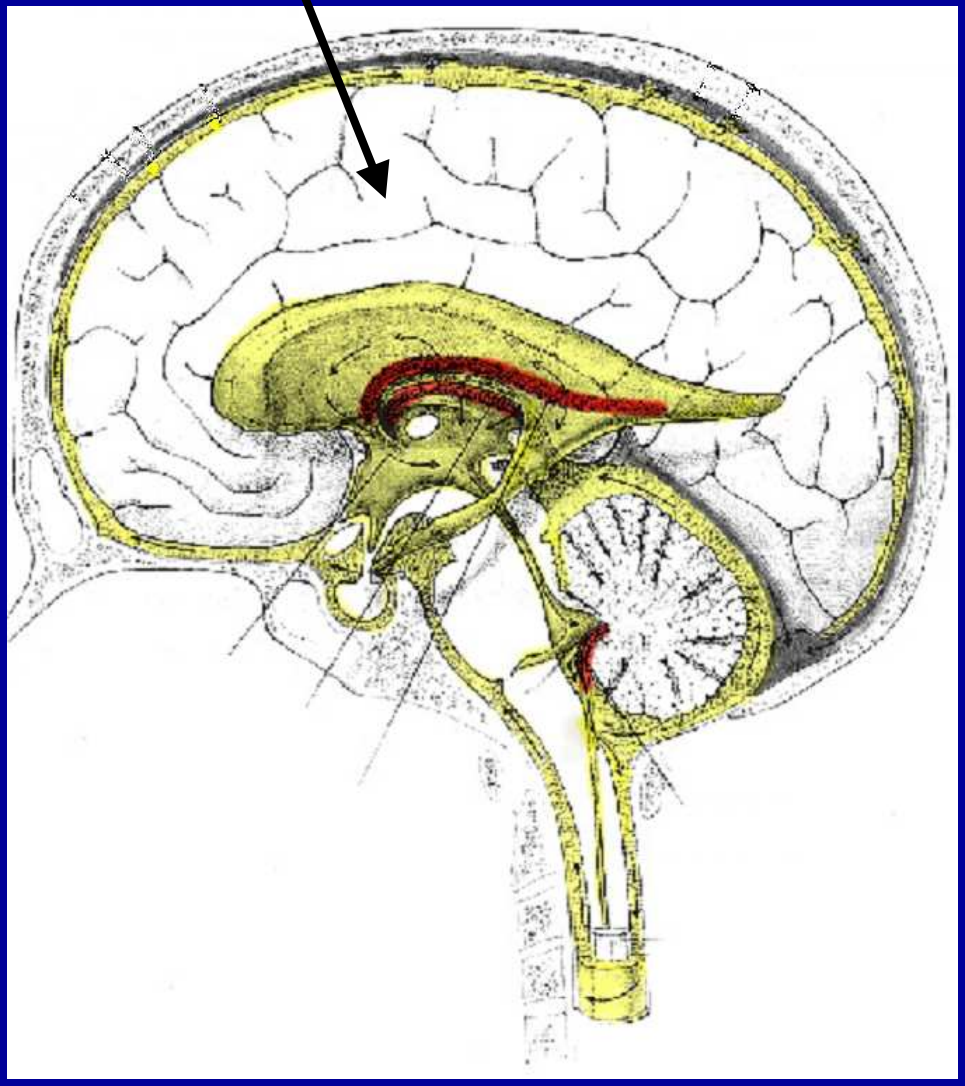
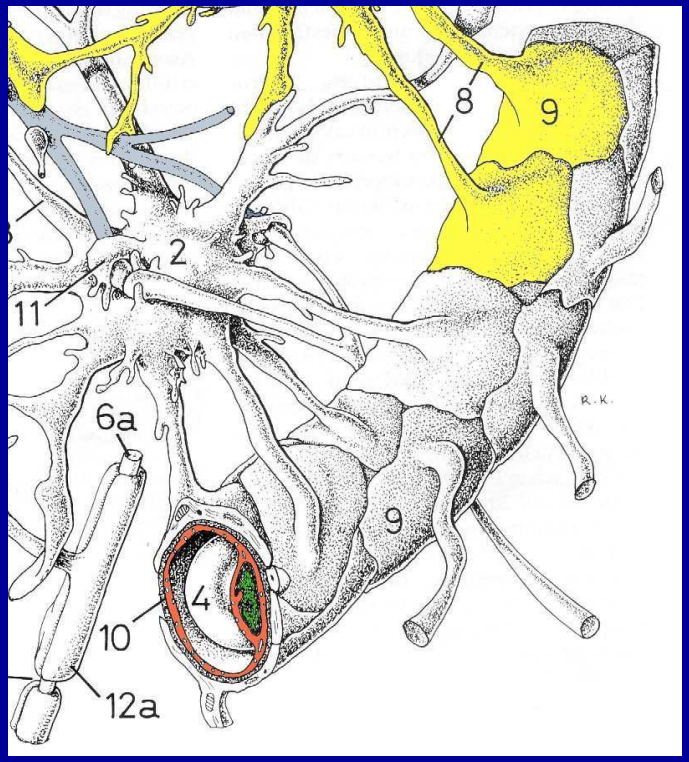
The Blood-Brain Barrier (BBB)



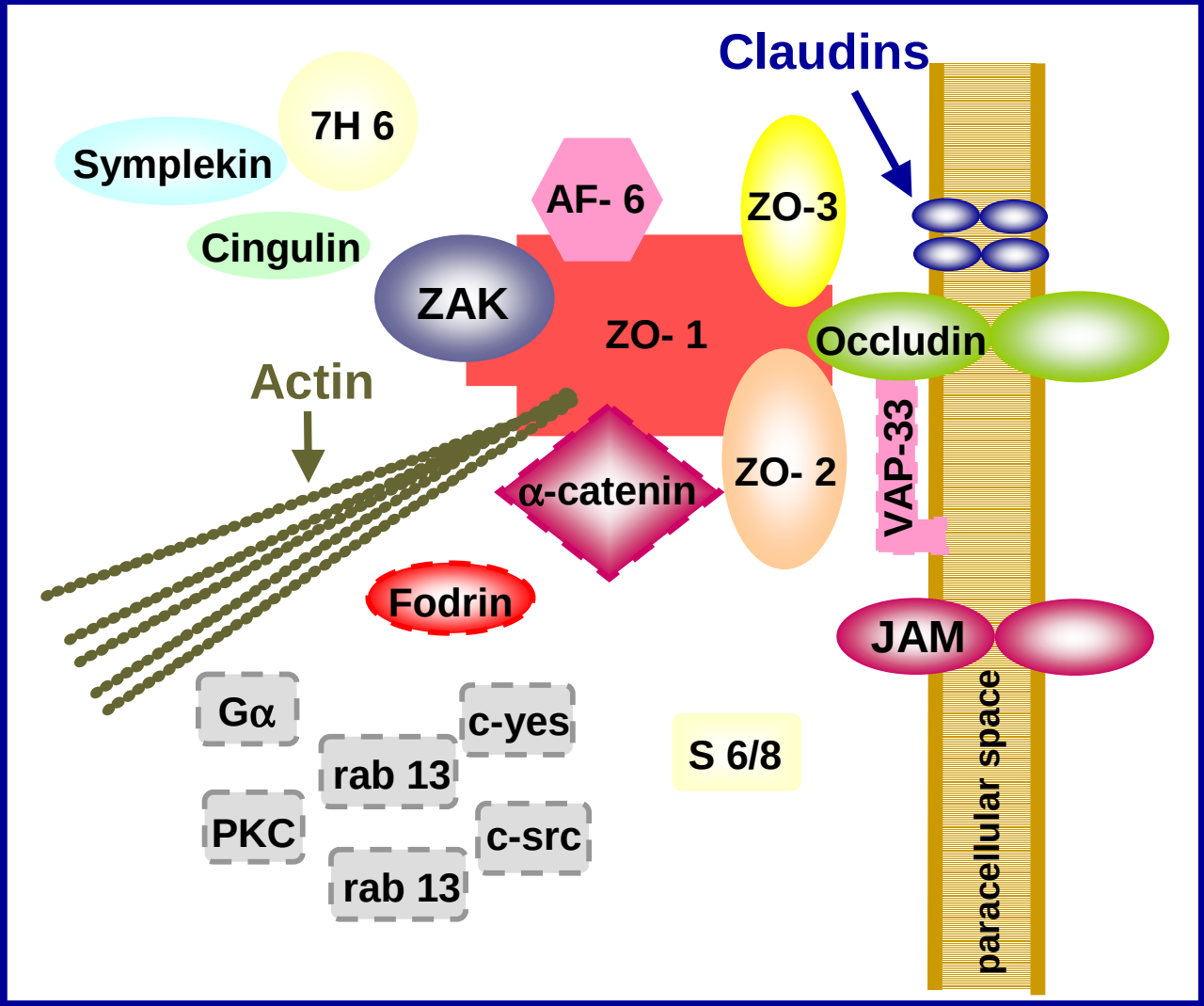
Endothelial cells of the cerebral capillaries form the permeability barrier



Function of the BBB

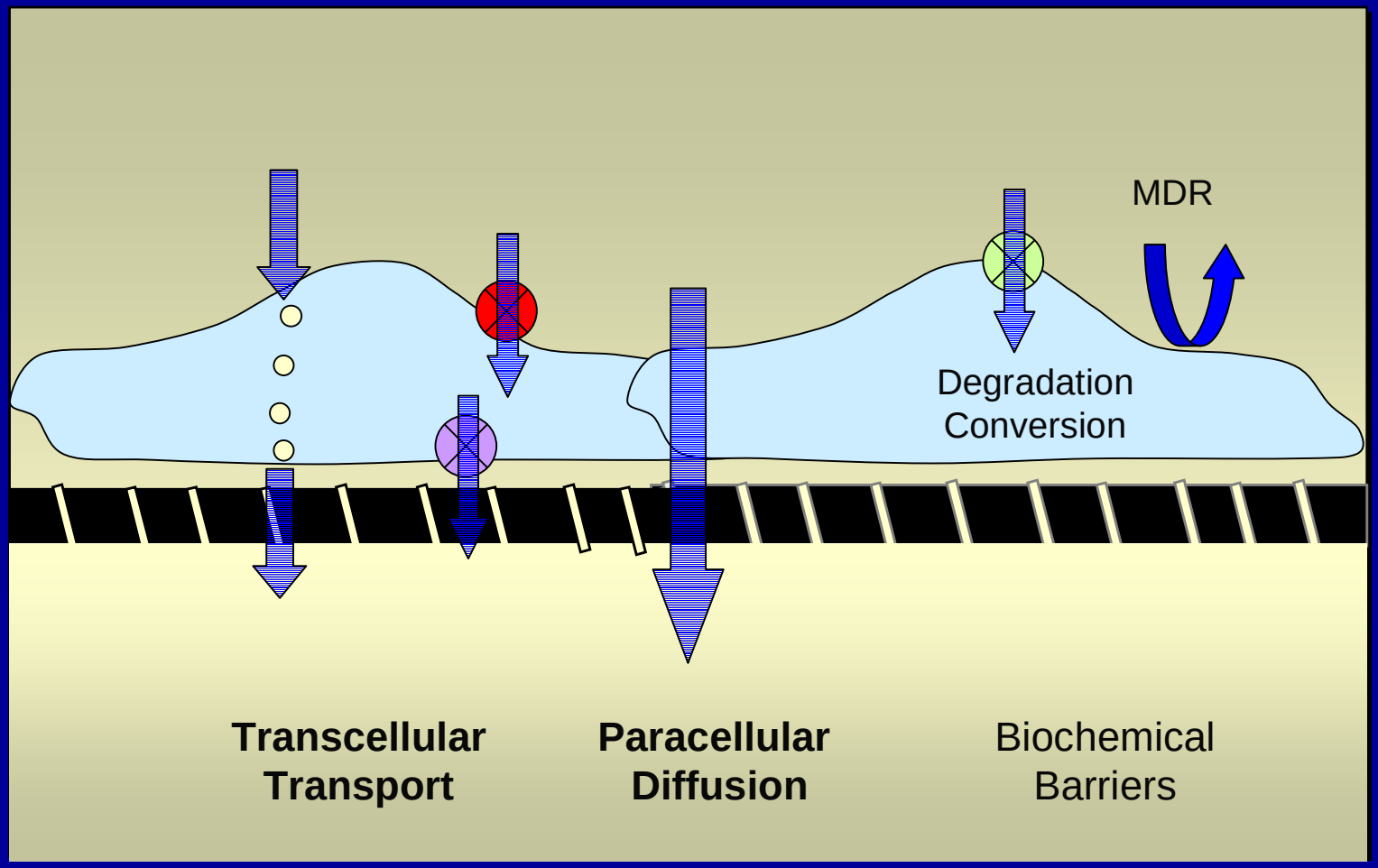
- maintenance of a constant solute/ion environment: „homeostasis" of the CNS
- essential for proper brain function
- control of substance flow between brain tissue and circulating blood
- controlled import of nutrients into the CNS
- protection against toxins

Proteins at tight junctions



Fanning et al., 1999, J Am Soc Nephrol 10: 1337-1345

Transport mechanisms at an endothelial cell monolayer



in vitro approach: motivations

- reduction of in vivo complexity
- precise determination of field parameters
- reproducible exposure conditions
- facilitated field and temperature monitoring

Rat brain capillary endothelial cells (RBEC)

removal of meninges

homogenizing

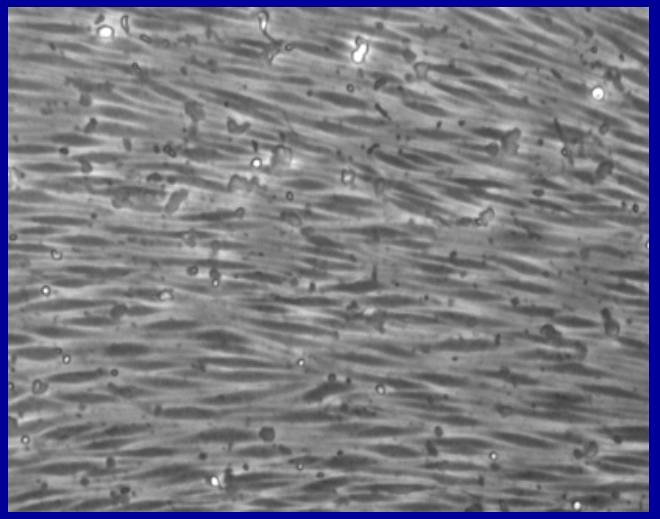
1st enzymatic digest

isolation of capillary vessels

2nd enzymatic digest

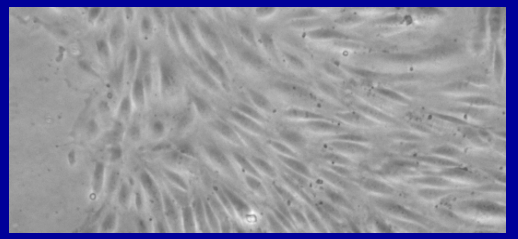
isolation of endothelial cells

sowing and culturing

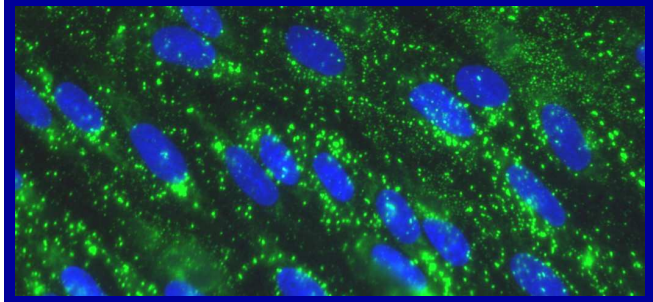


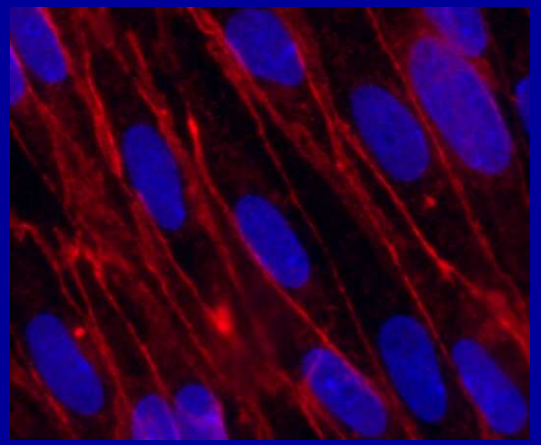
RBEC monolayer

characterization of RBEC



- + squamous morphology
- + von-Willbrand-Factor-VIII
- + vimentin
- + tight junction proteins: ZO-1, occludin
- - smooth muscle actin, GFAP, CD11b
- ---> minimal cell contamination





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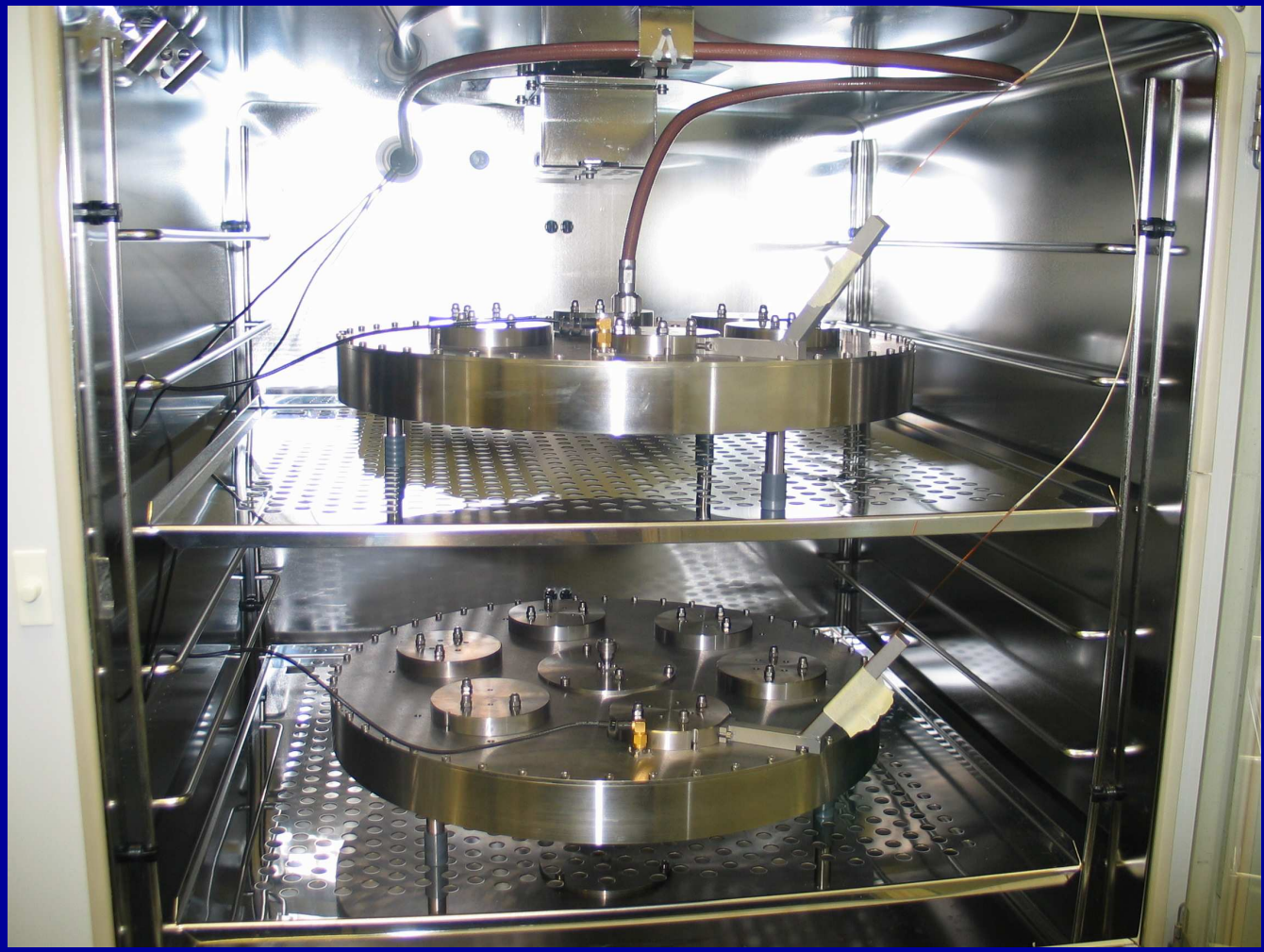
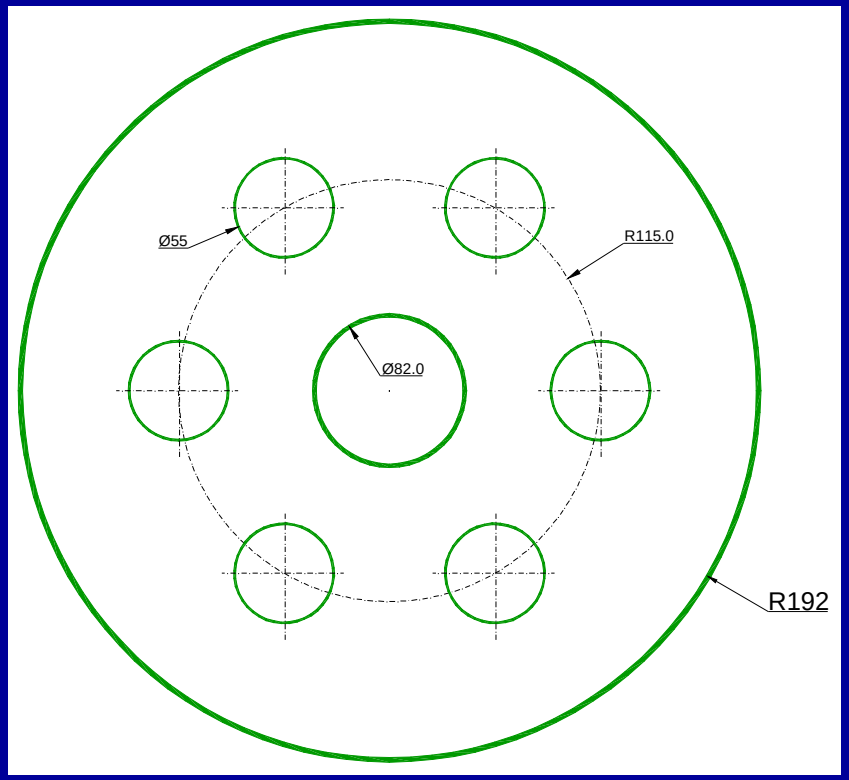
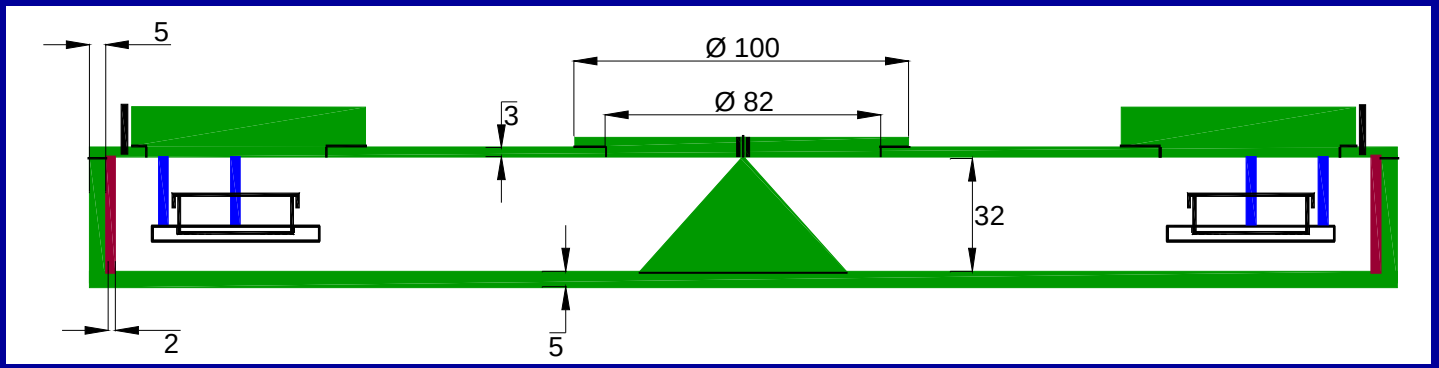
the exposure device

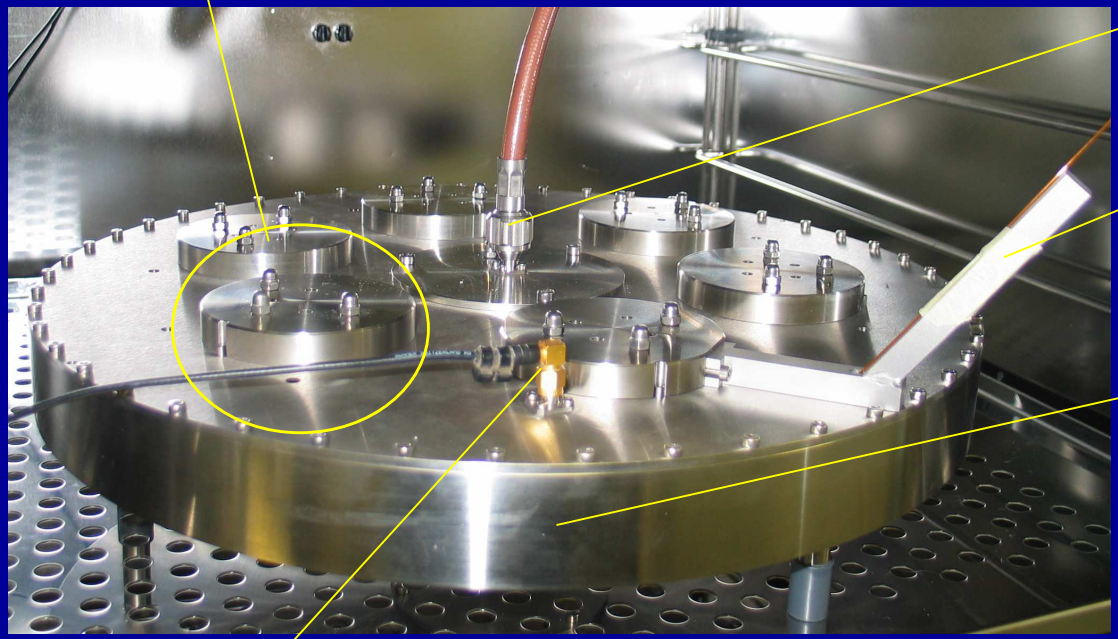


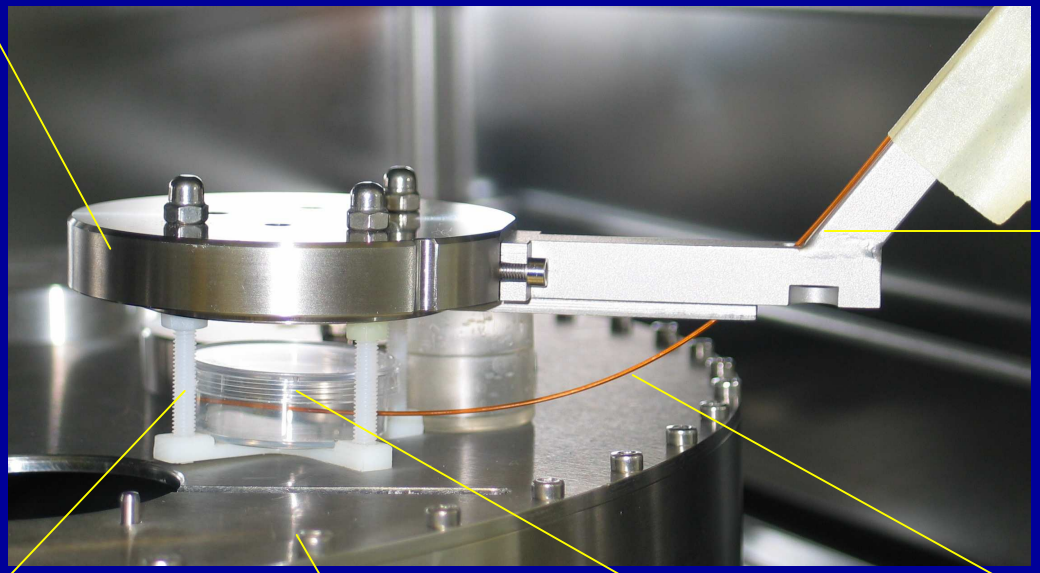
assembly of radial waveguide



- 6 petridishes
- 40 cm diameter, 9 cm height
- sample holder centres petridishes
- temperature probe
- field probe




cap
HF-Inlet
support for
fiberoptic probe
radial waveguide
field sensor
connector


cap
support for fiberoptic probe
fiberoptic temperatureprobe
tray for petridish
petridish
radial waveguide

GSM-Exposure setup:

- amplifier
- signal generator
- wave guides
- fiberoptic temperature probes
- incubator

exposure parameters

- two radial wave guides
  - 2 x 6 samples, thermistor probe, field antenna
  - parallel exposure & sham exposure
- generic UMTS signal
- generic GSM1800 signal
- permanent exposure
  - 3d duration
  - 4 different exposure levels (~0.4-8 W/kg)

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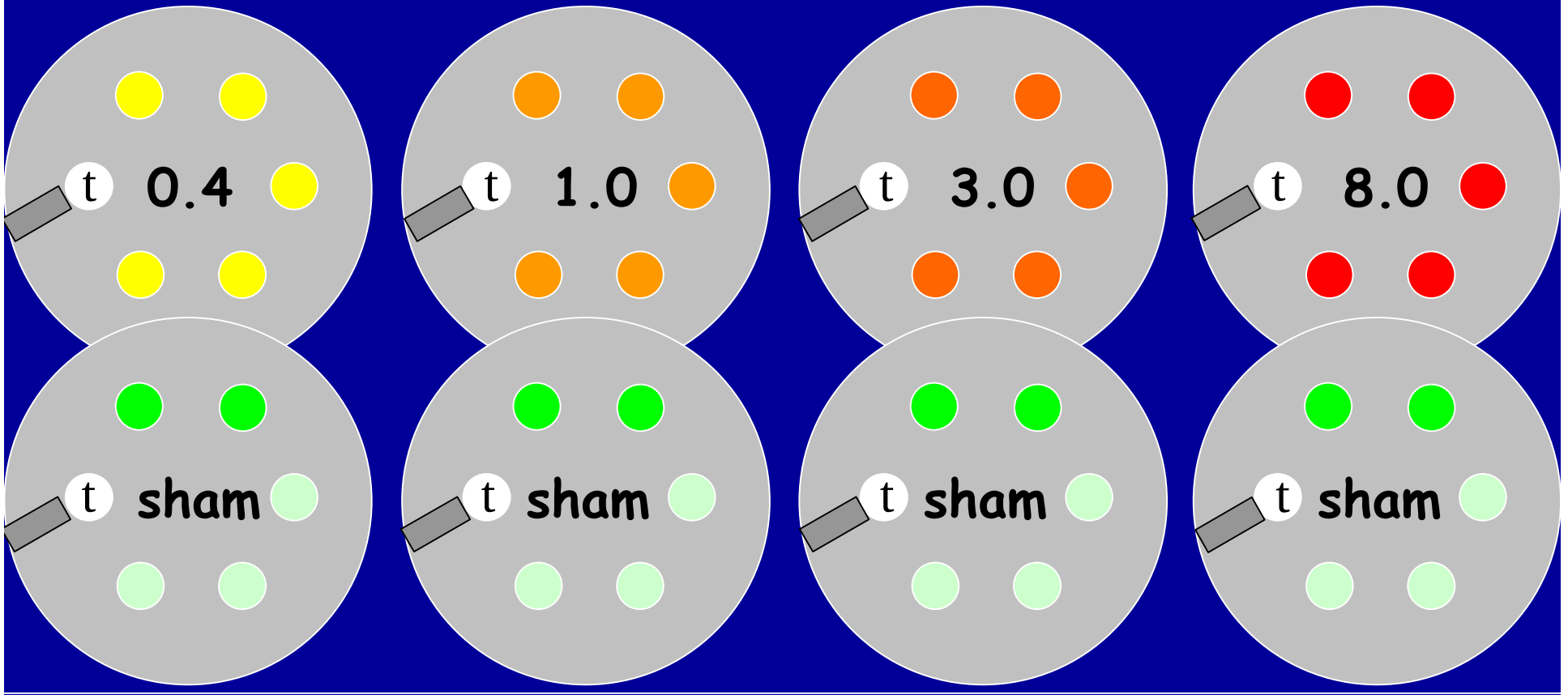
exposure groups: GSM1800 / UMTS @0.4-8.0 W/kg (72h)

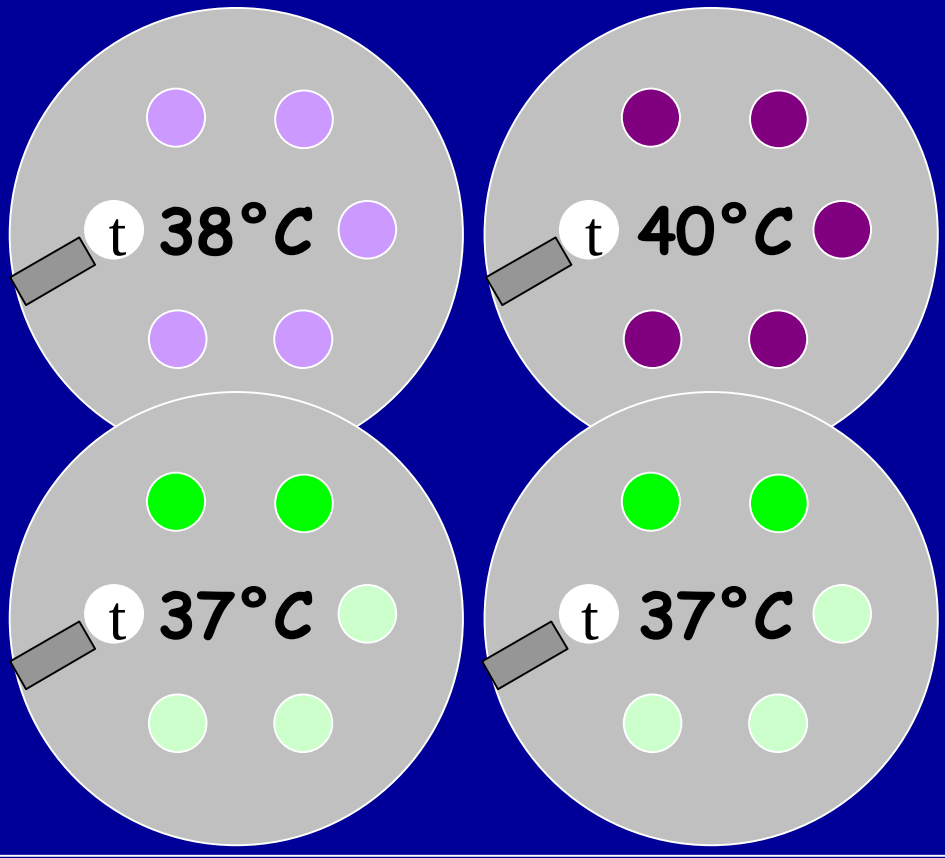


biological replicates:

5+2 GSM	5+2 GSM	5+2 GSM	5+2 GSM
5+2 UMTS	5+2 UMTS	5+2 UMTS	5+2 UMTS

temperature control groups:
38°C / 40°C (72h)

max. temperature
@ 8W/kg: 38°C



biological replicates:

5+2 38°C

5+2 40°C

Σ = 70 Chip Arrays

RNA isolation protocol

- lysis of RBEC immediately after termination of exposure (< 5 min.)
- RNA isolation from RBEC:
  Qiagen RNeasy Micro-Kit
  store samples @ -70°C
- quality control: *Agilent Bioanalyzer*
- *RNA-conc: min. 1µg/µL*

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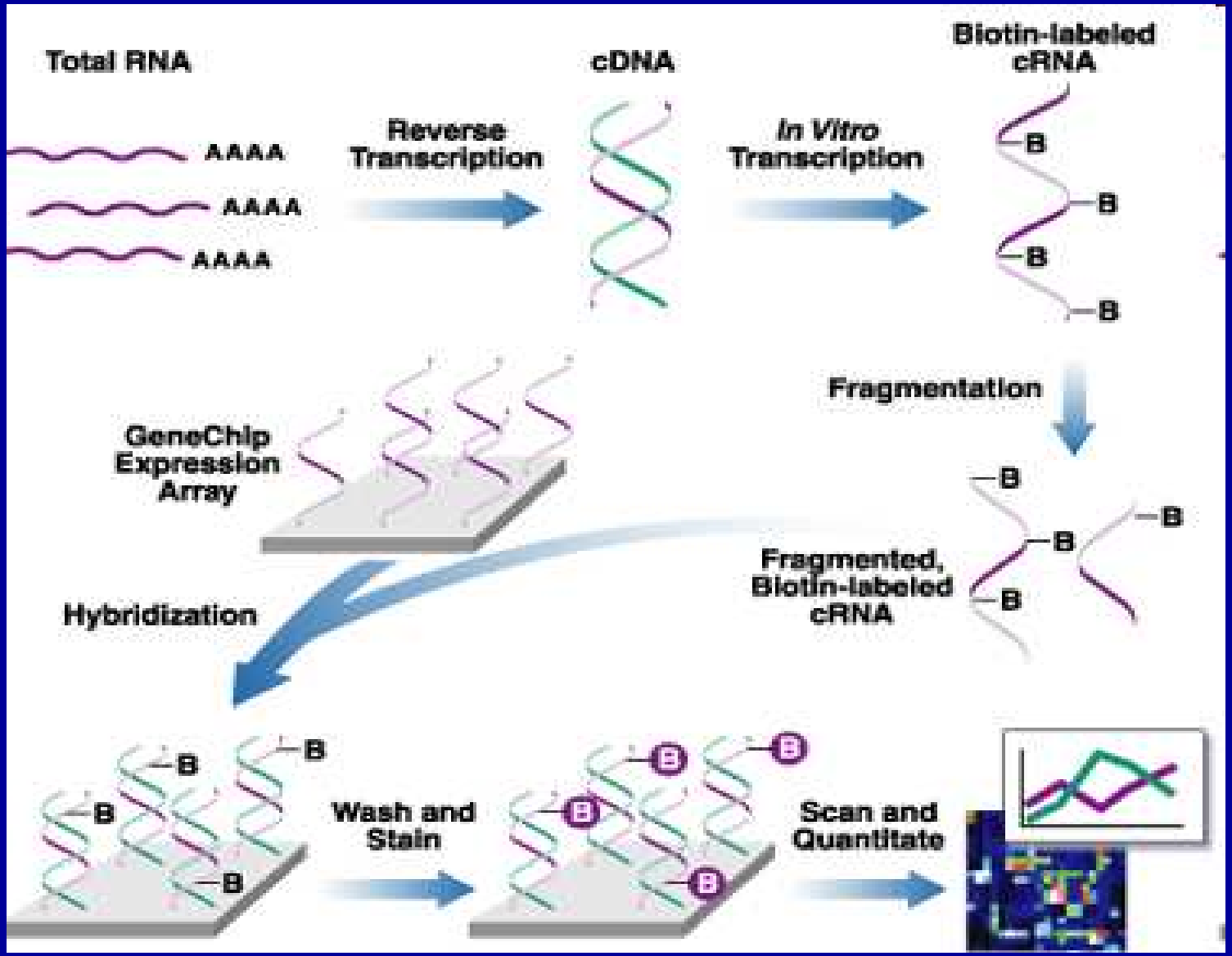
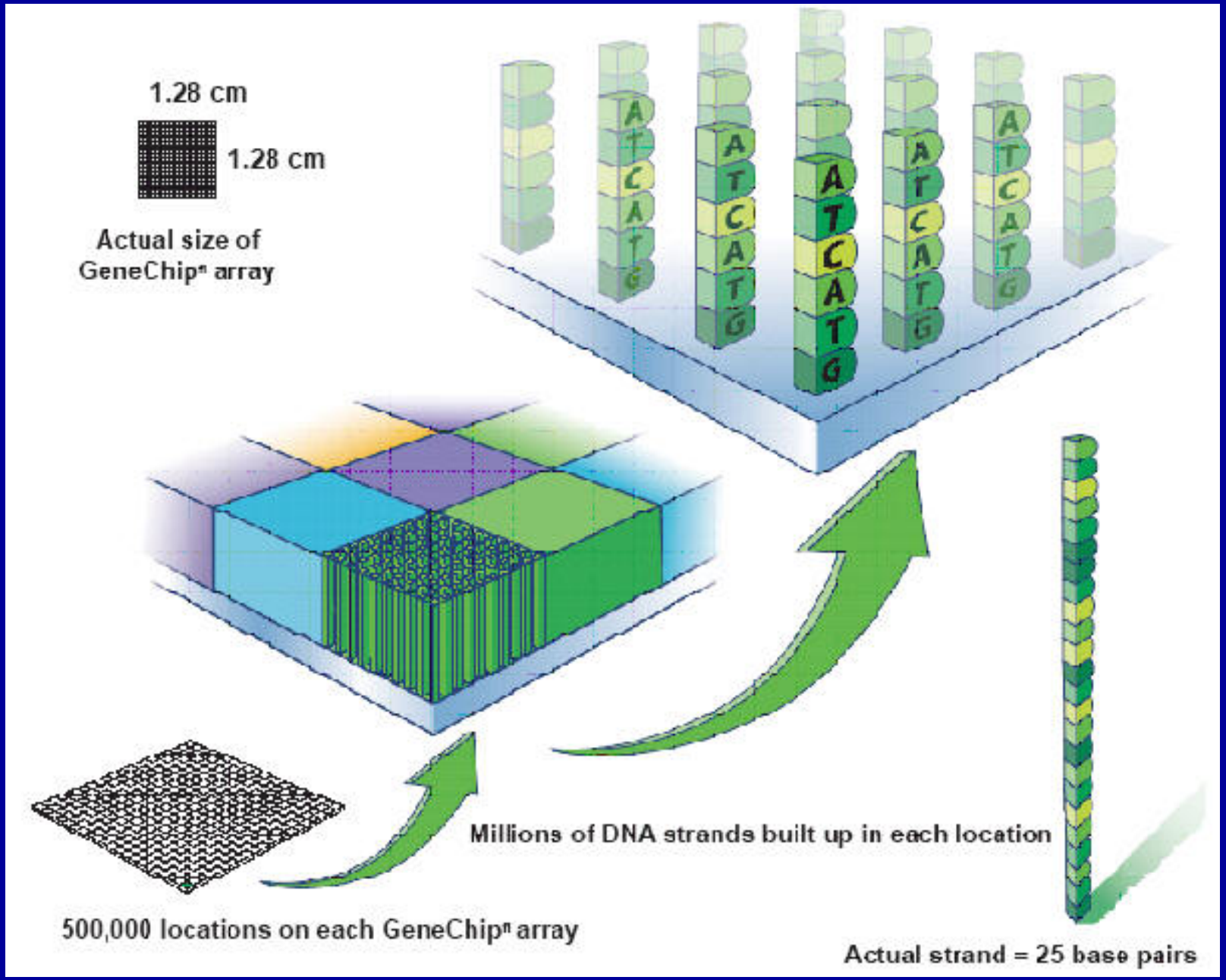
RNA-analysis with chip-microarrays



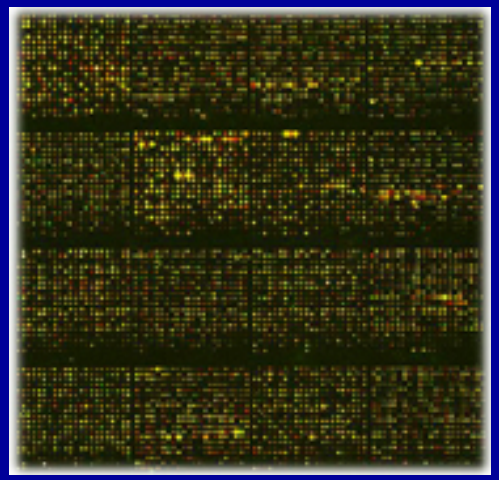
Quelle: http://www.dkfz.de/gpcf/uploads/pics/AffyPrincipleWorkingScheme.jpg



Quelle: http://keck.med.yale.edu/affymetrix/genechip%20tile.jpg

protocol for chip-arrays

- *reverse transcription RNA -> cDNA*
- *in vitro transcription cDNA -> cRNA+biotinlabelling*
- *fragmentation of cRNA*
- *hybridization: Affym. GeneChip® Rat Genome 230 2.0 Array*
- *washing and staining*
- *array scanning*



Affymetrix GeneChip®
Rat Genome 230 2.0 Array

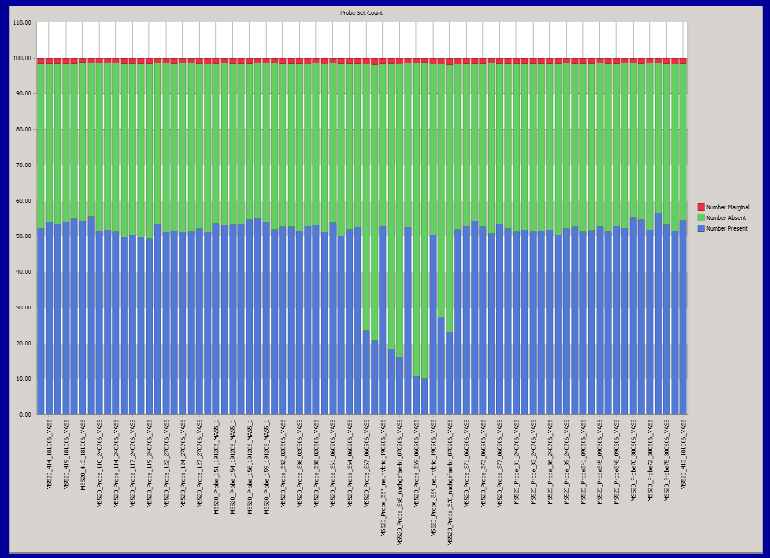
- data tables showing the signal intensities of the various probe sets
- 28000 genes on the chip!

~28,000 genes

Quelle: http://privatewww.essex.ac.uk/~harry/images/affymetrix-genechip-hgu133p.gif

filtering of ‚absent calls'

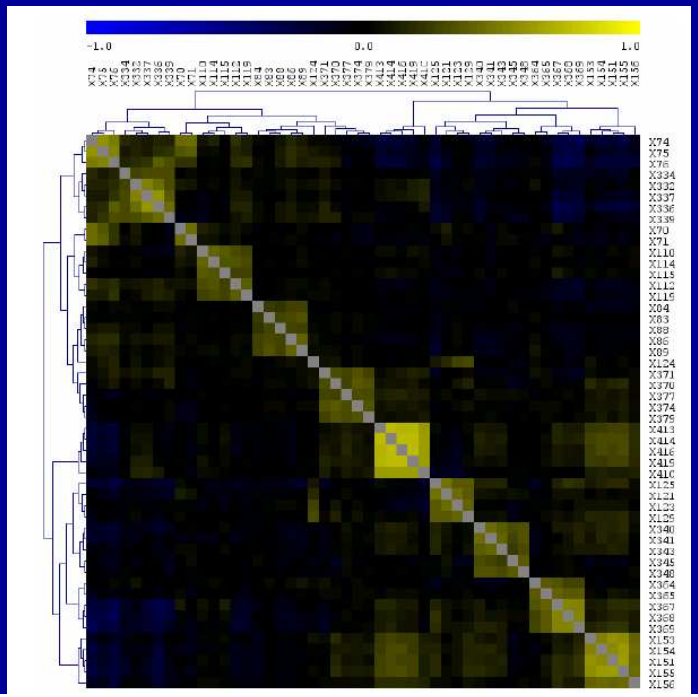
- MAS 5.0 (Microarray Suite, Affymetrix)
- normalization of signal levels
- of 28000 genes on the chip,
  18663 could be detected reliably
  („present" calls in ≥ 3 of 5 chips per
  experimental group)



18,663 genes

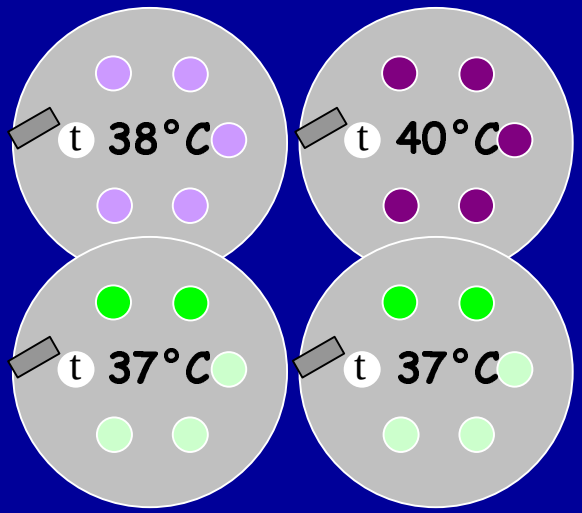
filtering of genes with fold-change < 1.4

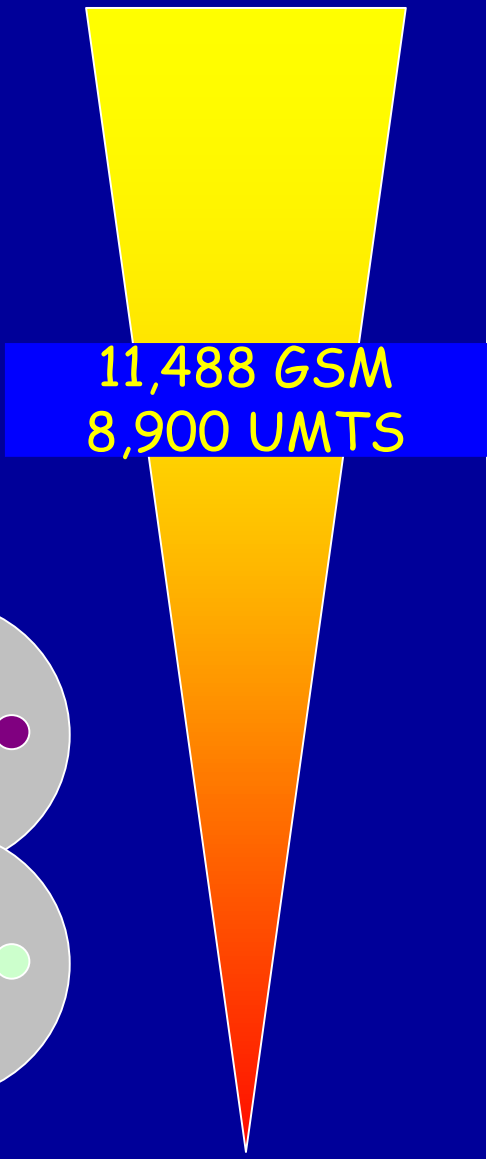
- of 18663 present genes, 14287 showed at least 1.4x change in gene expression compared to sham exposed RBEC



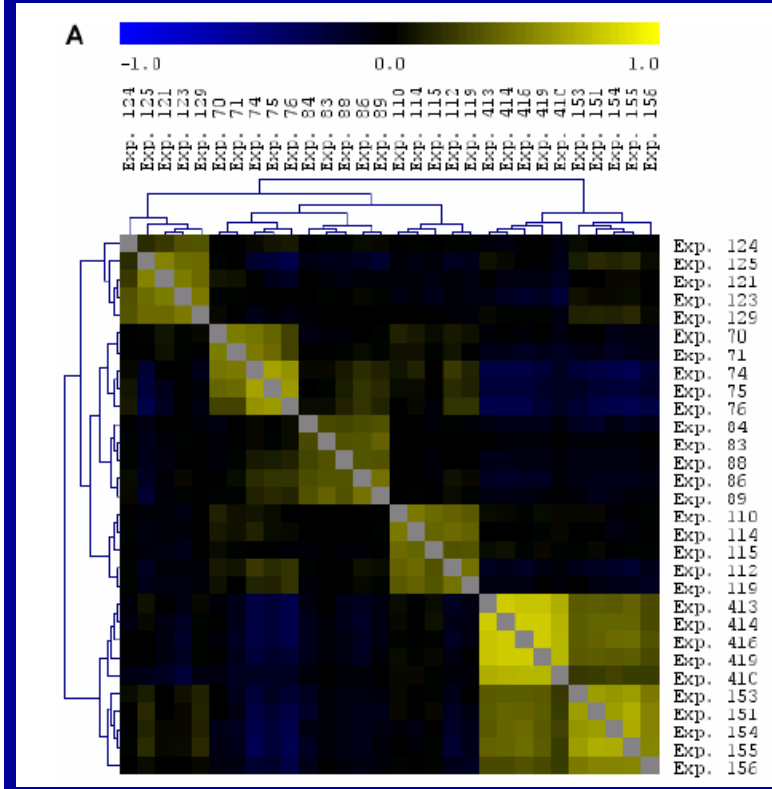
filtering by t-test vs. temperature controls

- of 14287 differentially expressed genes, differential expression of 11488 genes (GSM) or 8900 genes (UMTS) was not merely due to temperature increase ($p < 0.05$).

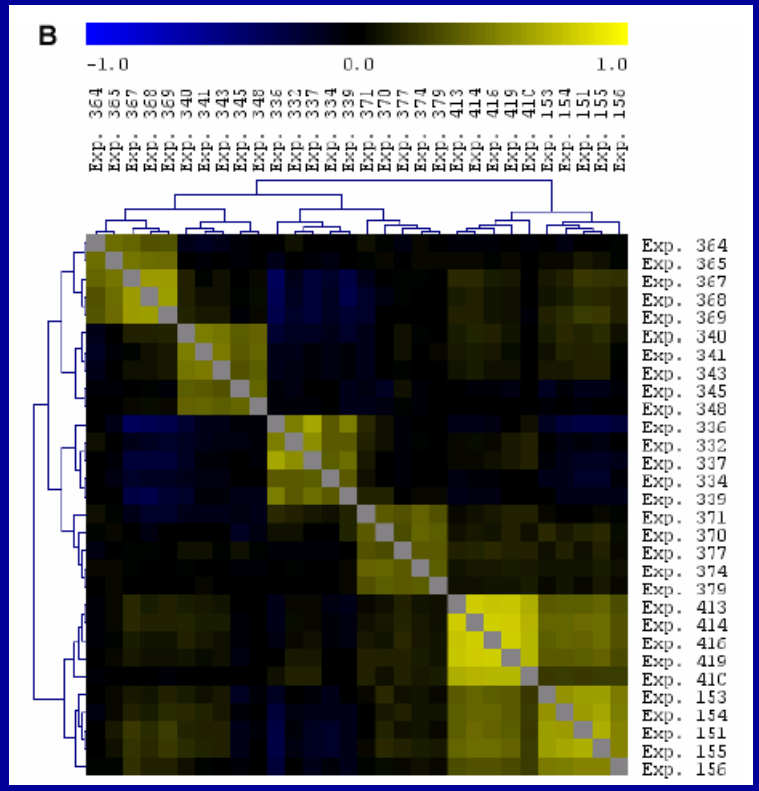




correlation analysis



UMTS



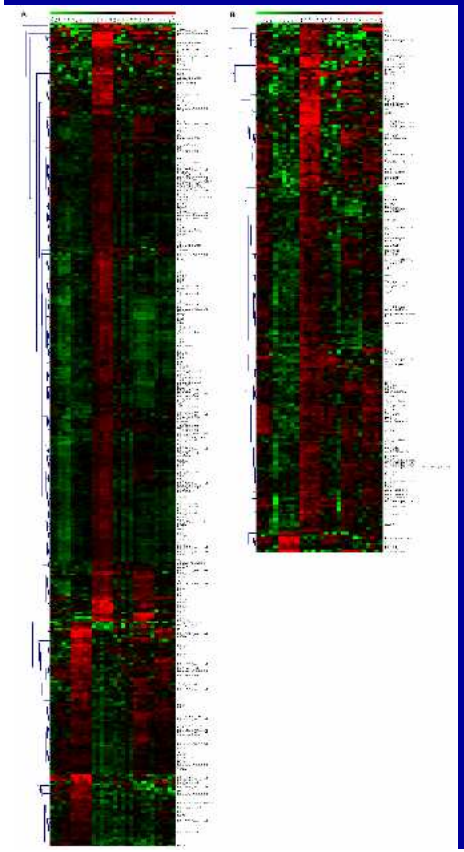
GSM

good clustering of experimental groups after filtering for present genes, 1.4x fold-change, temperature.

Discriminatory Genes Analysis:

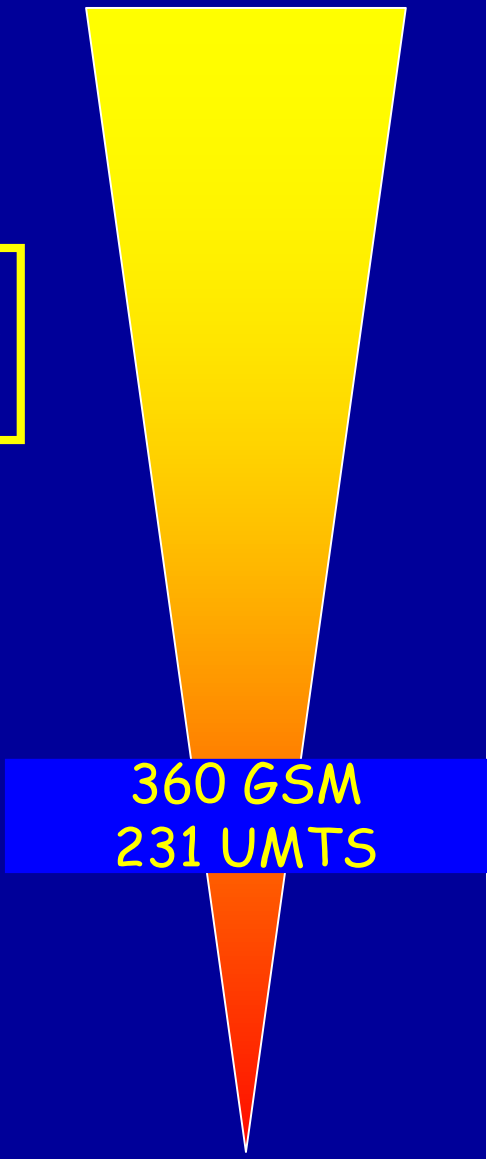
SAM (Significance Analysis of Microarrays)

1W/kg vs. 3W/kg

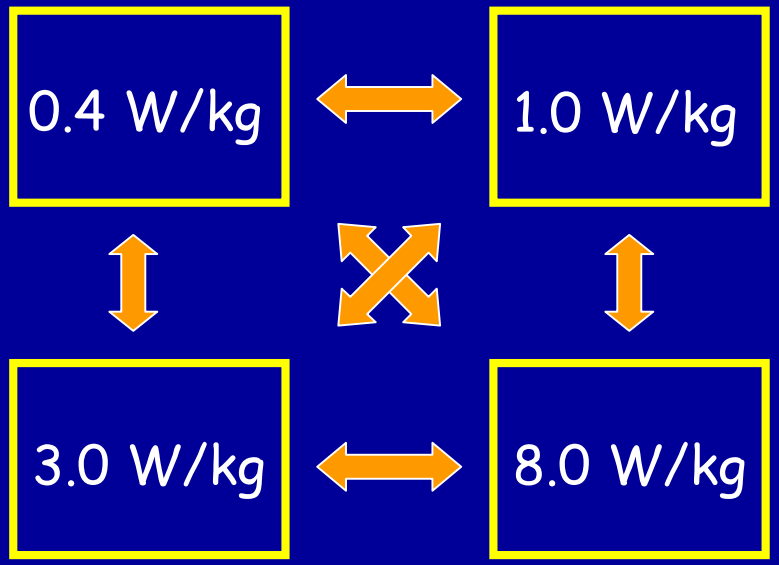


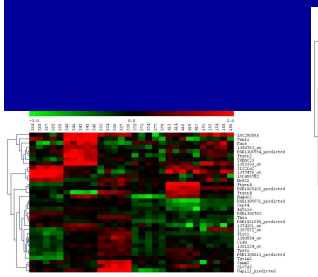
1.0 W/kg ⟷ 3.0 W/kg

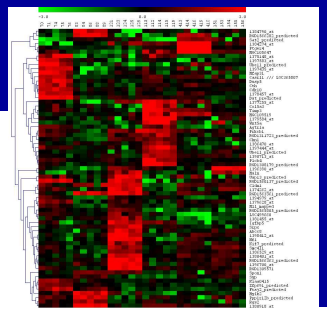
- GSM1800: of 11488 genes, 360 genes were identified by SAM as differentially expressed between 1 W/kg group and 3 W/kg
- UMTS: 231 of 8900 genes
- parameter: no false positives

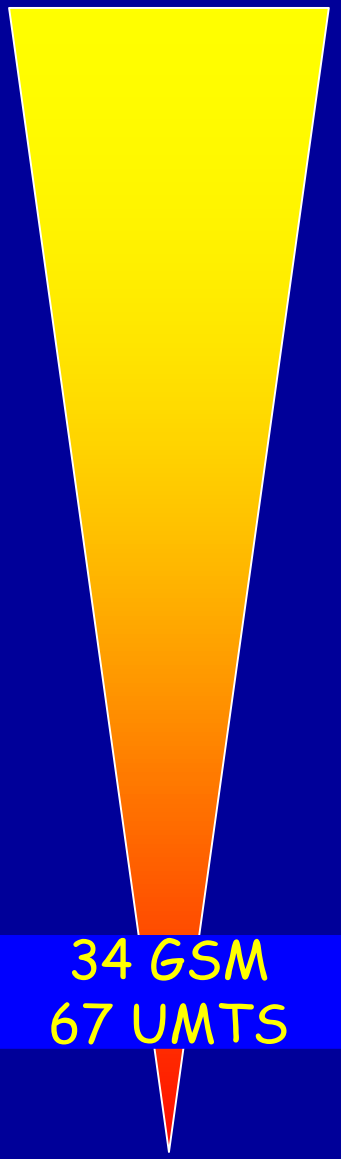


SAM: 4x cross-wise comparison
0.4 W/kg – 8.0 W/kg







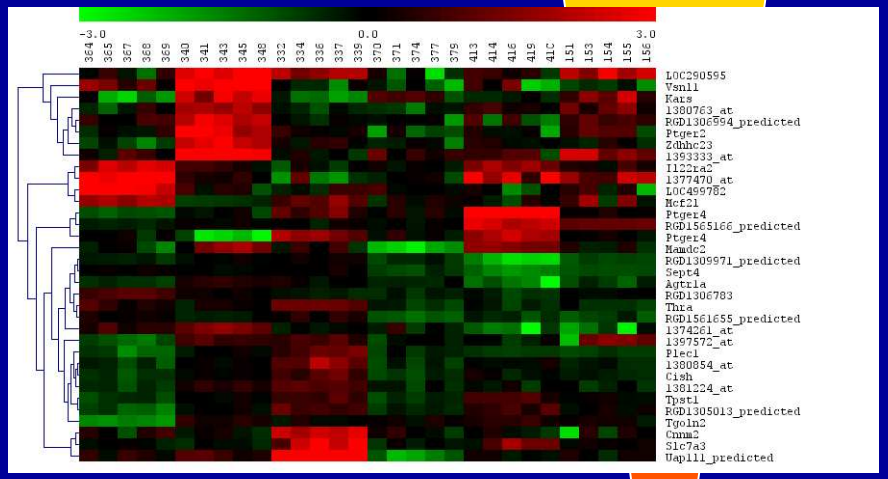


34 GSM
67 UMTS

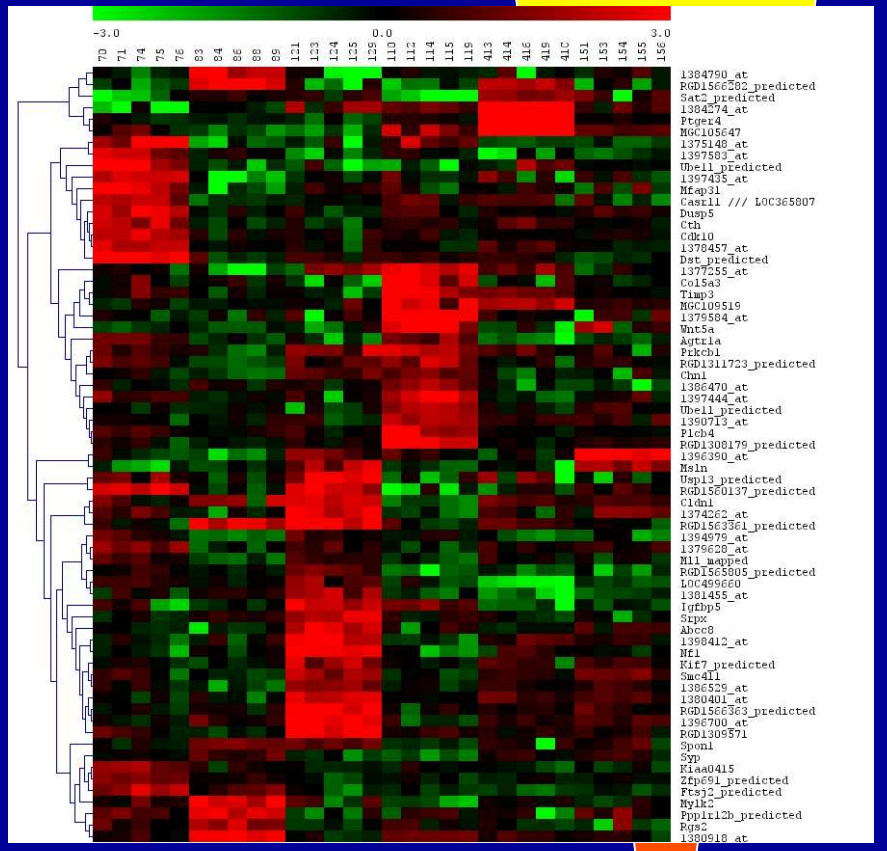
bioinformatic evaluation

- processing of raw data with MAS5.0 probe level algorithm (= normalization)
- filtering absent genes (n>2)
- computing ratio exposed vs. control (sham)
- filtering genes by fold changes (min. +/- 1.4)
- t-test ($p<0.05$) exposed samples vs. temperature controls (filters genes that only changed expression due to temp. increase)
- SAM discriminatory genes analysis 1W/kg vs. 3W/kg
- SAM discriminatory genes analysis between all signal intensities
- Pathway analysis with discriminatory genes

gene lists after crosswise SAM analysis



34 GSM



67 UMTS

„manual" selection of BBB related genes EXAMPLES

integrin alpha 1
activated leukocyte cell adhesion molecule
angiotensin II receptor, type 1 (AT1A)
procollagen, type V, alpha 3
procollagen, type XII, alpha 1
tropomyosin 1, alpha
procollagen, type V, alpha 1
nidogen 1
a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 8 (predicted)
actin, beta;similar to Actin, cytoplasmic 2 (Gamma-actin);actin, gamma, cytoplasmic 1
cadherin 23 (otocadherin)
catenin (cadherin-associated protein), alpha 1
heat shock protein 70kDa 12B (predicted)
integrin alpha 1
matrix metallopeptidase 14 (membrane-inserted)
similar to RIKEN cDNA 1810022C23;peroxisomal delta3, delta2-enoyl-Coenzyme A isomerase
solute carrier family 16 (monocarboxylic acid transporters), member 13
solute carrier family 39 (metal ion transporter), member 6
solute carrier family 39 (zinc transporter), member 14 (predicted)
solute carrier family 4, sodium bicarbonate cotransporter, member 7
solute carrier family 5 (sodium-dependent vitamin transporter), member 6
solute carrier family 6 (neurotransmitter transporter, creatine), member 8
tight junction protein 1 (predicted)
transforming growth factor, beta 2
transforming growth factor, beta 3
transforming growth factor, beta receptor II
tumor necrosis factor receptor superfamily, member 21 (predicted)
plakophilin 1 (predicted)
a disintegrin and metallopeptidase domain 11 (predicted)
vinculin (predicted)
caspase 12
caspase 1
contactin 3
laminin, beta 2
procollagen, type XVIII, alpha 1
chemokine (C-X-C motif) ligand 10
MAP kinase-activated protein kinase 2
mitogen activated protein kinase 8 interacting protein
mitogen activated protein kinase kinase kinase kinase 2 (predicted)
mitogen-activated protein kinase 7
endothelin converting enzyme 1
syndecan 2
solute carrier family 27 (fatty acid transporter), member 4
solute carrier family 33 (acetyl-CoA transporter), member 1
solute carrier family 37 (glycerol-6-phosphate transporter), member 4
solute carrier organic anion transporter family, member 4a1
actinin, alpha 1
ATP-binding cassette, sub-family C (CFTR/MRP), member 8
ATP-binding cassette, sub-family D (ALD), member 4
insulin-like growth factor 2 receptor
integrin beta 3 binding protein (beta3-endonexin)
matrix metallopeptidase 2
phospholipase A2, group VI
phospholipase A2, group VI
transforming growth factor, beta 2
transforming growth factor, beta 2
tumor necrosis factor receptor superfamily, member 1a
actinin, alpha 1
presenilin 2
caspase 4, apoptosis-related cysteine peptidase
procollagen, type XVIII, alpha 1

procollagens (extracell. matrix)

solute carrier fam. (var. transporters)

ABC-Proteins (multidrug resistance)

caspase 1,4,12 (apoptosis)

claudin 1 (tight junctions)

[illegible] family 19, member 1
[illegible] oxide synthase trafficker
insulin-like growth factor 1 receptor
solute carrier family 27 (fatty acid transporter), member 1
solute carrier family 16 (monocarboxylic acid transporters), member 6
solute carrier family 17 (anion/sugar transporter), member 5
heat shock 70kD protein 1A;heat shock 70kD protein 1B (mapped)
fibronectin 1
phospholipase A2, group VI
thrombospondin 1
ATP-binding cassette, sub-family A (ABC1), member 7
scavenger receptor class B, member 1
claudin 1
phospholipase A2, group VI
von Willebrand factor
claudin 1;McKusick-Kaufman syndrome protein
ATP-binding cassette, sub-family D (ALD), member 4
heat shock protein 4
coagulation factor VIII
claudin 22 (predicted)

BBB selection + SAM 1 vs. 3 W/kg

Gene Symbol	Affy Probe Set ID	Gene Name	GSM	UMTS	0.4 W/kg	1.0 W/kg	3.0 W/kg	8.0 W/kg
	1382189_at	syndecan 2	X		-10,70	-5,24	1,79	6,92
Pkp1_predicted	1385182_at	plakophilin 1 (predicted)	X		-1,49	-1,19	1,45	-1,34
	1398476_at	vinculin (predicted)	X		-1,68	1,04	1,71	-1,33
Tgfb2	1388011_a_at	transforming growth factor, beta 2	X		1,08	1,05	1,78	1,06
Casp1	1369186_at	caspase 1	X		1,17	1,48	-1,25	-1,48
Gene Symbol	Affy Probe Set ID	Gene Name	GSM	UMTS				
Tpm1	1395350_at	tropomyosin 1, alpha		X	-1,27	-2,28	2,85	1,48
Abcc8	1369632_a_at	ATP-binding cassette, sub-family C (CFTR/MRP), mem		X	-1,33	-1,49	5,70	1,39

Pathway analysis: GO (gene ontology)

and further biological annotation lists - TreeRanker©

- cellular component
  this may be an anatomical structure or a gene product group
- biological process
  series of events accomplished by one or more ordered assemblies of molecular functions
- molecular function
  describes activities, such as catalytic or binding activities, that occur at the molecular level.

for further information:

http://www.geneontology.org/

significantly enriched pathways

(1 vs. 3 W/kg)

- cell communication
- signal transduction
- protein binding
- adherens junction
- focal adhesion
- cell-matrix junction
- cell-substrate adherens junction
- basloateral plasma membrane

- CAVE: rat genome annotation still incomplete

- ✓ establishment of an isolation method for RBEC
- ✓ characterazation of RBEC
- ✓ installation of exposure device and determination of field parameters
- ✓ exposure of RBEC
- ✓ RNA isolation
- ✓ chip-arrays for differential gene expression
- ✓ bioinformatic evaluation of gene-chip data

- RT-PCR validation
- identification of protein targets

closing remarks

- cells react differently to GSM1800 and UMTS
- clear clustering of most samples from one treatment group
- expression changes oberved from –15x to +13x
- no general trend of gene expression parallel to SAR increase
- selection of candidates for qRT-PCR is ongoing

- Lehrst. f. theor. Elektrotechnik
  Dr. Joachim Streckert, Dr. Andreas Bitz
  AG Prof. Hansen, *BU Wuppertal*

- IFG (integrierte funktionelle Genomik)

  Dr. Kurt Sieberns, H. Stegemann, H. Lahl, Dr. M. Eisenacher
  IZKF, *Uniklinik Münster*

- Miltenyi Biotec

  Dr. Corinna Scholz, Dr. Jan Schäferkordt

- Bundesamt für Strahlenschutz
  Dr. Monika Asmuß

thank you for your ...