25 years

1987-2012

3R Research Foundation – ecopa- Swisstox

Program and Abstracts

3R Sessions and Posters, Room Basic 2nd Floor

Continuing Education, 19-20 November, 2012

Technopark Zürich

3R Proceedings

of the joint 25th Anniversary Meeting 2012

November 19 – 20, 2012

Technopark, Zürich, Switzerland

Accredited as a Continuing Professional Education event for directors of animal experimentation studies and animal experimenters in Switzerland (more information on page 12).

organized by

3R Research Foundation

www.forschung3r.ch

and

Swiss Laboratory Animal Science Association

www.sgv.org

in cooperation with the invited two societies

european consensus-platform for alternatives

www.ecopa.eu/

and

Swiss Society of Toxicology

www.swisstox.ch/swisstox-en

Acknowledgements

Sponsoring

An event of this kind requires substantial funding.

- The Administrative Board of the 3R Research Foundation Switzerland authorized the generous support of all four 3R sessions. see: http://www.forschung3r.ch/en/information/stiftungsrat.html

- Catering is possible due to the benefits derived from the companies present at this meeting:

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SF3R:	Peter Maier	
	Ernst Diener	
Swisstox	Franziska Boess	
	Friedlieb Pfannkuch	
ecopa	Lisbeth Knudsen	

Many thanks

- To all the speakers for coming and providing slides for the proceedings
- To the colleagues from the SF3R Evaluation Committee: see http://www.forschung3r.ch/en/information/experten.html
- To the colleagues who chair the 3R Sessions
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Catering:	Restaurant Villaggio im Technopark, Compass Group

Preface

What, where, who?

Refinement goes together with Replacement: Welcome to the 25th Anniversary Meeting jointly organised by the *Swiss Laboratory Animal Science Association* and *3R Research Foundation Switzerland*.The slogan chosen, "Refinement goes together with Replacement", not only reflects our co-operation, it is also intended to point beyond the day. Refinement is a key issue in animal experiments for research and testing and the 3R Research Foundation Switzerland has supported a number of Refinement-oriented projects over the past 25 years. Replacement becomes possible once we know more about actual processes that occur in animal experiments. The Foundation is Switzerland's premier institution to promote and support scientific research projects aimed at replacing animal testing.

Together we make a difference: The people who can implement the 3Rs are those who work with animals and design research studies, including studies required to ensure the safety of humans, animals and our environment. We particularly welcome the *Swiss Society of Toxicology*, Swisstox's, participation in designing the programme and in leading some of the presentations in the session *"The 3R's in Toxicity and Safety Testing"* (see pages 27-39).
Implementation of the 3Rs occurs in a global context and we rely upon international exchange of information and insights. As a member of ecopa, the *European Consensus Platform for 3R Alternatives*, 3R Research Foundation Switzerland extends a warm welcome to the representatives of Europe's national consensus platforms.* In three sessions, *"Progress of the 3Rs in Europe"*, *"Support of the 3Rs"* and "The 3Rs as a European Consensus", they will provide information on recent developments outside Switzerland (see pages 47-69).

Academic research: 3R Research Foundation Switzerland has been making a difference in academic research. Projects from various fields will be presented in the session *"3Rs in Research and Development"* (see pages 15-26). The *Poster Exhibition* (see pages 81-112) provides ample proof that 3R-relevant research is very much alive, with 18 different laboratories currently at work on the implementation of 3R-relevant methodologies.

Research promotion: Over 25 years ago, forward-looking politicians and scientists created 3R Research Foundation Switzerland as a Public-Private-Partnership organisation. Since then, the Swiss taxpayers, through the Federal Veterinary Office (FVO), and Switzerland's pharmaceutical research industry have supported 134 projects selected from 143 draft projects and 456 applications. Stringent selection criteria and results published in peer-reviewed journals have led to a measurable increase in 3R-relevant knowledge.

Outlook: 3R issues have been and remain highly topical, with new methodologies in life sciences promising further positive developments towards our ideal of Replacement. The increasing number of applications for 3R relevant research projects deemed worthy of our support and is a strong indicator that the research community has come on board. I do hope and trust that the Foundation will be able to access additional funding to sustain researchers in their full implementation of 3R-relevant ideas.

Thank you for your interest in the 3Rs and enjoy the lasting benefits of this event.

Peter Maier
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Scientific Adviser
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E-Mail (personal): peter_maier@bluewin.ch or *peter.maier@uzh.ch*

*Consensus between between four kinds of stakeholders, i.e. academic circles, the authorities, the chemical/pharmaceutical industries, and animal protection agencies..

“Refinement goes together with Replacement”

Contents

All 3R Sessions are taking place in the Conference Room Basic 2nd floor .

All 3R Sessions will be transmitted by video into the Conference Room Pascal 3rd floor.

Use this opportunity in order to attend selected 3R lectures only.

Program Overview

Monday 19.11 2012 Auditorium SGV

	Empathy in the animal kingdom	
09:00	WELCOME	SGV and SF3R
09:10 10:10	Plenary Lecture "Empathy in the animal kingdom"	S. D. Preston (University of Michigan, USA)
10:10 10:40	Social modulation of pain as evidence for empathy in mice	D.J. Langford (University of California, San Francisco, USA)
	COFFEE BREAK- INDUSTRIAL EXHIBITION	
11:10 11:40	Empathy is moderated by genetic background in mice	G.P. Lahvis (Oregon Health and Science Univ., USA)
11:40 12:10	Empathy and pro-social behaviour in rats	P. Mason (University of Chicago, USA)
	LUNCH- INDUSTRIAL EXHIBITION	
	Pain perception in fetuses and neonates	
13:40 14:00	Developmental differences between precocial and altricial animals.	R. Zeller (University of Basel, CH)
14:00 14:45	Pain perception in human fetuses and neonates	N. Powell (University of Birmingham, UK)
14:45 15:30	Pain perception in rodent fetuses and neonates	G.A .Barr (Children's Hospital of Philadelphia,USA)
	COFFEE BREAK- INDUSTRIAL EXHIBITION	
	Pain behaviours and assessment in rodents	
16:00 16:50	Rodents grimace scale (mouse and rat), their use to evaluate postoperative analgesics	JS Mogil (McGill University, Canada)
16:50 17:05	SGV AWARDS 2012	SGV
17:05 17:30	Nest building and burrowing	P. Jirkof (University of Zürich, CH)
18:00	**SGV General Assembly at 18:00**	
20:00	**Gala Dinner Restaurant Metropol**	

Monday 19.11 2012 SF3R

Room Basic (2nd Floor) and Video in Room Pascal (3rd Floor)

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10:25 10:50	MRI its impact on Refinement and Reduction	N. Beckmann NIBR, Basel
	COFFEE BREAK - INDUSTRIAL EXHIBITION - POSTER VIEWING	
11:10 11:40	Bacterial Meningitis: Brain Injury and Regenerative Therapy *in vitro*	S. Leib Inst. for Infectious Diseases, Univ. of Berne
11:40 12:10	From pigs to cells: Prediction of virulence of classical swine fever virus *in vitro*	N. Ruggli IIVI, Mittelhäusern
	LUNCH - INDUSTRIAL EXHIBITION - POSTER VIEWING	
13:40 14:05	*In vitro* induction of stage conversion in the parasite *Neospora caninum*	A. Hemphill Inst. of Parasitology, Univ. of Berne
14:05 14:30	25 years 3R-project funding, effects on the 3Rs	S. Schindler Animalfree Research, Zürich
	The 3R's in Toxicity and Safety Testing	Chair: Fransiska Boess + Friedlieb Pfannkuch
14:30 14:35	Swiss Tox and the 3Rs	F. Pfannkuch Swiss Society of Toxicology
14:35 15:00	New 3R relevant methods in drug development	F. Boess F. Hoffmann-La Roche, Basel
15:00 15:25	New tests for (nano)particles - *In vitro* evolution	M. Geiser Inst. of Anatomy, University of Berne
	COFFEE BREAK - INDUSTRIAL EXHIBITION - POSTER VIEWING - *InSphero* LAB TOUR	
16:00 16:30	Impact of the International Conference on Harmonization (ICH) on the use of animals in Regulatory Toxicology	F. Pfannkuch Swiss Society of Toxicology
16:30 17:00	Proofing the relevance of the Ex Vivo Eye Irritation Test (EVEIT)	F. Spöler RWTH Aachen, Germany
17:00 17:30	Criteria for acceptance of alternative methods in the approval process of chemicals	D. Oggier Bundesamt für Gesundheit, Bern
18:30	**Aperitif Hotel St. Gotthard**	
19:00-	**25 Years Anniversary Dinner** (with tickets only)	

Tuesday 20.11 2012 Auditorium SGV

	Replace-Refine-Reduce Lecture	Chair Mark Deurinck and Peter Maier
09:00 09:30	From Cells to functional cardiovascular implants	S. Hoerstrup (University Hospital and University of Zürich)
	Genetic reference population of mice	
09:30 10:15	Plenary Lecture: "A Paradigm Shift in Mouse Genetics: Merging Reductionist and Holistic Approaches to Model Pervasive Diseases in Human Populations"	R. Williams (UT Center for Integrative and Translational Genomics, USA)
10:15 10:45	The use of genetic reference population in metabolic diseases	J. Auwerx (EPFL, CH)
10:45 11:10	The use of genetic reference populations in infectious diseases	K. Schughart (Helmholtz-Zentrum f. Infektionsforschung, D)
	COFFEE BREAK- INDUSTRIAL EXHIBITION	
11:40 12:00	Transportation as major life event in rodents: *Effects on welfare and limits of adaptation*	K. Kramer (University of Amsterdam, NL)
12:00 12:20	The impact of animal facility routines on welfare and stress of laboratory rodents: *in-house transport*	MJ Castelhano-Carlos (Life and Health Sciences Research Institute, P)
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	Experimental procedures: from basics in surgery to new challenging techniques	
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14:20 14:45	Cannulation procedures in rodents	D. Bouard (Charles River Laboratories, F)
14:45 15:10	Chronic implants for long-term neural interfacing with the brain and spinal cord	A. Jackson (University of Newcastle; UK)
	COFFEE BREAK- INDUSTRIAL EXHIBITION	
15:40 16:40	Rodent laparoscopy: refinement for rodent drug studies and model development, and monitoring of neoplastic, inflammatory and metabolic diseases	SW Baran (Veterinary Bioscience Institute, USA)
16:40 17:05	Spinal cord surgery: multi-system neurorehabilitative strategies to restore motor functions	G. Courtine (Ecole Polytechnique Fédérale Lausanne, CH)
17:05 17:30	New tracers and techniques in Bio-imaging	Y. Seimbille (Center for Biomedical Imaging, EPFL-HUG, CH)

Tuesday 20.11 2012 SF3R

Room Basic (2nd Floor) and Video in Room Pascal (3rd Floor)

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10:10 10:40	Do we still need teratomas? Alternatives to the pluripotency assay	G. Weitzer Medical University Vienna, Austria
	COFFEE BREAK - INDUSTRIAL EXHIBITION - POSTER VIEWING - *InSphero* LAB TOUR	
11:00 11:30	Novel *in vitro* human cell model to detect kidney carcinogens using transcriptomics and systems biology	M. Ryan University College Dublin, Ireland
11:30 11:55	State of the Art: Safety assessment of cosmetics in the EU	Odile de Silva L'Oreal, Paris, France
11:55 12:20	Assuring Safety Without Animal Testing	R. Stierum TNO, Zeist, The Netherlands
	LUNCH - INDUSTRIAL EXHIBITION - POSTER VIEWING - *InSphero* LAB TOUR	
	Support of the 3Rs	Chair: Odile de Silva + Adrian Smith
13:50 14:20	Directive 2010/63/EU on the protection of animals used for scientific purposes	S. Louhimies European Commission, Brussels
14:20 14:45	Implementation of the EU directive in Finland-how did the different stakeholders react	T. Heinonen University of Tampere, Finland
14:45 15:10	EPAA and Academic Research	K. D. Bremm EPAA, Platform of Science, Brussels
	COFFEE BREAK - INDUSTRIAL EXHIBITION - POSTER VIEWING - *InSphero* LAB TOUR	
15:30 15:50	Horizon 2020: Advancing safety science and health research with modern, non-animal tools	T. Seidle Humane Society International London, U.K.
15:50 16:10	Interrelationships between the National Science Foundation and the 3R Platform in the Netherlands	J. de Boer ZonMw, The Netherlands
	The 3Rs as a European consensus	Chair: Lisbeth Knudsen + Thomas Hartung
16:10 16:20	***ecopa***: Who, What, Where? Progress in Denmark, DAKOPA	L. E. Knudsen University of Copenhagen, Denmark
16:20 16:30	Progress in Austria, *zet*	K. Sommer BioMed-zet Life Science, Linz, Austria
16:30 16:40	Progress in Finland, *Fincopa*	T. Heinonen FICAM, University of Tampere, Finland
16:40 16:50	Progress in Germany, Stiftung *set*	Ch. Buda Stiftung-set, Frankfurt, Germany
16:50 17:00	Progress in France, FRANCOPA	Ph. Hubert INERIS, Verneuil-en-Halatte, France
17:00 17:10	Progress in the Netherlands, NKCA	S. Deleu RIVM, The Netherlands
17:10 17:20	Progress Norway, *Norecopa*	A. Smith Norecopa, Oslo, Norway
17:20 17:30	Progress in Switzerland, SF3R	P. Maier University of Zürich, Switzerland
17:45 18:15	ecopa General Assembly 2012	L. E. Knudsen University of Copenhagen, Denmark

Welcome address to the joint 25th Anniversary Meeting

25 years of 3R Research Foundation Switzerland

Dear Researchers

I am delighted to welcome you to the Joint 25th Jubilee Meeting – Refinement goes together with Replacement! With this scientific event we are celebrating our jointly achieved success for better research with fewer animals. Today and tomorrow we shall also be given a brief outlook on our future success.

Three organizations are involved in this training event: the two jubilarians, the Swiss Laboratory Animal Association and the 3R Research Foundation Switzerland, the Swiss Society of Toxicology, and the European 3Rs organization ecopa is our guest. At the 3Rs, too, it is only constructive cooperation that enables us to achieve sustainable success.
Implementation of the 3R principles is an integral part in animal experimentation today. This was not the case some years ago. It took untiring perseverance by all of us to prove that animal welfare and scientific research are not contradictory to each other.

In its 25 years of support for research in the interest of animal welfare, the 3R Research Foundation has initiated around 130 research projects. You will learn more at this conference with a representative selection of sustainable projects. Many successful projects led to persuasive solutions with which animal experiments can be reduced, improved or even replaced altogether. This knowledge is available in publications and also on the website of the 3R Research Foundation (www.forschung3r.ch) – it is just waiting to be used and developed further.

Research funding has been provided by the Federal Veterinary Office and Interpharma, the association of research-based pharmaceutical companies in Switzerland. Regarding the scientific and technical expertise, the expert committee of the Foundation, in which specialists from academia and industry cooperate in their specific fields of expertise, ensures that the submitted grant applications receive a competent and balanced assessment. Both the Foundation and the researchers who benefit from this support are deeply grateful for this engagement.

Some highly promising results have been achieved, but there is still a lot to do to ensure that the 3Rs becomes widely established as the guiding set of principles in day-to-day research. Your research expertise and your daily work with your animals will enable you to establish the 3Rs in your specialty field. The 3R Research Foundation is prepared to continue to supporting you to the best of its ability.

With this in mind, I wish you two days full of new ideas for implementing the 3R principles in your own field of work, and I hope that you will also enjoy meeting and exchanging ideas and experiences with other researchers.

Best regards
Christine Egerszegi-Obrist, Council of States
President of the 3R Research Foundation

The 25 Year Jubilee of the Swiss Laboratory Animal Science Association - Schweizerische Gesellschaft für Versuchstierkunde (SGV)

Dear Participant,

The SGV was founded in 1987, six years after the new Animal Welfare Ordinance was adopted. It was the answer of researchers concerned by the use of animals in biological and biomedical sciences. The same year, the Confederation and the pharmaceutical industry created the 3R Research Foundation.

Twenty-five years later, the two organisations are still active. Their responsibilities and roles in Science and Society have significantly grown through the years.

The 3R Research Foundation has contributed to all the 3 Rs defined by Russell & Burch with a high priority on Replacement. Meanwhile, the SGV has contributed to Refinement by a constant and systematic continuing training program. Fifteen years ago, our annual meetings were easy to organize; today the situation has dramatically changed. Years ago we were a few dozen of people; today hundreds of participants are coming from all parts of the country. Today, not only do we learn new concepts and views on lab animals from experts coming from abroad but we can network on many topics during our annual two-day meeting, another way to strengthen the 3Rs.

How did such spectacular participation to our meetings happen? The answer is straightforward, by personal and voluntary involvement of both the Education Committee and the SGV Board with the support of all SGV members. I take this opportunity to thank very gratefully the two boards for their dedicated and constant work. Without them, no annual meetings, no thrilling talks, no commercial exhibitions, no interactions with authorities, no networking at the European level with the FELASA on issues like international recommendations and other guidelines. The voluntary work is precious and we need to care for it because involvement of such people impact directly on a realistic and efficient 3R implementation in the labs.

I would like to welcome all colleagues from the 3R Research Foundation, the Swiss toxicology Society and the representatives of ECOPA. I wish to all of you a very fruitful meeting with a lot of exciting news and exchanges.

We open the first session with a topic, which was not even mentioned 25 years ago: “empathy in the animal kingdom”. This topic is emblematic of our both Jubilees. We can see empathy as the emotional dimension of our involvement in the 3Rs, a concept full of logical reasoning. If animals are emphatic between them and maybe with us, we as *Homo sapiens*, have the moral obligation to apply to our laboratory animals the 3R stance.

With my Very Best,
Marcel Gyger
President of SGV

Important Information

Two Types of Certificates

Certificate of attendance
A CERTIFICATE OF ATTENDANCE will be sent to all participants via email a few weeks after the meeting. This certificate of attendance is not the certificate for continuing education and therefore should not be presented to the Swiss veterinarian authorities as such.

Continuing education certificate
Study directors and Experimenters working in Switzerland can obtain the continuing education certificate **ONLY** if their badge is scanned at the registration desk at the end of the meeting.

That is:
If you participate to one meeting day, please come at the end of that day.
If you participate to both days, please come at the end of the second day.
If you cannot stay a whole day, you have to scan you badge when you leave to receive a 0.5 day certificate (for this you must follow 3 hours conference).

3R Sessions in two Conference Rooms

All 3R Sessions are taking place in the Conference Room Basic 2nd floor .
All 3R Sessions will be transmitted by video into the Conference Room Pascal 3rd floor.
Use this opportunity in order to attend selected 3R lectures only.

InSphero LAB TOUR (more information on page 111)

Learn how 3D microtissues can help to reduce animal testing, including life demonstration of microtissue models

- 4 slots available: Mon 19 Nov: 16:30
  Tue 20 Nov: 11:30 / 13:50 / 15:40
- Free participation
- Register on site (poster board) or in advance at www.insphero.com/registration
- Meeting point at InSphero poster (3R poster area)

Monday 19.11 2012

Plenary Lecture Auditorium

9:10 - 10:10

The Ultimate and Proximate Bases of Empathy and Altruism

Stephanie D. Preston, University of Michigan, USA

Empathy and altruism often top the list of things that make us uniquely human—crowning achievements of our evolution from simple animals to complex, compassionate, cognitive creatures. However, considerable evidence suggests that both are deeply-rooted reflections of our evolutionary history as social, caregiving mammals.

Empathy as perception-action. Neuroscientific views assume that empathy evolved from a basic and efficient perception-action neural architecture that maps the actions and emotions of others on to our own representations for performing those actions, for feeling those emotions. Thus, when I see you reach for a mug and wince in pain, my neural regions that encode for reaching and grasping are spontaneously activated (as in “mirror neuron” theories). In addition, my neural regions that encode for my experience of pain are activated along with distributed conceptual representations for mugs, reaching, drinking and you (as in the “Perception Action Model” (PAM)). At the most basic level, this spreading activation allows me to understand your action.

In addition, because these are “embodied” representations, I can experience this perceptual event “intersubjectively”—as if I am also doing the reaching or feeling the pain—this concordance being what most describe as “empathy.” However, while this overlap between your experience and my neural activity is necessary for understanding, I do not always subjectively feel this concordance. Thus, empathy is really better construed as a form of emotion perception that simply reflects a higher level of awareness of the link between self and other, which can go on to produce many different interpersonal states (e.g., understanding, empathy, sympathy, personal distress, joyful sharing).

As the other speakers will attest, this basic perception-action process and affective sharing have been proven to across species, even motivating animals to alleviate the suffering of others under the right conditions (i.e., when the distress is maintained within limits and the observer clearly knows what to do, is not endangered, and can enact the response). Thus, what is unique in humans is not the capacity for empathy, but the ability to apply it in more abstract, strategic, and planful situations—but even these more sophisticated processes still operate through activation of the lower-level personal representations for the relevant state (as in “simulation theory”). While significant data support the perception-action model of empathy, we still do not fully know why sometimes people help without feeling empathy and feel empathy without offering support. How can we explain such discrepancies?

Altruism as offspring care. Current research and theory in my lab supports a caregiving model of altruism that is based upon extensive data in rodents and monkeys (and should extend to other social, caregiving mammals). According to this model, the neural circuits that evolved to maintain contact with helpless altricial offspring can also be activated in response to nonoffspring under the right conditions. Thus, as the rodent mother darts out to retrieve newborn pups to the safety of the nest, humans rush into dangerous situations to retrieve strangers from danger. This seemingly heroic compulsion to act reflects fundamental features of the caregiving system, which was designed to prevent action when the observer fears novelty or danger but potentiates action when the observer perceives distressed, vulnerable targets that they know how to help. This caregiving model intersects with the perception-action model since observers need the latter to first accurately decode and be motivated by the state of the target. However, heroic responses are characterized by their fast response, which skips over the intervening subjective sense of empathy or sympathy—emotions that seem to prevail when the observer is not an active participant in the event and the target is not in imminent danger (as in classic empathy experiments using videos or narrative targets of longer-term need).

By studying empathy and altruism from a scientific and neural perspective, we can see how even the crowning achievements of humanity reflect our adaptive and ancient propensity to be sensitive to the state of others—creating an important link not only between me and you, but also between us and them.

Relevant Citations

1. Preston, S.D. and F.B.M. de Waal (2002). The communication of emotions and the possibility of empathy in animals, in *Altrusim and Altruistic Love: Science, Philosophy, and Religion in Dialogue*, S. Post, et al., Editors. 2002, Oxford University Press: Oxford. 284-308.

2. Preston, S.D. and F.B.M. de Waal (2002). Empathy: Its ultimate and proximate bases. *Behavioral and Brain Sciences*, *25*: 1-71.

3. Preston, S.D. and F.B.M. de Waal (2011). Altruism, in *The Handbook of Social Neuroscience* J. Decety and J.T. Cacioppo, Editors. 2011, Oxford University Press: New York. 565-585.

4. Preston, S.D. and A.J. Hofelich (2012). The many faces of empathy: Parsing empathic phenomena through a proximate, dynamic-systems view of representing the other in the self. *Emotion Review*, *4*: 24-33.

5. Preston, S.D. (under review). The origins of altruism in offspring care.

Room Basic (2nd Floor) and Video in Room Pascal (3rd Floor)

Session I

The 3Rs in Research and Development Chair: Thomas Lutz and Peter Maier		
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10:25 10:50	MRI its impact on Refinement and Reduction	N. Beckmann NIBR, Basel
	COFFEE BREAK - INDUSTRIAL EXHIBITION - POSTER VIEWING	
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14:05 14:30	25 years 3R-project funding, effects on the 3Rs	S. Schindler Animalfree Research, Zürich

10:15 - 10:25

Refinement goes together with Replacement

Peter Maier, 3R Research Foundation Switzerland and University of Zürich, Switzerland

Refinement versus Replacement ?

"Principles of Humane Experimental Technique"

- describes the application of humane principles to the technique of experimentation with animals.
- was only incidentally concerned with the humane treatment of (laboratory) animals.

  already at that time considered as a prerequisite to conduct reliable animal experiments. See: Prof. Alastair Worden, ed.: Handbook on the Care and Management of Laboratory Animals (UFAW, 1947).
- "to establish protocols to reduce and refine animal experimentation and intervention and to replace such experimentation by alternative methods of equivalent validity."

Refinement goes together with Replacement

- 1959. Russell and Burch were fully aware that there are "areas of overlap between these categories".

Existing ambiguity:

- Alternative method of what?
  - an alternative (refined) animal experiments
  - an alternative to an animal experiment (Replacement)
- The frequently used term „3R methods" actually stands for „3R-relevant methods" (it`s not the method itself, much more a new combination of it)
- The 3Rs are often misread as a synonym for replacement methodologies.

Refinement and Replacement follow the same rules

2 R

- based on scientific principles

  (however there is no 3R Research discipline as such)
- requires updated methods specific for each research area
- development is a continuing process (development, application and dissemination)
- improves
  - animal welfare,
  - experimental design
  - quality of results

Research goes together

Refinement and Replacement cross-fertilize each other?

as a rule (derived from hundreds of applications for funding)

- valid proposals for Replacement derive from animal experimenters
- relevant Replacement methods are based on pathways assumed out of data from animal experiments
- inspirations for new pathways or procedures are initiated by stressful work with animals

Cooperation between Refinement and Replacement

3R

SGV SSEAL

- 20th and 25th Anniversary Meeting jointly organised
- SGV organising Continuing Education courses on Refinement throughout the year
- 3R Experts from SF3R are involved in the basic professional education (1/2 day about 3Rs)
- bridges the distance between animal experimenters and non-animal alternatives
- SF3R funds projects about Refinement and Replacement

Open field in Research and Development

R+D

- Knowledge of in vivo and in vitro

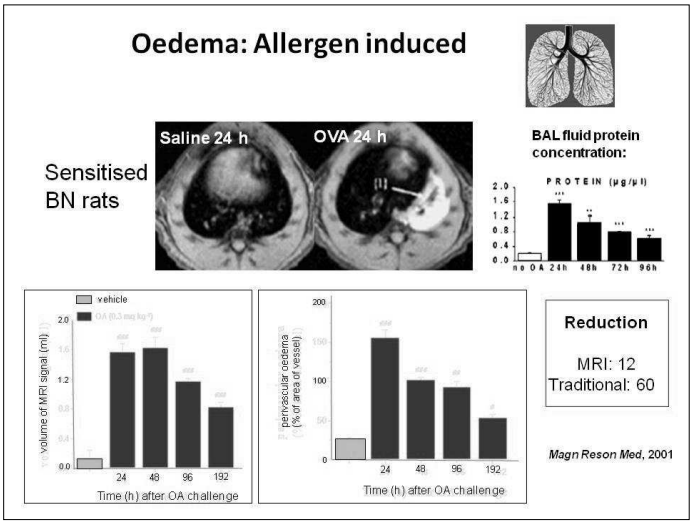
or

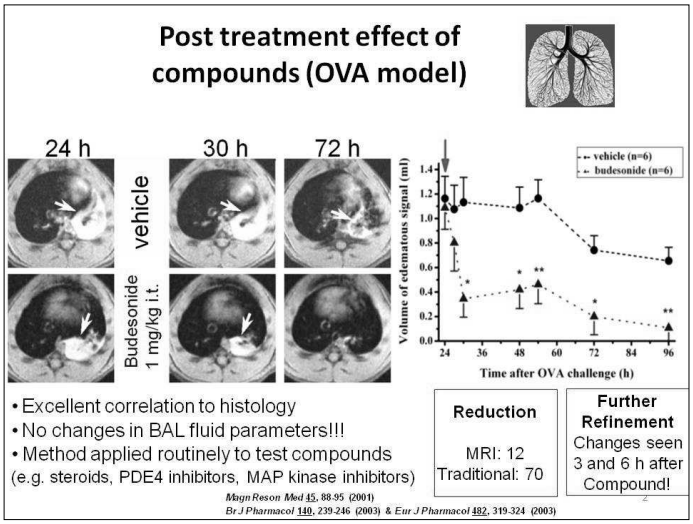
- Cooperation with other labs
- Numerous fields of applications:
  - Drug Research (N. Beckmann)
  - Biomedical Sciences (S.L. Leib)
  - .....
  - Virology
  - Parasitology
  - Toxicology
- Refinement leads to partial Replacement
- Replacement leads to partial Reduction
- Immediate application
- Impact measurable?

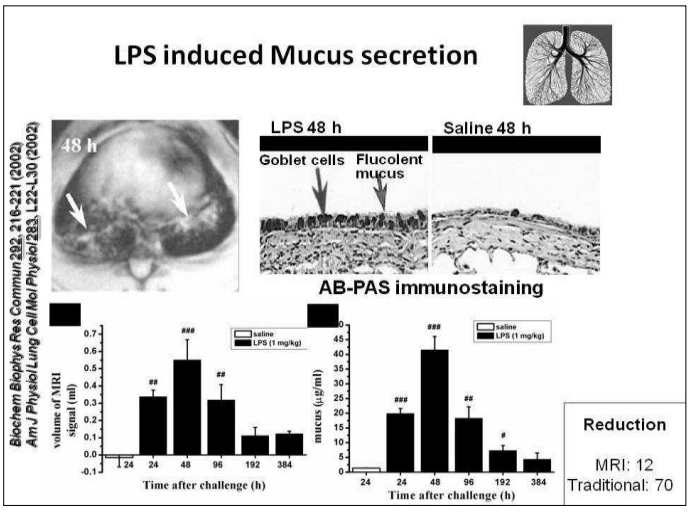
10:25- 10:50

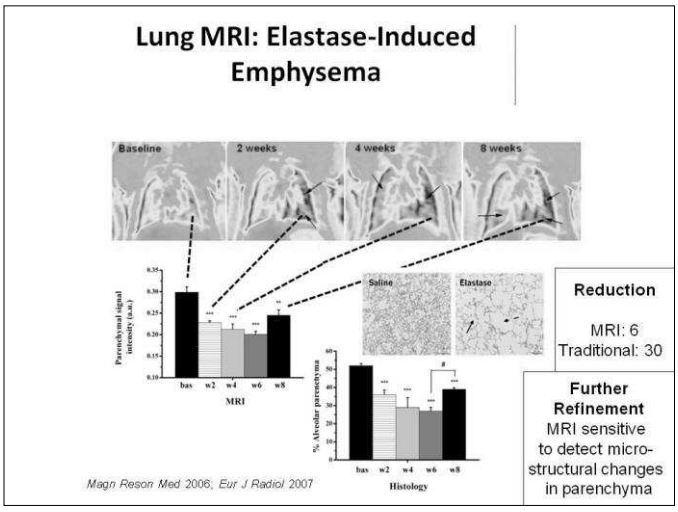
MRI its impact on Refinement and Reduction

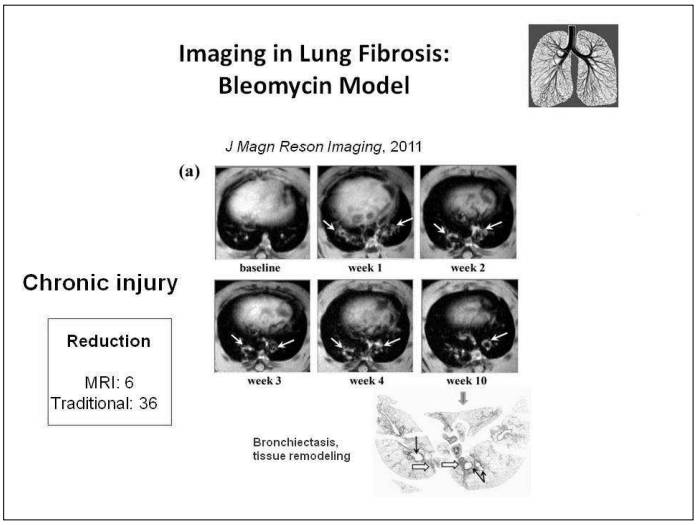
Nicolau Beckmann, Novartis Institute for BioMedical Research, Basel, Switzerland

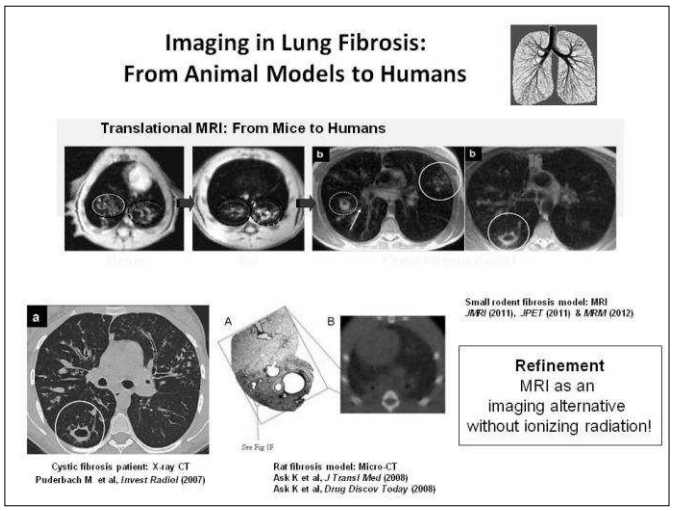


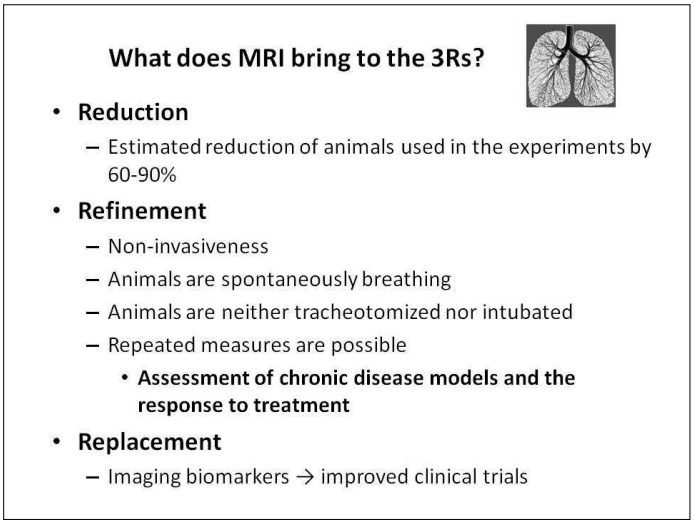












1. Bleomycin-Induced Lung Injury in Mice Investigated by MRI: Model Assessment for Target Analysis (A. L. Babin, C. Cannet, C. Gérard, P. Saint-Mezard, C. P. Page, H. Sparrer, T. Matsuguchi, and N. Beckmann). Magn. Reson. Med. 67, 499-509 (2012).

2. Non-Invasive Assessment of Bleomycin-Induced Lung Injury and the Effects of Short Term Glucocorticosteroid Treatment in Rats Using MRI (A. L. Babin, C. Cannet, C. Gérard, D. Wyss, C. P. Page, and N. Beckmann). J. Magn. Reson. Imaging 33, 603–614 (2011).

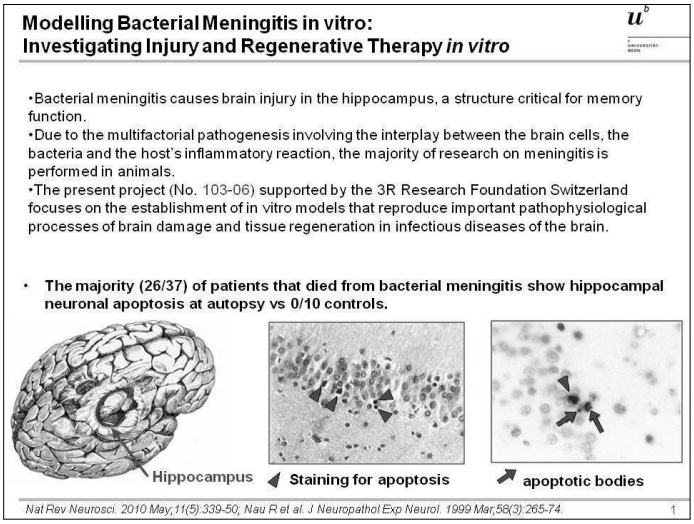
3. Probing Cerebrovascular Alterations in Alzheimer's Disease Using MRI: From Transgenic Models to Patients (N. Beckmann). Curr. Med. Imag. Rev. 7, 51-61 (2011).

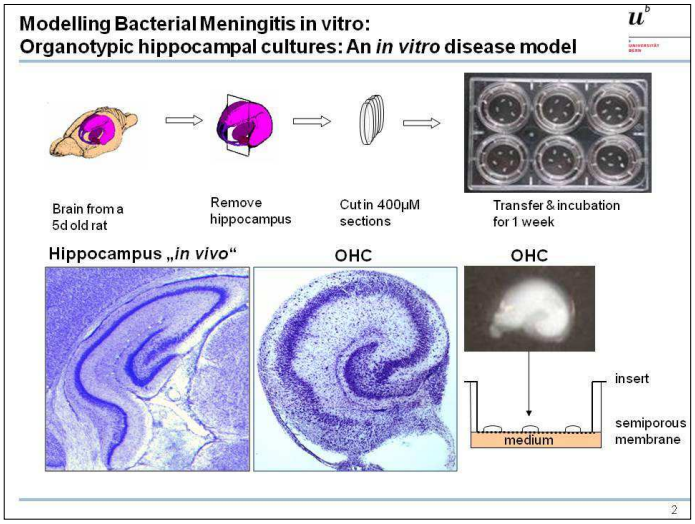
4. Non-invasive MRI detection of cerebral amyloid angiopathy related microvascular alterations utilizing superparamagnetic iron oxide particles in APP transgenic mouse models of Alzheimer's disease: Application to passive Aβ immunotherapy (N. Beckmann, C. Gérard, D. Abramowski, C. Cannet, and M. Staufenbiel). J. Neurosci. 31, 1023-1031 (2011); doi:10.1523/JNEUROSCI.4936-10.2011.
5. A View on Imaging in Drug Research and Development for Respiratory Diseases (C. J. A. van Echteld and N. Beckmann). J. Pharmacol. Exp. Ther. 337, 335-349 (2011).
6. Spatially resolved assessment of serotonin-induced bronchoconstrictive responses in the rat lung using 3He ventilation MRI under spontaneous breathing conditions (K. Mosbah, V. Stupar, Y. Berthezène, N. Beckmann, and Y. Crémillieux). Magn. Reson. Med. 63, 1669-1674 (2010).
7. Optica l and Magnetic Resonance Imaging as Complementary Modalities in Drug Discovery. (B. S. Sandanaraj, R. Kneuer, and N. Beckmann). Fut. Med. Chem. 2, 317-337 (2010).
8. ENaC-mediated effects assessed by magnetic resonance imaging in a rat model of hypertonic saline-induced airways hydration (F.-X. Blé, C. Cannet, S. Collingwood, H. Danahay, and N. Beckmann). Br. J. Pharmacol. 160, 1008-1015 (2010).

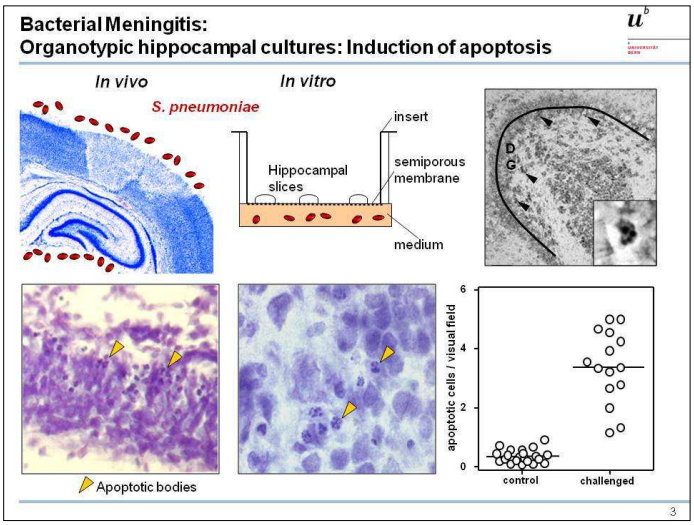
11:10-11:40

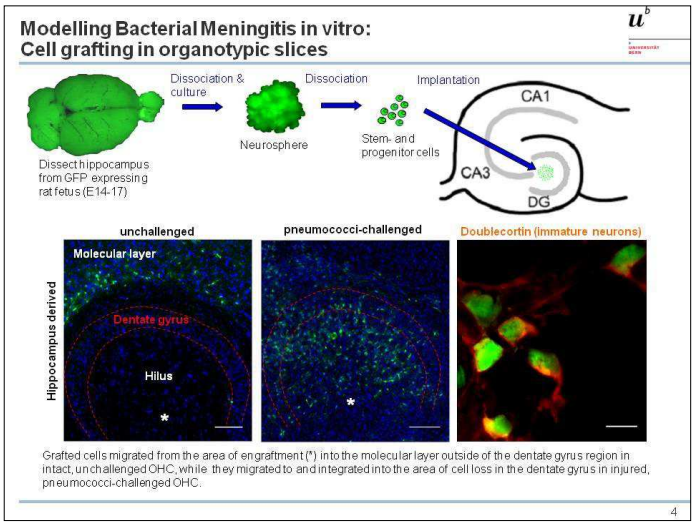
Bacterial Meningitis: Brain Injury and Regenerative Therapy *in vitro*

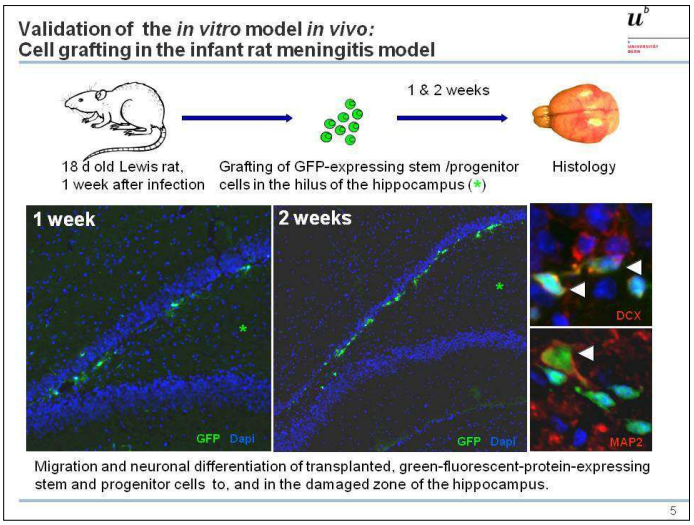
Stephen L. Leib, Institute for Infectious Diseases & Cluster for Regenerative Neuroscience, Departement for Clinical Research, University of Bern, Switzerland

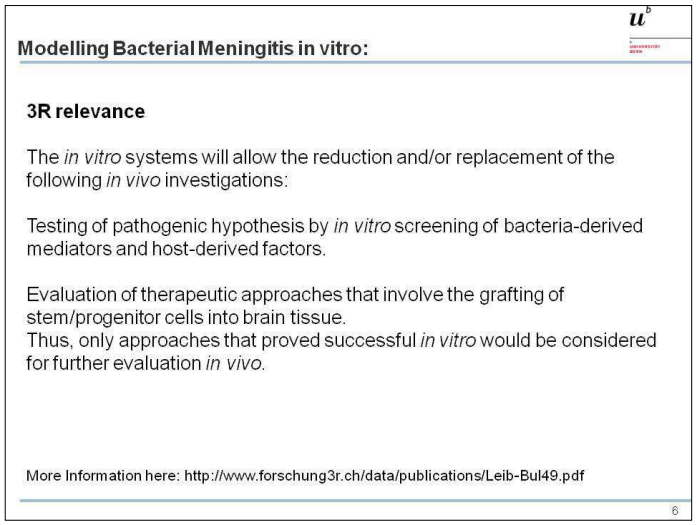












1. Grafted neuronal precursor cells differentiate and integrate in injured hippocampus in experimental pneumococcal meningitis. Hofer S, Magloire V, Streit J, Leib SL., Stem Cells. 2012 Jun;30(6):1206-15.
2. Bacterial meningitis impairs hippocampal neurogenesis. Hofer S, Grandgirard D, Burri D, Fröhlich TK, Leib SL.,J Neuropathol Exp Neurol. 2011 Oct;70(10):890-9.
3. An in vitro model of central nervous system infection and regeneration: neuronal stem cells as targets of brain damage and regenerative therapies in bacterial meningitis., Leib SL. ALTEX. 2007;24 Spec No:90-2.
4. Apoptosis of hippocampal neurons in organotypic slice culture models: direct effect of bacteria revisited.,Gianinazzi C, Grandgirard D, Simon F, Imboden H, Joss P, Täuber MG, Leib SL., J Neuropathol Exp Neurol. 2004 Jun;63(6):610-7.

11:40 – 12:10

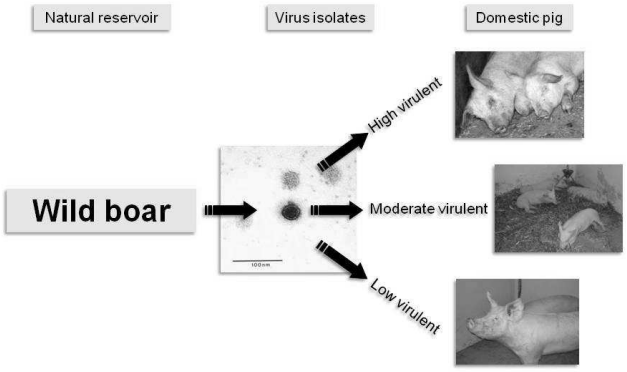
From pigs to Cells: Prediction of virulence of classical swine fever virus *in vitro*

Nicolas Ruggli, Laboratory of Virology, Institute of Virology and Immunoprophylaxis, Mittelhäusern, Switzerland

Classical swine fever (CSF)

- Highly contagious disease of pigs caused by classical swine fever virus (CSFV)
- Peracute (100% mortality), acute haemorrhagic or chronic disease, depending on the virus strain
- Notifiable to the WOAH (OIE)
- Non-vaccination policy
- Important economic impact: culling of infected and seropositive animals, trade restrictions
- Virus reservoir: wild boar

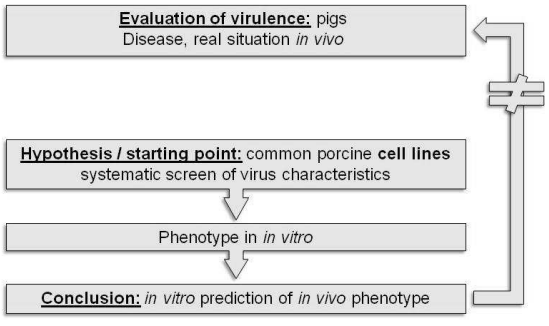
Problem: CSFV isolates of different virulence

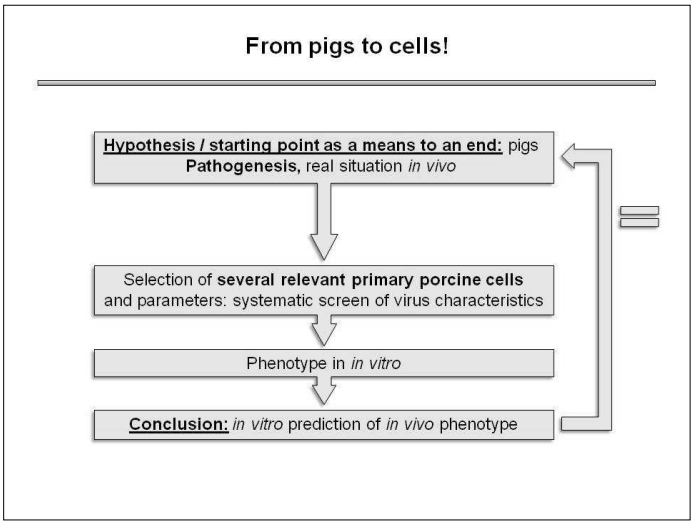


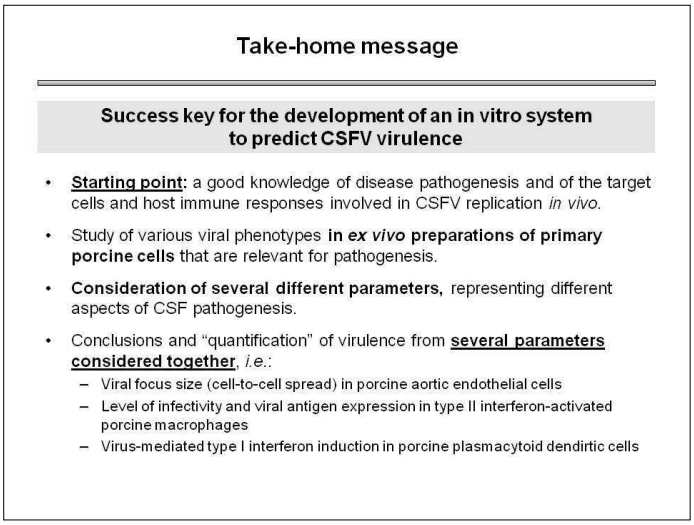
Motivation for 3R: to study virulence in diagnostics and research without animal experimentation.

Diagnostics / surveillance: **predict virulence of new isolates**	*Fundamental Research:* **Identify virulence determinants**
• Switzerland and most European countries are currently free from CSF. • Permanent risk of disease reintroduction through wild boar and illegal meat trade • Low virulent CSFV isolates represent the major problem for disease surveillance. • For appropriate control measures, there is a need for prediction of virulence of emerging wild boar isolates *in vitro*.	• The viral molecular determinants of virulence are unknown. • Mutant CSFV can be constructed using reverse genetics. • So far, the virulence phenotype of mutant viruses could only be studied by experimental infection of pigs. • There is a need for prediction of virulence of genetically engineered mutant CSFV using high throughput screening methods *in vitro*.

From cells to pigs?







Abbreviations:

CSFV: classical swine fever virus
WOAH: World Organisation for Animal Health (OIE)

References:

Leifer, I., R. Eymann-Häni, A. Summerfield, N. Ruggli. 2012. Measurement of classical swine fever virus virulence using macrophage, endothelial, and dendritic cell cultures. *Submitted for publication*.
Eymann-Häni, R., I. Leifer, K.C. McCullough, A. Summerfield, N. Ruggli. 2011. Propagation of classical swine fever virus in vitro circumventing heparan sulfate-adaptation. J. Virol. Methods 176, 85-95.

3R-Info-Bulletin No 44, Editor: Peter Maier. 2010. From pigs to cells: Virulence of classical swine fever virus in vitro circumventing heparan sulfate-adaptation (Nicolas Ruggli, Summerfield Artur, Rita Eymann-Häni)
Ruggli N, A. Summerfield. 2007. Establishment of an in vitro system for the prediction of the degree of virulence of classical swine fever virus isolates. ALTEX 24, 98-9.

Links

BVET:	http://www.bvet.admin.ch/
IVI:	http://www.bvet.admin.ch/ivi/index.html
3R project 105-06 :	http://www.forschung3r.ch/fr/projects/pr_105_06.html
WOAH:	http://www.oie.int/

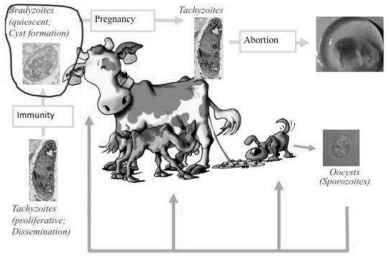
13:40 - 14:05

In vitro induction of stage conversion in the parasite *Neospora caninum*

Andrew Hemphill, Institute of Parasitology, University of Bern, Switzerland

1. Neospora caninum & neosporosis

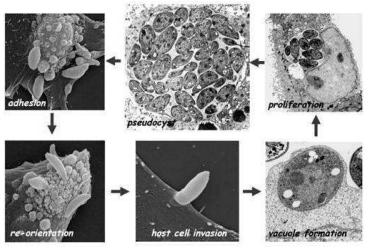
- Single-celled protozoan parasite, belonging to the Apicomplexa
- Global distribution
- Major cause of abortion in cattle (neosporosis)
- Veterinary problem
- Economically important
- Intensely studied areas:
  - Epidemiology
  - Vaccines
  - Chemotherapy



2. In vitro culture of N. caninum tachyzoites

- Culture in standard media at 37 C, 5% CO_2
- Most mammalian cells can act as host cells
- Adherent monolayers or cell suspensions

In vitro culture of the tachyzoite stage is easily established, yielding proliferative and infective parasites.



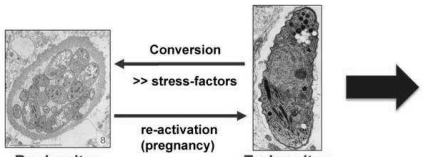
Applications:

- Generation of parasite antigens for immunodiagnostic applications and/or vaccination purposes.
- Studies on the cell- and molecular biology of *Neospora caninum*
- In vitro screening of novel, potentially interesting drugs
- Identification of novel drug targets

3. Neospora caninum bradyzoites

Reasons for developing *N. caninum* bradyzoite culture:

- **Large numbers of tissue cysts** are required for:
  - **immunological studies** on oral infection of carnivores
  - studies on the **life cycle**
  - **oocyst production in dogs** for subsequent infection of intermediate hosts (cattle)
- **Small laboratory animal models** for tissue cyst production (immune-compromised mice, gerbils) have proven **unreliable** and **large numbers of animals** are required.



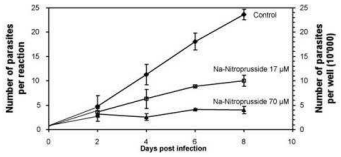
Bradyzoites
- dormant
- resistant
- persistant
- orally infective

Tachyzoites
- virulent & proliferative
- immunopathology
- disease
- congenital infection

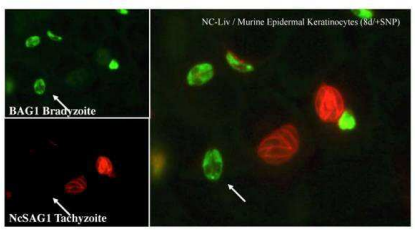
Physiological stress- inducers could potentially act as triggers for initiating stage conversion in cell culture

4. Exogenous NO induces stage conversion

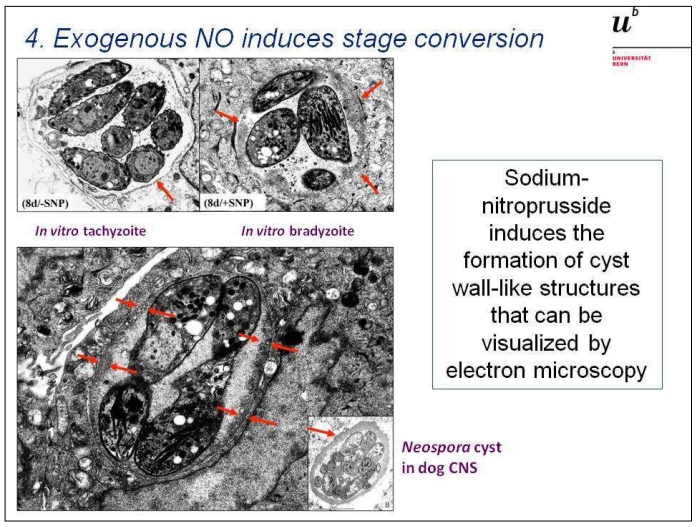
Quantitative real time PCR:

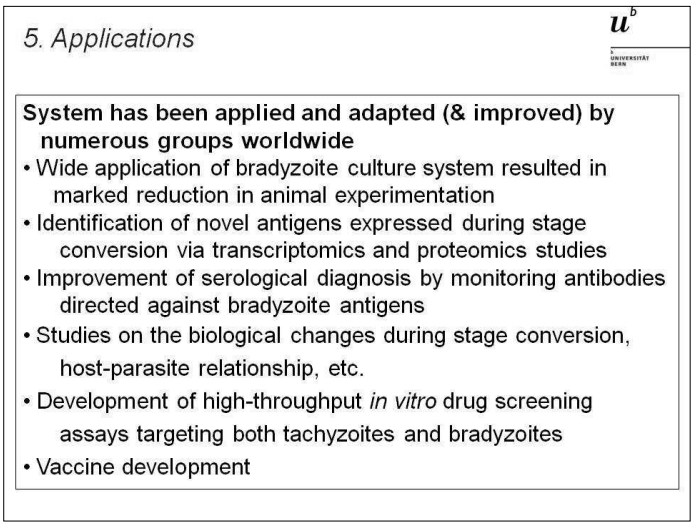


Sodium-nitroprusside inhibits parasite proliferation in a dose-dependent manner



Sodium-nitroprusside induces expression of the bradyzoite-specific antigen BAG1 and other antigens





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Vonlaufen, N., Guetg, N., Naguleswran, A., Müller, N., Bjorkman, C., Schares, G., von Blumroeder, D., Ellis, J., and Hemphill, A. 2004. In vitro induction of *Neospora caninum* bradyzoites in Vero cells reveals differential antigen expression, localization, and host-cell recognition of tachyzoites and bradyzoites. Infection and Immunity 72: 576-583.

Hemphill, A. 2007. Generation of parasite cysts in cultured cells instead of living animals. Altex 24:29-31

Guionaud, C., Hemphill, A., Mevissen, M., Alaeddine, F. 2010. Molecular characterization of *Neospora caninum* MAG1, a dense granule protein secreted into the parasitophorous vacuole, and associated with the cyst wall and the cyst matrix. Parasitology 137: 1605-1619

14:05 - 14:30

25 years 3R-project funding, effects on the 3Rs

Stefanie Schindler, Animalfree Research, Zürich

Structure

Part 1: Quantitative analysis- general information

(e.g. number of projects, costs, and duration, locations of the projects)

Part 2: Qualitative analysis – special information

(e.g. comparison of original aims and achieved goals, publications, practical effects of the project, «success»)

1

Results: Examples

Projekt duration	1 year: 29 1.5 years: 5 2 years: 47 3 years: 40 4 years: 4 5 years: 2	Outside Switzerland	13
Applicants that were funded twice	11	Cooperations: inside Switzerland	5
Three times	1	Cooperations: outside Switzerland	9

2

Concerned **species** (several projects concern more than one main species)	mouse rat rabbit dog primate gerbil Fish Cattle/pig Others/not clear	45 20 7 5 2 1 6 10 29
Organs/tissues (several approaches combine more than one tissue)	immune system respiratory tract and lung circulation digestive tract liver Nervous system barriers (skin, endothelium, epithelium) Musculoskeletal system Entire organism hormones genome kidney	36 5 8 6 5 26 12 10 20 4 6 1

Special information: work in progress

To be determined:	Did publications come from the project? Did they specifically adress the purpose of the 3Rs?
Were the project goals achieved? What were the reasons why they were not achieved?	Did the project result in implementations into national or international regulations?
Is the method still in use a) In the developing lab? b) In other labs	Was the funding of the foundation instrumental for the project to happen?
Did the implementation of the method contribute to saving animals or improve their situation?	Were there areas of research that were particularly successful?

4

Conclusions

The process of project funding of the Foundation 3R is highly professional

The presented numbers and data are most probably an understatement: only clearly provable numbers and data are considered

There are multiple aspects of an impact on the 3Rs that are not measurable nor quantifiable

It is not possible to raise 100% objective information on the success of a given project

5

Conclusions (2)

In many cases, a direct connection between the funding/the project and an effect on animal experiments is difficult to establish. This is partly due to the principles of funding

1) Mainly basic/fundamental research
2) Preferably new approaches/methods
3) Funding (usually) no more than three years

Animal numbers: a 1:1 replacement is rarely possible, and if,

1) 1 in vitro assay does not necessarily stand for one replaced animal experiment (laboratories make more tests once they have switched to in vitro)
2) It is not legitimate to calculate replaced experimental numbers into an indefinite future

6

Monday 19.11 2012

Room Basic (2nd Floor) and Video in Room Pascal (3rd Floor)

Session II

The 3R's in Toxicity and Safety Testing

Chair: Franziska Boess and Friedlieb Pfannkuch

14:30 14:35	Swiss Tox and the 3Rs	F. Pfannkuch Swiss Society of Toxicology
14:35 15:00	New 3R relevant methods in drug development	F. Boess F. Hoffmann-La Roche, Basel
15:00 15:25	New tests for (nano)particles - *In vitro* evolution	M. Geiser Inst. of Anatomy, University of Berne
COFFEE BREAK - INDUSTRIAL EXHIBITION - POSTER VIEWING - *InSphero* LAB TOUR		
16:00 16:30	Impact of the International Conference on Harmonization (ICH) on the use of animals in Regulatory Toxicology	F. Pfannkuch Swiss Society of Toxicology
16:30 17:00	Proofing the relevance of the Ex Vivo Eye Irritation Test (EVEIT)	F. Spöler RWTH Aachen, Germany
17:00 17:30	Criteria for acceptance of alternative methods in the approval process of chemicals	D. Oggier Bundesamt für Gesundheit, Bern

14:30 - 14:35

Swiss Tox and the 3Rs

Friedlieb Pfannkuch, President of the Swiss Society of Toxicology

Email: friedlieb.pfannkuch@bluewin.ch

Who needs our Experts?

Swiss Society of Toxicology

- Universities
  - Basel, Zurich, Geneva, (Lausanne)
  - Center for Xenobiotic Risk Research - XeRR
  - Swiss Centre of Applied Oekotoxicology
  - Swiss Centre of Applied Human Toxicology - SCAHT
- Governmental Agencies, Regulatory Authorities
  - Federal Agencies (eg. BAG, Swissmedic, BLW, EAWAG,...)
- Pharmaceutical Industry
  - Novartis, Roche, Actelion, Basilea, ...
- (Agro-)Chemical Industry
  - BASF, Syngenta, Huntsman, Lonza, Dow Chemicals, ...
- Food & Food Additives
  - Nestlé, DSM, ...
- Contract Research Organizations; Consultancies
  - Harlan
  - Consultancy Networks (e.g. Green Tox, ...)

1

Extrapolation to Man

Swiss Society of Toxicology

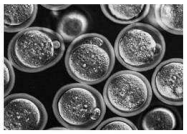
- The target of most animal studies is the human!
- Predictivity for humans of effects produced in laboratory is insufficient and this is evident for efficacy as well as for safety
  - e.g. only 1 out of ~100 molecules at the start of drug development or 14 clinical drug candidates finally reaches the market / patient
  - e.g. 20% of the failure of drug candidates due to toxicity occur only after administration in clinical trials

2

Mid-term Perspective

Swiss Society of Toxicology

- Ask precise questions about whether sensitive physiological processes are disturbed
  - Do not expose animals to high doses, inducing a multitude of effects
- Move to (high-throughput) alternative methods based on identified mode of action / pathways of toxicity
  - Human cells, fish eggs, invertebrate species etc.
  - Computational methods / *in silico* expert systems

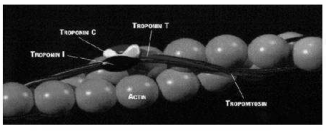


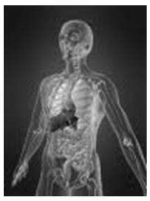
3

Far Future Perspective

Swiss Society of Toxicology

- Speed up development of batteries of sensitive and specific biomarkers of efficacy and safety
- Shift from animal to studies in humans
  - e.g. micro-dosing, single-dose trials at sub-therapeutic doses or into the anticipated therapeutic range, multiple-dose trials





4

Outlook

Swiss Society of Toxicology

- Reliability of extrapolation from animals to humans has to be systematically improved
- Further develop the scientific basis for reduction / replacement of animal studies and for exploratory trials in humans
- All parties have to contribute to public risk awareness and acceptance by improved information policy

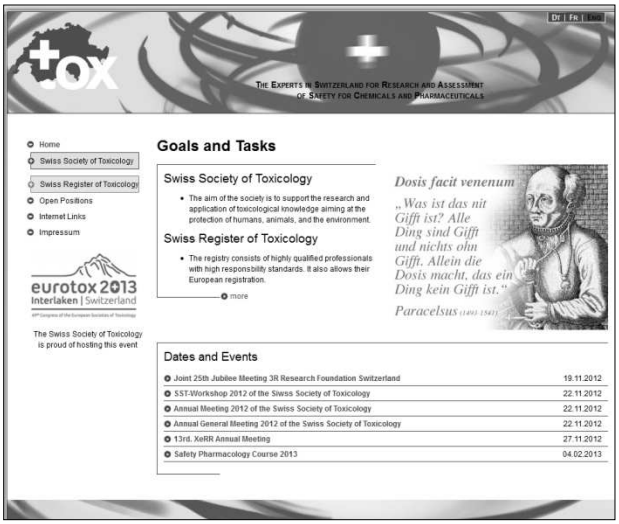
5

The Swiss Society of Toxicology ...

Swiss Society of Toxicology

- Congratulates the 3R Research Foundation Switzerland to the achievments reached over the past 25 years
- Supports the future activities of the 3R Research Foundation Switzerland
- Is strongly committed to the 3Rs

6

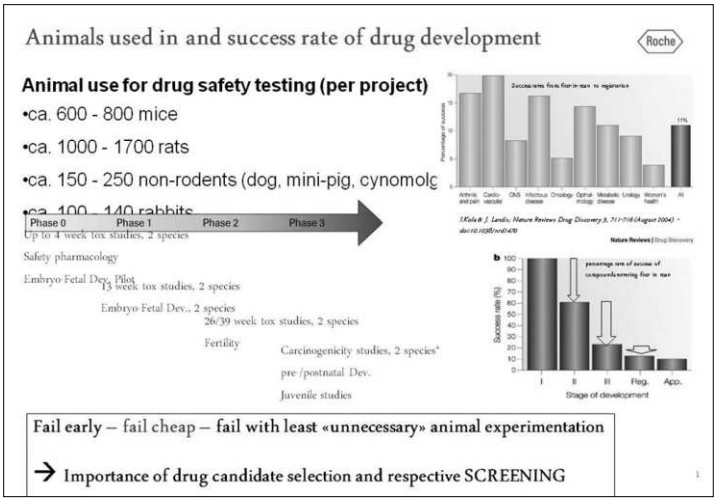


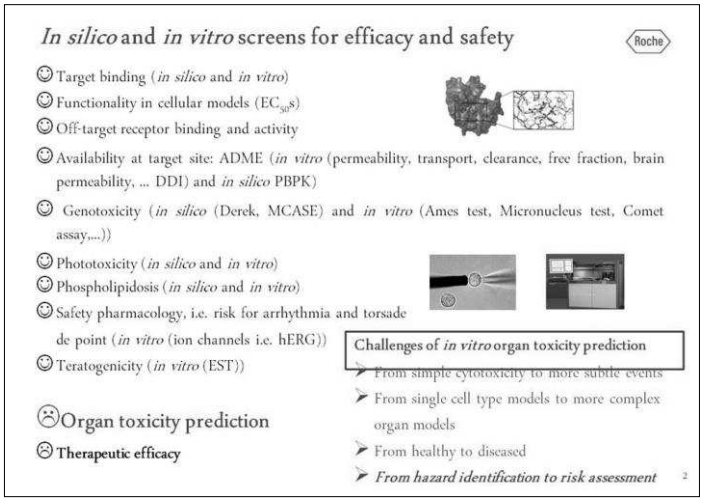
Schweizerische Gesellschaft für Toxikologie - SGT
Société Suisse de Toxicologie - SST
Swiss Society of Toxicology - SST
www.swisstox.ch

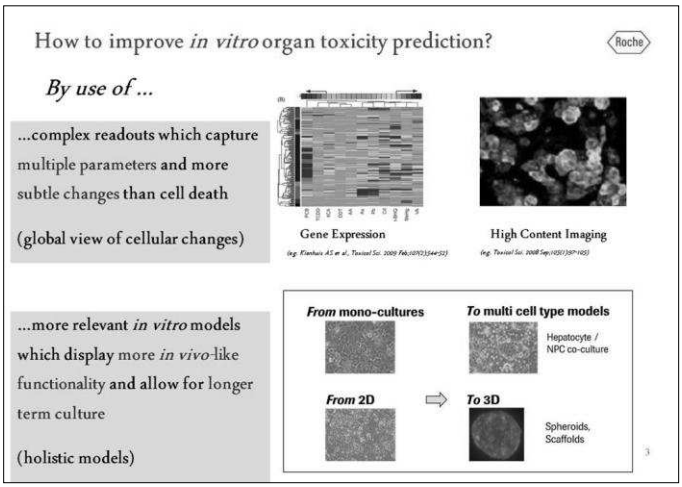
14:35 - 15:00

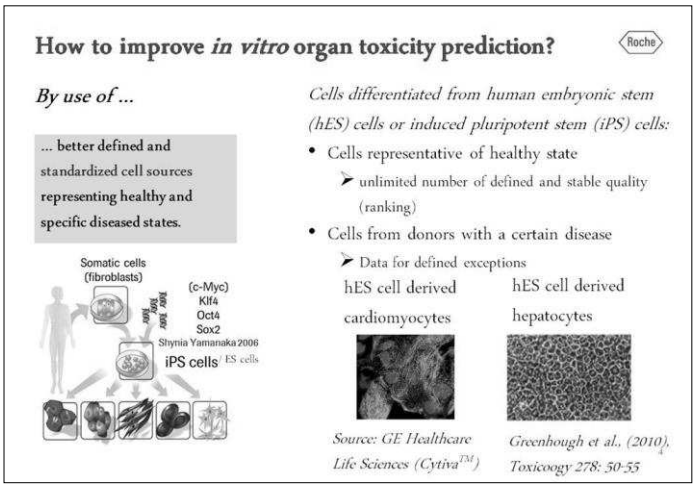
New 3R relevant methods in drug development

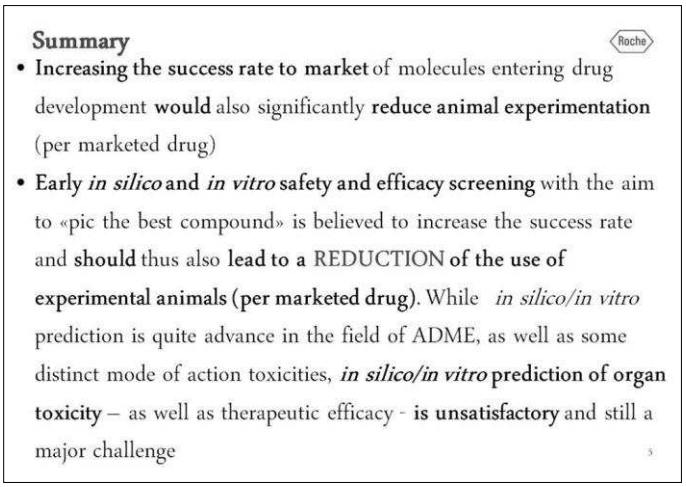
Franziska Boess, Non-clinical Drug Safety, F. Hoffmann-La Roche, Basel, Switzerland

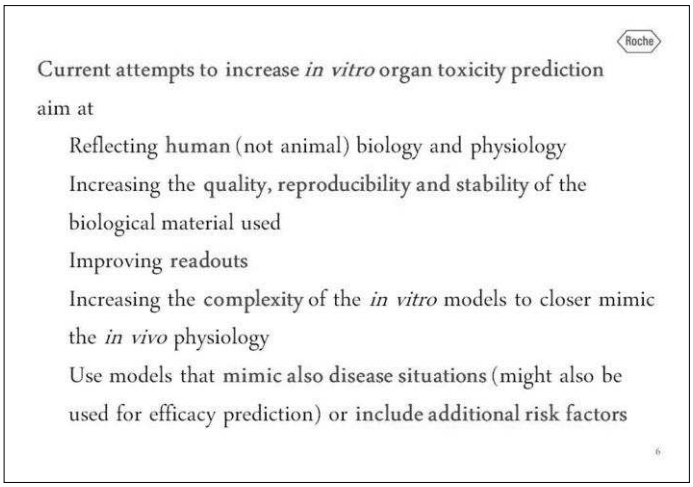












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- A. S. Kienhuis et al.*:* "Parallelogram approach using rat-human *in vitro* and rat *in vivo* toxicogenomics predicts Acetaminophen-induced hepatotoxicity in humans?" *Toxicological sciences* 107(2): 544-542 (February 2009), *doi: 10.1093/toxsci/kfn237*
- J. J. Xu et al.: "Cellular Imaging Predictions of Clinical Drug-Induced Liver Injury" *Toxicological Sciences* 105(1): 97-105 (September 2008), *doi:10.1093/toxsci/kfn109*
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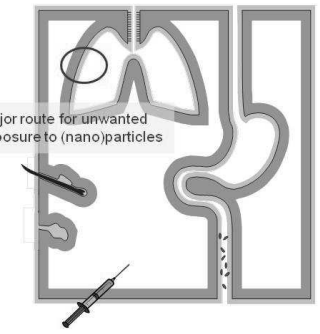
15:00 - 15:25

New tests for (nano)particles - *in vitro* evolution

Marianne Geiser Kamber, Institute of Anatomy, University of Berne, Switzerland

Interaction of particles with the organism: routes of entry, barriers

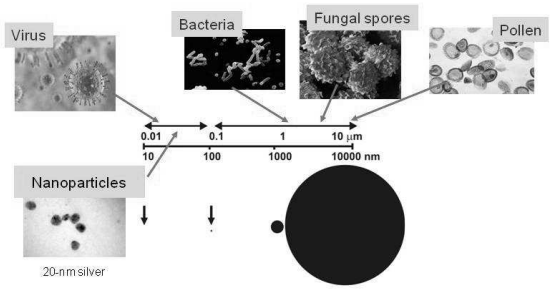
- Outer surface
  - Skin
- Inner surfaces
  - Respiratory tract
  - Digestive tract
- Injection
  - Blood circulation



M. Geiser – 25 years 3R, October 2012

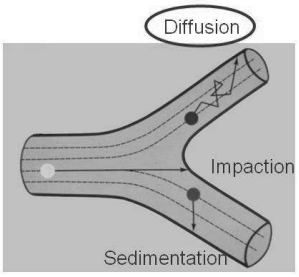
Aerosols: particles suspended in air

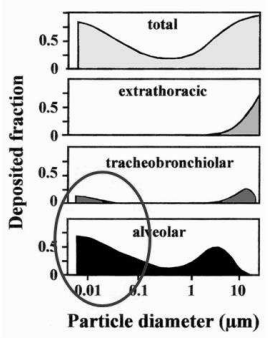
- Inhalable particles (≤ 10 μm, PM_{10})



M. Geiser – 25 years 3R, October 2012

Deposition of inhaled particles: nanoparticles deposit by diffusion in the entire respiratory tract



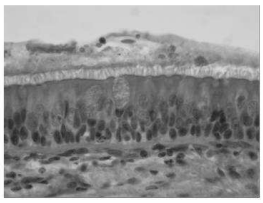


From Schulz et al., Lenfant Series, 2000

M. Geiser – 25 years 3R, October 2012

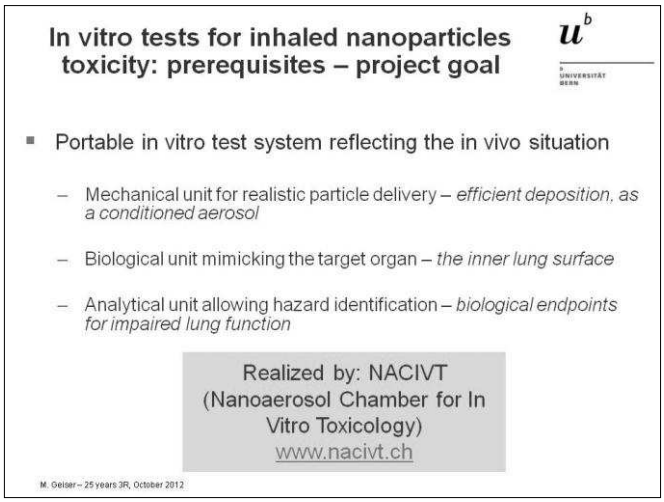
The respiratory epithelium: highly specialized cells

- ★ Ciliated cells – *Movement*
- ◆ Goblet cells – *Secretion*
- ✦ Basal cells – *Cell replacement*
- Surface lining layer with mucus
- Basement membrane



Trachea

M. Geiser – 25 years 3R, October 2012



References:

1. Anderson JO, Thundiyil JG, Stolbach A, 2012. Clearing the Air: A Review of the Effects of Particulate Matter Air Pollution on Human Health. *J Med Toxicol* 8, 166-175.
2. Fulcher ML, Gabriel S, Burns KA, Yankaskas JR, Randell SH, 2005. Well-differentiated human airway epithelial cell cultures. *Methods Mol Med* 107, 183–206.
3. Geiser M, Kreyling WG, 2010. Deposition and Biokinetics of Inhaled Nanoparticles. *Particle Fibre Toxicol* 7:2 doi:10.1186/1743-8977-7-2.
4. Mertes P, Praplan AP, Künzi L, Dommen J, Baltensperger U, Geiser M, Weingartner E, Ricka J, Fierz M, Kalberer M, 2012. A compact and portable deposition chamber to study nanoparticles in air-exposed tissue. *J Aerosol Med Pulm Drug Deliv J Aerosol Med Pulm Drug Deliv,* in press

Links:

5. WEF Global Risk Reports 2007, 2008 and 2009, http//www.weforum.org/pdf/globalrisk/2009.pdf
6. Woodrow Wilson database, http://www.nanotechproject.org/inventories/consumer

16:00 - 16:30

Impact of the International Conference on Harmonization (ICH) on the use of animals in Regulatory Toxicology

Friedlieb Pfannkuch, c/o F. Hoffmann-La Roche Ltd., Basel, Switzerland
Email: friedlieb.pfannkuch@bluewin.ch

The Issue

- Industry refers to the fact that **alternatives to animal testing are widely used**
  - However, numbers of animals used in experiments increase
- Can we substantiate and **demonstrate the impact of 3R activities**?
  - Our approach is the use of average portfolio assumptions for Pharmaceutical Companies

1

ICH - A Unique Approach

- **International Conference on Harmonization** was created in 1990
- Agreement between the **EU, Japan and the USA** to harmonize different regional requirements for registration of medicinal products
- Unique because joint effort by **regulators and** associated **pharmaceutical industry** trade associations
- **www.ich.org**

2

Assumptions on Drug Development

for a (mid-size to big) Pharmaceutical Company

Development Phase	Portefolio (compounds / year)
0 / I	**15**
II	**8** (incl. 2 Biopharmaceuticals)
III	**3** (incl. 2 Biopharmaceuticals)
Filing	**2**

- Experimental activities and the need of animals per year is estimated under 'normal' conditions

3

Impact of ICH Guidelines M3(R2), S9, S2(R1)

for a (mid-size to big) Pharmaceutical Company per year

Animal Species	Animal Numbers
	Before ICH Guideline (Revisions)
Rodent	10'150
Rabbit	960
Non-Rodent	1'110
Primates	90
Total	**12'310**
	Savings following ICH Guidelines M3(R2), S9 and S2(R1)
Rodent	2'160
Rabbit	500
Non-Rodent	48
Primates	45
Total	**2'753**
Savings	**22%**

- Not considered: Reduced toxicity packages due to use of exploratory clinical trials

4

ICH – Further Progress to be Expected …

- S6(R1) Addendum to **Biotechnology-derived Pharmaceuticals**
  - Impact on animal savings: (?)
- M7: **Safety Evaluation of Genotoxic Impurities**
  - Heavily relying on *in silico* and *in vitro* testing
- S10: **Photosafety Testing**
  - Impact on animal savings: (?)
- S1(R1): **Carcinogenicity testing**
  - Proposals to reduce the number of rodent bioassays

5

Summary and Conclusions

- The International Conference on Harmonization (ICH) is the most appropriate platform for refinement, reduction and replacement of animal testing in regulatory toxicology
- Reliability of extrapolation from animals to humans has to be systematically improved and the scientific basis for exploratory clinical trials has to be further developed
- Non-clinical safety may still remain the target for criticism, however the potential for essential reduction of animal testing is somewhere else

6

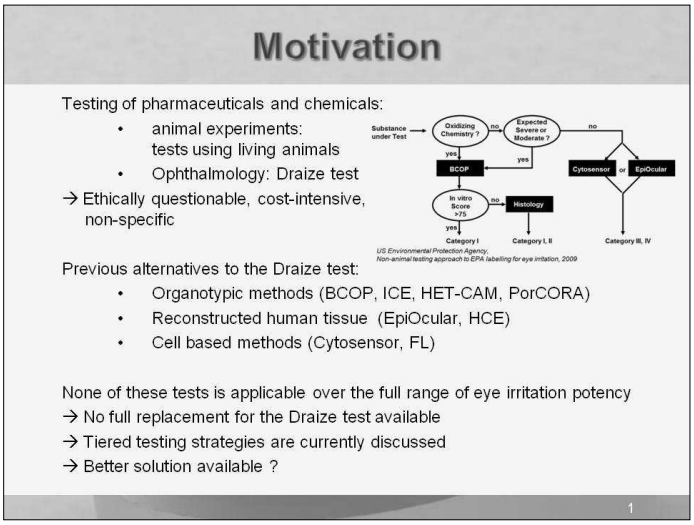
http://www.ich.org/

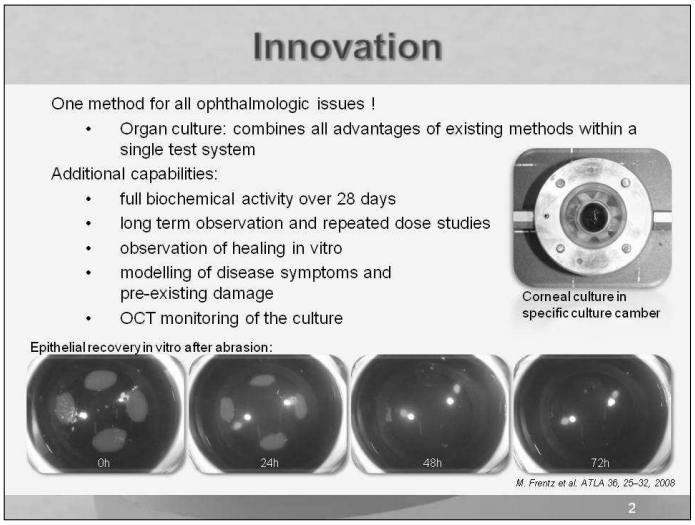
16:30 - 17:00

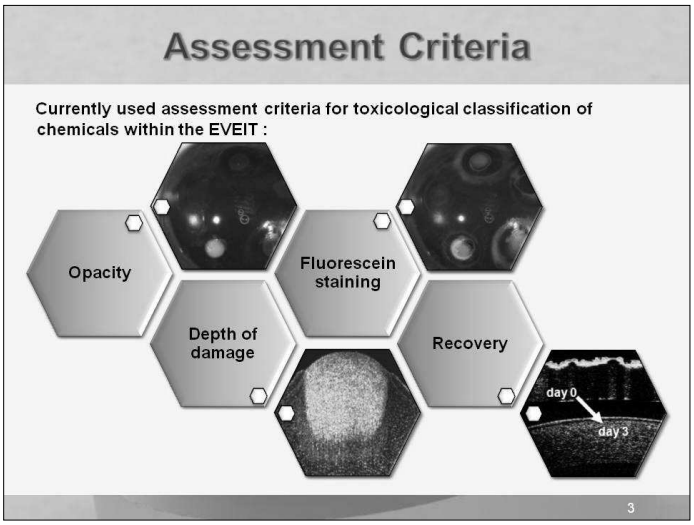
Proofing the relevance of the Ex Vivo Eye Irritation Test (EVEIT)

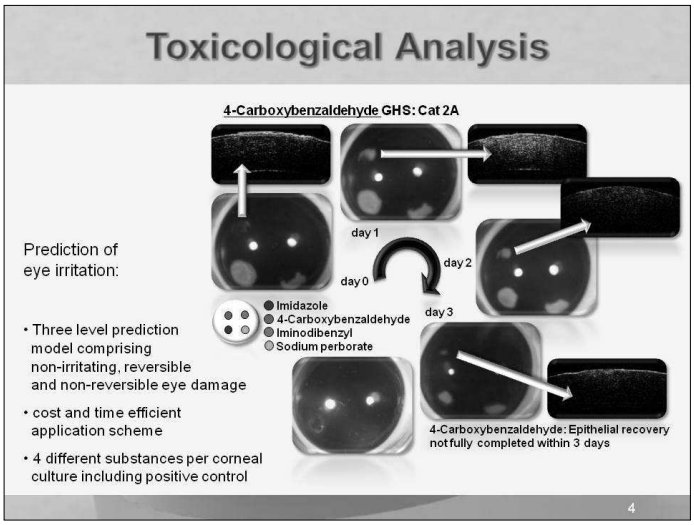
Felix Spöler[1], Stefan Kray[1], Oya Kray[1], Norbert Schrage[2], Claudia Panfil[2]

[1] RWTH Aachen University, Institute of Semiconductor Electronics, Germany
[2] Aachener Centre for Technology Transfer in Ophthalmology, Germany









Toxicological Results

Substance under test	GHS	EVEIT
2-Methoxy-ethyl-acrylat	Cat 1	Cat 1
Benzalkonium chloride (10%)	Cat 1	Cat 1
CTAB (10%)	Cat 1	Cat 1
Methyl-thioglycolate	Cat 1	Cat 2
Sodium Lauryl Sulphate (15%)	Cat 1	Cat 1
Triton X-100 (10%)	Cat 1	Cat 2
2,6-Dichlor-benzoyl-chlorid	Cat 2a	Cat 1
Acetone	Cat 2a	Cat 2
Benzalkonium chloride (1%)	Cat 2a	Cat 1
Deoxycholic acid Na salt (10%)	Cat 2a	Cat 2
Methyl acetate	Cat 2a	Cat 2
Methyl-ethylketon	Cat 2a	Cat 2
N-Lauroylsarcosine Na salt (10%)	Cat 2a	Cat 2
Sodium hydroxide (1%)	Cat 2a	Cat 1
2-Methyl-Pentanol	Cat 2b	Cat 2
3-Chloropropionitrile	Cat 2b	Cat 2
Ethyl-2-methyl-aceto-acetate	Cat 2b	NC
1,2-Propane-diol	NC	NC
1,3-Dibromo-propane	NC	NC
2-Bromo-propane	NC	NC
4-Methyl-pentan-2-one	NC	NC
iso-Octyl- acrylate	NC	NC
Trichloro-acetic acid (3%)	NC	Cat 2
Toluene	NC	NC
Tween20	NC	NC

25 liquid and 13 solid substances were tested

A preliminary prediction model was applied

- 5 EVEIT-classifications were over predictive
- 5 EVEIT-classifications were **under predictive**
- 28 EVEIT-classifications correspond to GHS

EVEIT categorisation of solids compared to GHS categorisation

Substance under test	GHS	EVEIT
1-Naphtalene Acetic Acid	Cat 1	Cat 2
2,5-Dimethylhexanediol	Cat 1	Cat 2
Imidazole	Cat 1	Cat 1
Lauric acid	Cat 1	Cat 1
Sodium oxalate	Cat 1	Cat 1
Sodium Perborate Tetrahydrate	Cat 1	Cat 1
Quinacrine	Cat 1	Cat 1
4-Carboxybenzaldehyde	Cat 2a	Cat 2
Dibenzyl phosphate	Cat 2a	Cat 1
EDTA di-K salt	NC	NC
Iminodibenzyl	NC	NC
Magnesium carbonate	NC	NC
Tetra-aminopyrimidine sulfate	NC	NC

EVEIT categorisation of liquids compared to GHS categorisation

5

Summary

The Ex Vivo Eye Irritation Test (EVEIT) is applicable to analyse reversibility of ocular damage *in vitro*

- potential to directly distinguish GHS Categories 1 and 2
- potential to fully replace the Draize eye irritation and corrosion test
- new possibilities for *in vitro* pharmaceutical testing

Next steps:

- Analysis of transferability of experimental methods and toxicological results
- Entering the Presubmission process of the method at ECVAM
- Commercialisation of the test method is in progress

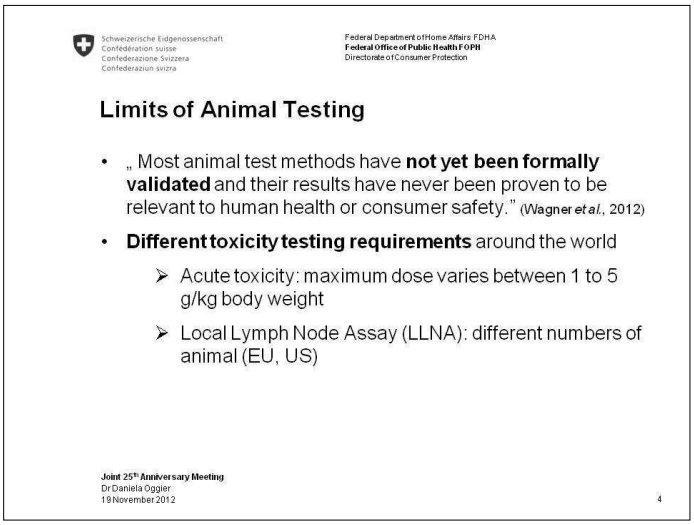
6

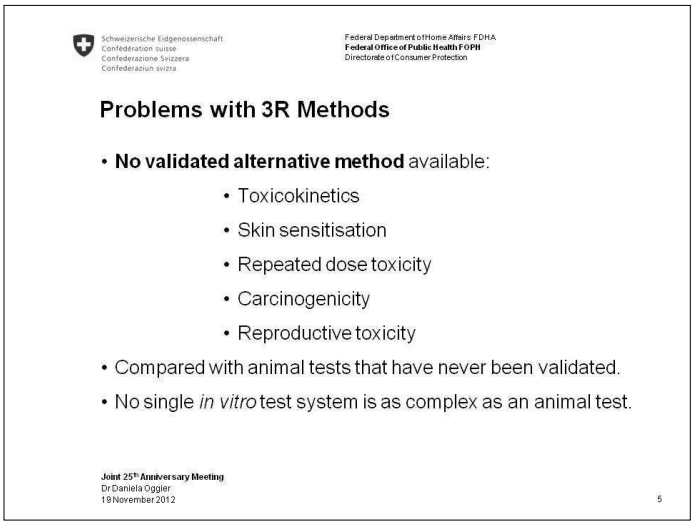
Additional information and further references concerning the Ex Vivo Eye Irritation Test (EVEIT) can be found on the homepage of the 3R Research Foundation Switzerland within the methods section:
http://www.forschung3r.ch/en/methods/method_M3.html

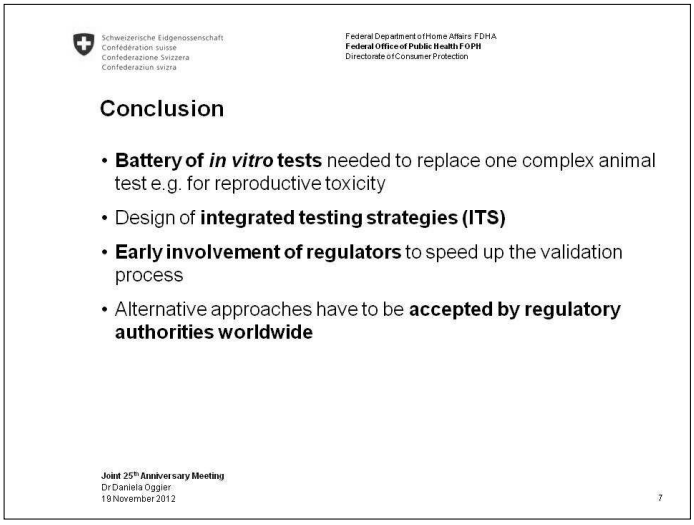
17:00 - 17:30

Criteria for acceptance of alternative methods in the approval process of chemicals

Daniela Oggier, Federal Office of Public Health FOPH, Directorate of Consumer Protection, Division of Chemical Products, Section of Risk Evaluation, Bern, Switzerland







Ferrario, D. and Rabbit, R.R. (2012). Analysis of the Proposed EU Regulation Concerning Biocide Products and its Opportunities for Alternative Approaches and a Toxicology for the 21st Century. *ALTEX* 29, 157-172.

Hartung, T. et al. (2004). A Modular Approach to the ECVAM Principles on Test Validity. *ATLA* 32, 467-472.

Schiffelers, M. J. et al. (2012). Regulatory Acceptance and Use of 3R Models: a Multilevel Perspective. *ALTEX 29*, 287-300.

Schiffelers, M. J. et al. (2007). Factors Stimulating or Obstructing the Implementation of the 3Rs in the Regulatory Process. *ALTEX 24*, 271-278.

Tralau, T. et al. (2012). Wind of Change Challenges Toxicological Regulators. *Environ Health Perspect.*, Online 7 August 2012

Wagner, K. et al. (2012). Inconsistencies in Data Requirements of EU Legislation Involving Tests of Animals. *ALTEX* 29, 302-332.

Homepages

ECVAM: http://ecvam.jrc.it/
OECD Test Guidelines: http://www.oecd-ilibrary.org/content/package/chem_guide_pkg-en
Euroecotox: http://www.euroecotox.eu/

Have a look at the 18 Posters about the present status of ongoing funded 3R relevant projects.

Description of the projects on page 79 – 110
or on the website of the 3R Research Foundation

http://www.forschung3r.ch/en/projects/index.html
including all funded 134 projects.

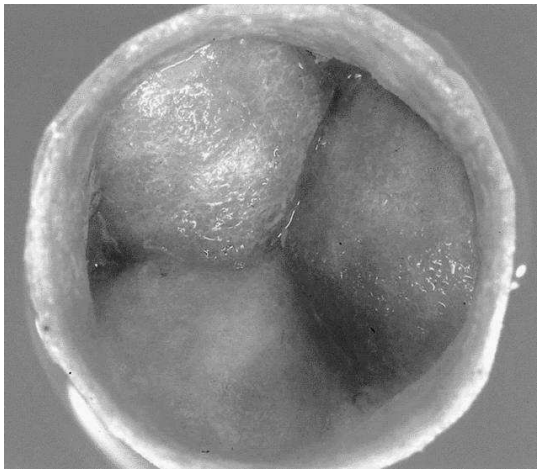
Tuesday 20.11 2012 Auditorium

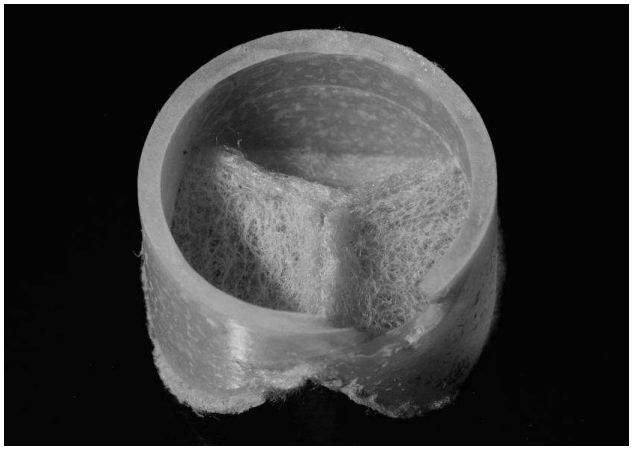
3R - Lecture 9:00 - 9:30

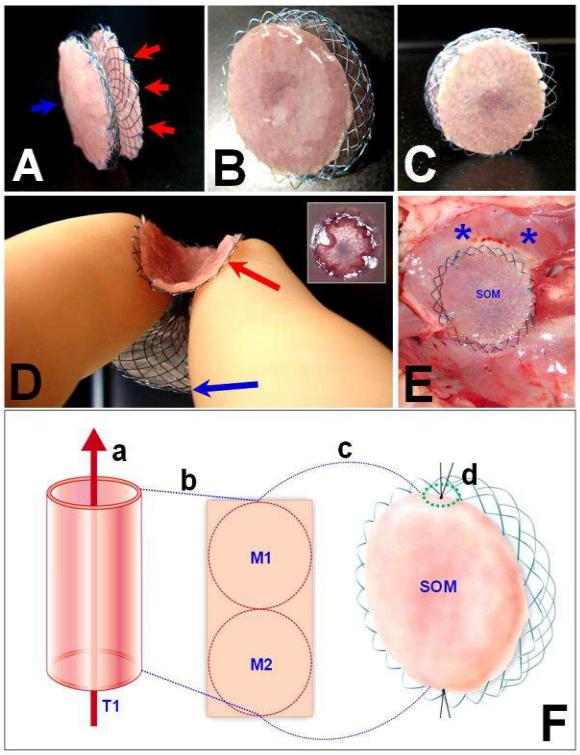
From cells to functional cardiovascular implants

Simon P. Hoerstrup, Cardiovascular Regenerative Medicine, University Hospital and University of Zürich, Switzerland

Cell-based therapy concepts comprising regeneration of damaged organs by e.g. transplanted stem cells and replacement of diseased/malformed structures by tissue engineered, living implants represent promising novel treatment modalities, ultimately aiming at “restitutio ad integrum” instead of repair. First successful clinical applications such as stem cell therapies of myocardial infarction and tissue engineering of autologous trachea have recently been demonstrated. Various cell sources including several categories of stem cells are being examined for cardiovascular applications. Our laboratory has been focusing on using marrow stromal derived stem cells (MSCs) as a versatile autologous cell source for tissue engineering of living cardiovascular structures such as arteries and heart valves. Beyond the “classical” in vitro tissue engineering approach, in vivo technologies using the cell attraction and remodeling potential of MSCs are currently investigated. For myocardial repair, the optimal cell delivery format using 3-D microtissue technology and the most suitable route for cell delivery (intracoronary vs. intramyocardial) after myocardial infarction are under investigation.



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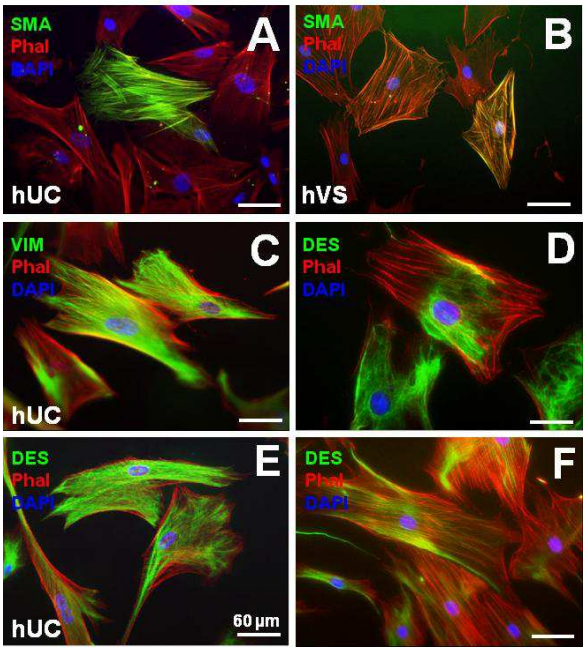
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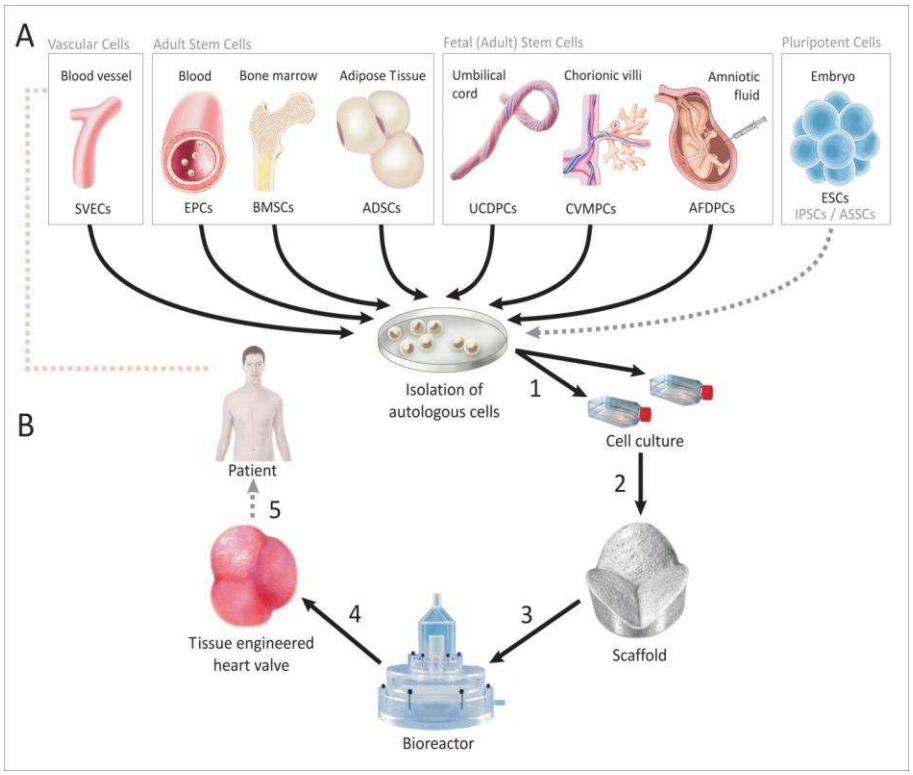
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Adult Stem Cells
Fetal (Adult) Stem Cells
Pluripotent Cells
Blood vessel
Blood
Bone marrow
Adipose Tissue
Umbilical cord
Chorionic villi
Amniotic fluid
Embryo
SVECs
EPCs
BMSCs
ADSCs
UCDPCs
CVMPCs
AFDPCs
ESCs
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B
Isolation of autologous cells
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Cell culture
Patient
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Tissue engineered heart valve
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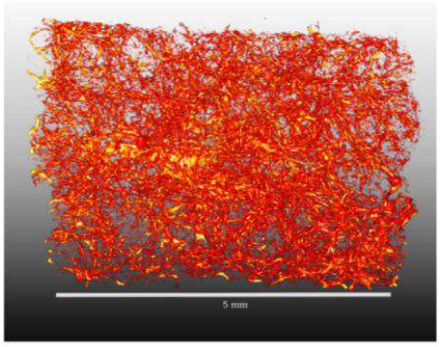
Scaffold
Bioreactor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Tuesday 20.11 2012

Room Basic (2nd Floor) and Video in Room Pascal (3rd Floor)

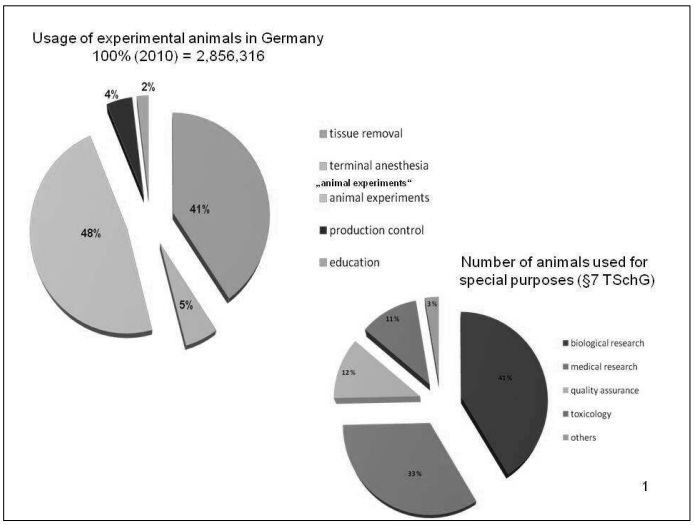
Session III

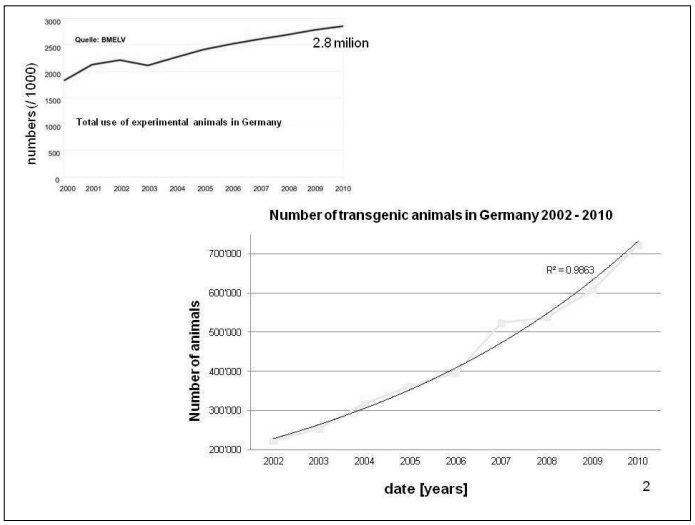
Progress of the 3Rs in Europe Chair: Simon Hoerstrup and Philippe Hubert		
09:35 10:10	Human neurons as alternatives to transgenic mice in neurobiology	M. Leist University of Konstanz, Germany
10:10 10:40	Do we still need teratomas? Alternatives to the pluripotency assay	G. Weitzer Medical University Vienna, Austria
	COFFEE BREAK - INDUSTRIAL EXHIBITION - POSTER VIEWING- *InSphero* LAB TOUR	
11:00 11:30	Novel *in vitro* human cell model to detect kidney carcinogens using transcriptomics and systems biology	M. Ryan University College Dublin, Ireland
11:30 11:55	State of the Art: Safety assessment of cosmetics in the EU	Odile de Silva L'Oreal, Paris, France
11:55 12:20	Assuring Safety Without Animal Testing	R. Stierum TNO, Zeist, The Netherlands
	LUNCH - INDUSTRIAL EXHIBITION - POSTER VIEWING- *InSphero* LAB TOUR	

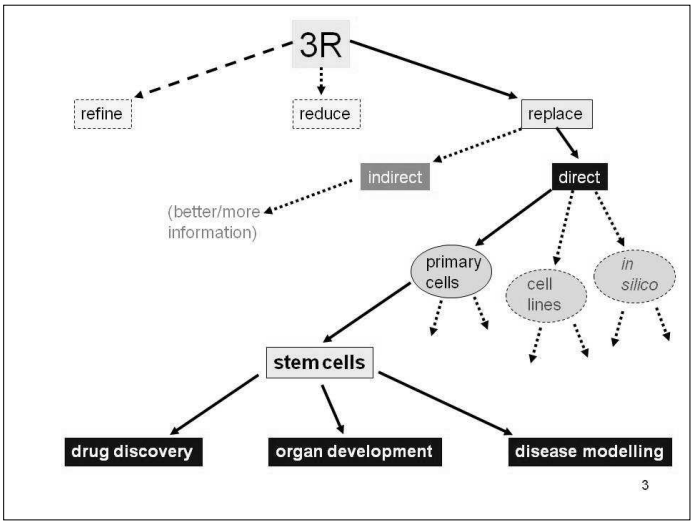
09:35 - 10:10

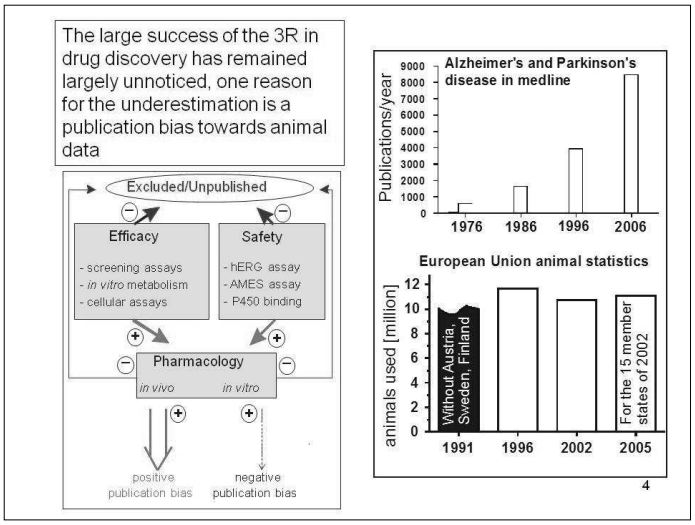
Human neurons as alternatives to transgenic mice in neurobiology

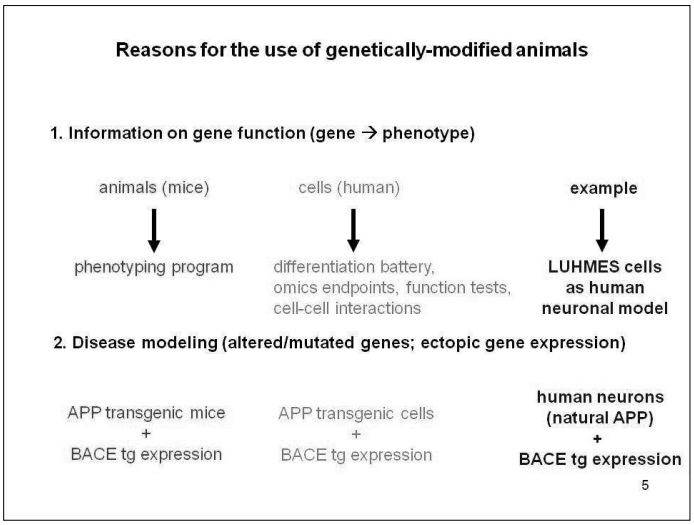
Marcel Leist, In vitro Toxicology and Biomedicine, University of Konstanz, Germany

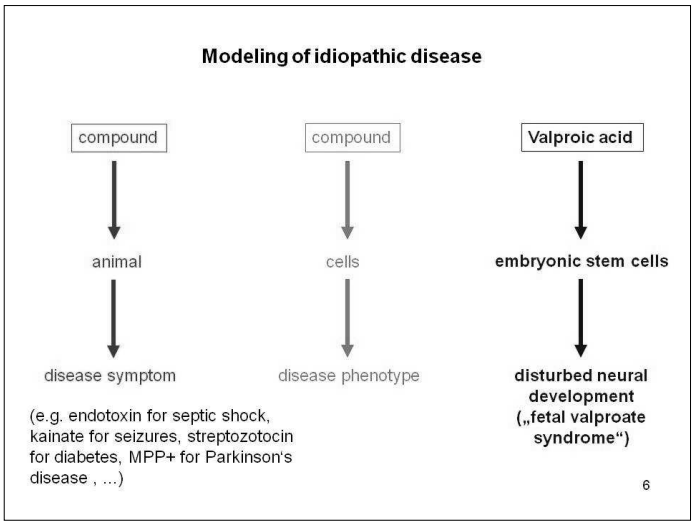












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2. Scholz D, Chernyshova Y, Leist M. Control of Aβ release from human neurons by differentiation status and RET signaling. Neurobiol Aging. 2012 Apr 23. [Epub ahead of print] PubMed PMID: 22534065.
3. Scholz D, Pöltl D, Genewsky A, Weng M, Waldmann T, Schildknecht S, Leist M. Rapid, complete and large-scale generation of post-mitotic neurons from the human LUHMES cell line. J Neurochem. 2011 Dec;119(5):957-71. doi:
4. 10.1111/j.1471-4159.2011.07255.x. Epub 2011 Apr 13. PubMed PMID: 21434924.

Abbreviations:

BACE, APP:	to proteins/genes involved in Alzheimer's disease
tg:	transgenic (animals or cells)
LUHMES:	a conditionally-immortalized human neuronal cell line (Lund Human Mesencephalic cell line)

10:10 - 10:40

Do we still need teratomas? Alternatives to the pluripotency assay

Georg Weitzer, Max F. Perutz Laboratories, Department of Medical Biochemistry, Medical University Vienna, Austria

Breaking a Butterfly on a Wheel

Reconsidering Pluripotency Tests

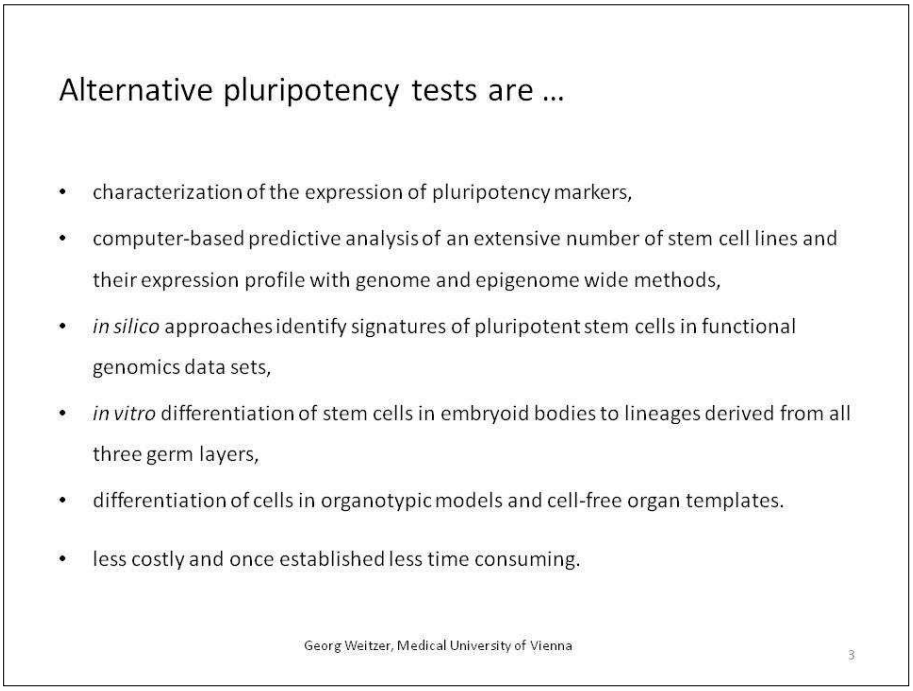
Christiane Buta[1], Robert David[2], Ralf Dressel[3], Mia Emgård[4], Christiane Fuchs[5], Ulrike Gross[6], Lyn Healy[7], Jürgen Hescheler[8], Roman Kolar[6], Ulrich Martin[9], Harald Mikkers[10], Franz-Josef Müller[11], Rebekka K. Schneider[12], Andrea E.M. Seiler[13], Horst Spielmann[14], Georg Weitzer[15].

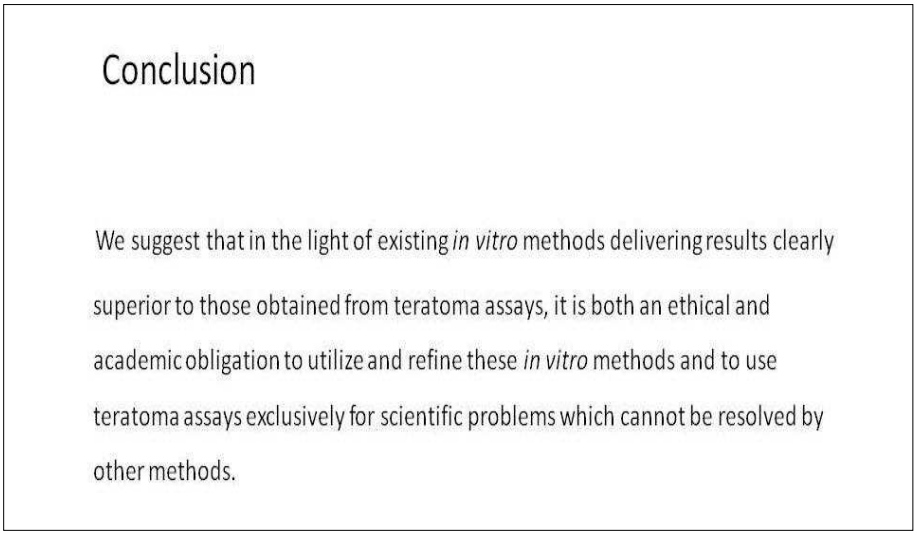
[1]SET Foundation Frankfurt am Main, Germany
[2]Ludwig-Maximilians-Universität, München Germany
[3]Universität Göttingen, Germany
[4]Cellartis, Göteborg and Karolinska Institute Stockholm, Sweden
[5]Universtity of Applied Sciences, Technikum Wien, Austria
[6]Akademie für Tierschutz, Neubiberg, Germany
[7]UK Stem Cell Bank, NIBSC, Hertfordshire, UK
[8]Universität Köln, Germany
[9]Medizinische Hochschule Hannover, Germany
[10]Universiteit Leiden, The Netherlands
[11]Universitätsklinikum Schleswig-Holstein, Campus Kiel, Germany
[12]RWTH, Aachen, Germany
[13]Federal Institute for Risk Assessment, Berlin, Germany
[14]Freie Universität Berlin, Germany
[15]Medical University of Vienna, Austria

Do we still need Teratoma Assays?

The induction of teratoma in mice by the transplantation of stem cells into extra uterine sites has been used to demonstrate pluripotency since the first description of cellular pluripotency in 1958. Since then, the teratoma assay has remained the assay of choice and has gained prominence during the recent hype surrounding human stem cell research for which stringent pluripotency tests, such as tetraploid-aggregation of embryos and stem cells are ethically unacceptable and *non licet*.

- Pluripotency tests are definitely required to classify the developmental potential of stem cells.
- Teratoma assays are currently required for the safety testing of stem cell-derived transplants, since pluripotency and tumorigenicity are closely related phenomena and this association constitutes a major safety issue and challenge for regenerative medicine.
- Notwithstanding previous *pro bono* arguments, teratoma assays are highly questionable because of little consistency in methodology despite their 'gold standard' status among many scientists.
- An equally important ethical rebuff against teratoma assays concerns animal welfare. The inherent value of all creatures demands a careful consideration of their dignity and rights.





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Stevens, L.C. (1958). Studies on transplantable testicular teratomas of strain 129 mice. J Natl Cancer Inst 20, 1257-1275.

11:00 - 11:30

Novel *in vitro* human cell model to detect kidney carcinogens using transcriptomics and systems biology

Michael P. Ryan, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Ireland

Introduction

- Overview of EU CarcinoGENOMICS project.
- Main aim to develop in vitro methods for assessing carcinogenic potential of compounds as an alternative to the 2-year rodent bioassay.
- Success story – Kidney cell model.
- Prediction of carcinogenicity, Phase I and II plus inter-laboratory prevalidation.
- Additional studies, cytomics – cilia; *in vitro –in vivo*; human renal cancer genes.
- Summary and conclusions

Carcinogenomics WP 3 Kidney Models :

Summary of reducing initial list of cell models

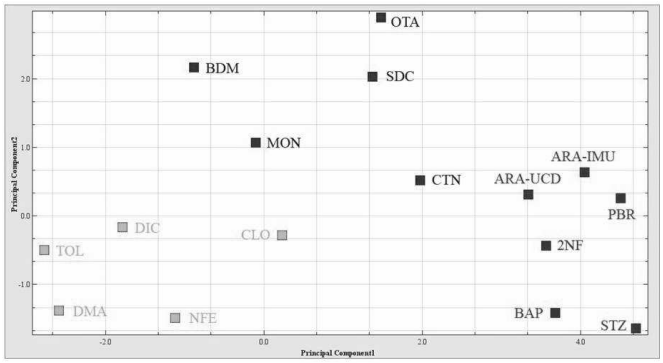
2 robust proximal tubular epithelial cell models selected and optimized based on:

Morphology and characteristics
Barrier function
Genetic stability
Transcription Profile
Metabolic Profile

Human - RPTEC/TERT1

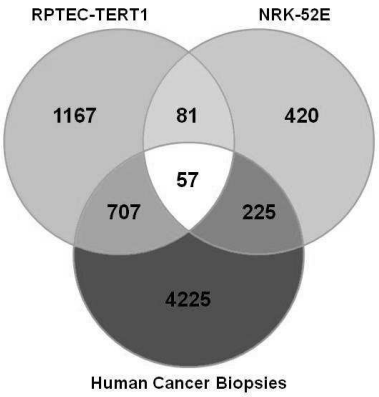
Rat - NRK-52E

Detection and separation of carcinogens by pathways



Carcinogenomics : Kidney Models Additional Studies

- Primary cilium – RPTEC/TERT1 cells.
- In vitro – in vivo comparison ; Ochratoxin
- Relationship of transcriptomic findings to human renal cancer.



Overall Summary : Kidney Model

- Results with both human and rat cells very good but pathway analysis suggest gene changes in human cells more relevant to known carcinogenic mechanisms.
- Excellent interlaboratory comparisons of transcriptomics with the RPTEC/TERT1 cells with correct carcinogen prediction despite cell culture problems in one lab.
- In vitro-in vivo rat correlations and comparisons to known gene changes in human renal cancer are very encouraging and exciting.
- Loss of cilia in RPTEC/TERT1 cells a possible additional in vitro assay for carcinogens.
- Kidney model for further development, as an alternative in vitro test system for carcinogens

Additional Information and contacts

- E-mail: michael.p.ryan@ucd.ie
- Web : www.ucd.ie/renal
- Web: www.carcinogenomics.eu

11:30 - 11:55

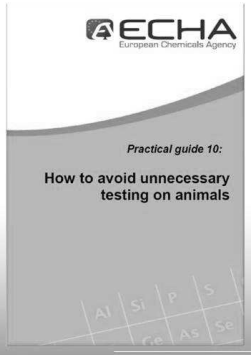
State of the Art: Safety assessment of cosmetics in the EU

Odile de Silva, L'Oreal, Paris, France

About Cosmetics Europe

- Cosmetics Europe (*new name of* Colipa) has been the voice of Europe's cosmetic, toiletry and perfumery industry since 1962.
- Membership consists of:
  - 27 national associations (representing circa 4000 SMEs)
  - 16 major international companies
  - 4 associated and 3 corresponding members
- More information:
  - www.Cosmetics Europe.eu

European Regulations applying to Cosmetics



REACH Regulation EC 1907/2006
- **Scientific use 2010/63/EU**
- **Test Methods EC 440/2008**

Strategy concerning animal welfare
- **Focus on reduction**
- **Need for alternative test strategy**

Cosmetics Regulation EC 1223/2009
- **7th amendment :2003/15/EEC**
- **Test Methods EC 440/2008**

Strategy concerning animal welfare
- **Focus on replacement**
- **Need for alternative test methods**

Key objectives of 7th Amendment

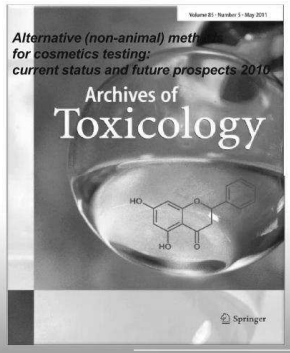
•Testing Ban
Prohibits animal tests in the EU to meet requirements of Cosmetics Directive

Finished products:
September 2004

Ingredients:
March 2009

•Marketing Ban
Prohibits marketing of cosmetics when product or ingredients tested on animals to meet requirements of Cosmetics Directive
Gradual prohibition (ingredients)
1st Deadline: *March 2009*
2nd Deadline: *March 2013*

Current status and Outlook 2011



•Review considered the toxicological endpoints important for the 2013 marketing ban deadline

•Revealed that the scientific basis to fully replace animal testing for the five toxicological key areas is still not fully established (*additional time beyond 2013 needed)*

•Confirmed that it could take at least another 7 – 9 years for the replacement of some of the current *in vivo animal* tests necessary for the safety assessment

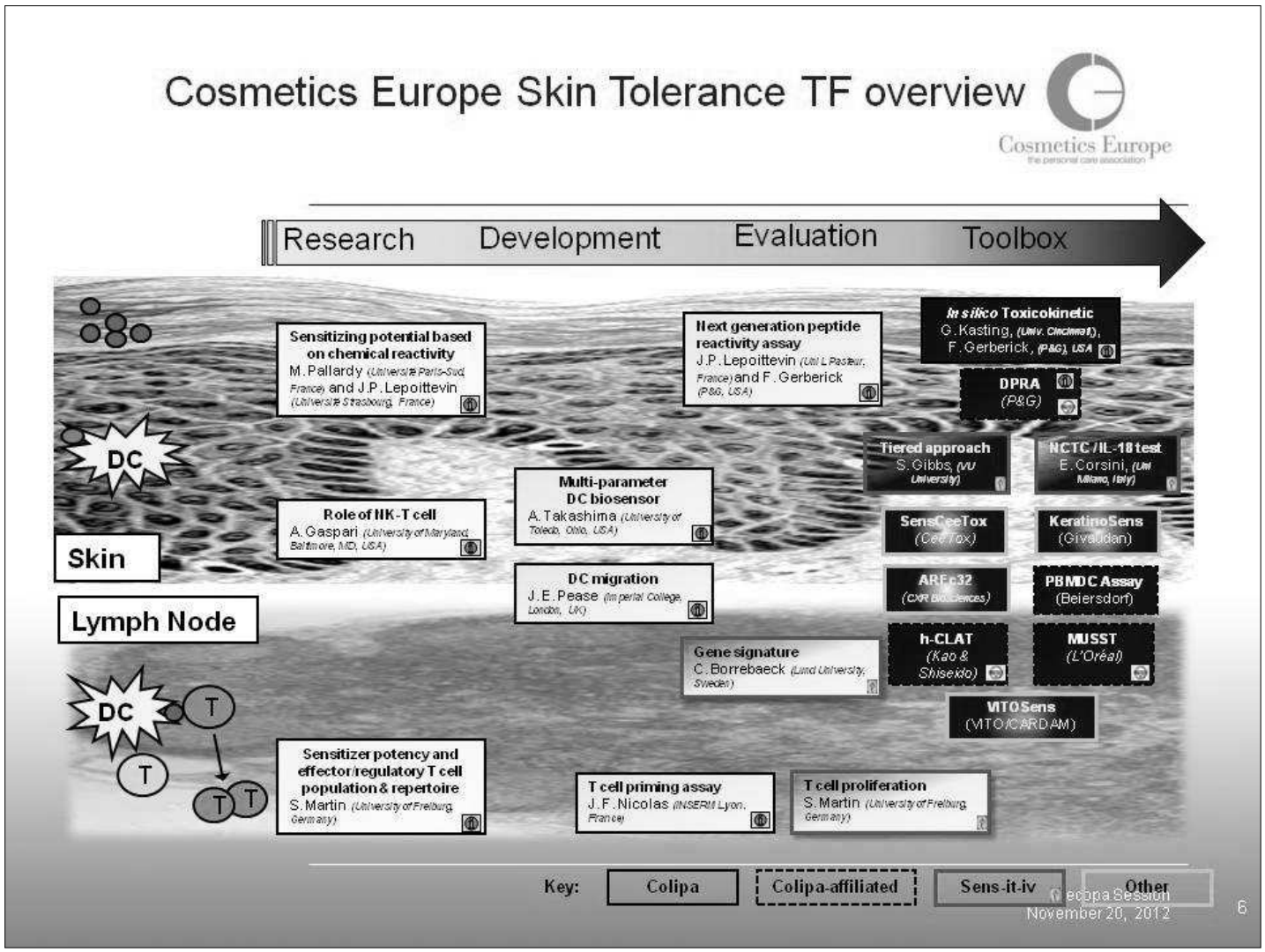
Next regulatory steps

The European Commission is currently assessing the impacts of the implementation of the full marketing ban by 2013 (environmental, animal welfare, economic and social) and on the basis of that assessment will decide whether or not to make a proposal in relation to the marketing ban.
The Commission will announce its final decision by the end of 2012.

1. Towards the Replacement of in vivo Repeated Dose Systemic Toxicity Testing - SEURAT-1 - Annual Report, Vol. 1 published in September 2011 & Annual Report Vol 2 published in september 2012

2. The reconstructed skin micronucleus assay (RSMN) in EpiDermTM: Detailed protocol and harmonized scoring atlas - Erica L. Dahla et al. ,Mutation Research 720 (2011) 42–52

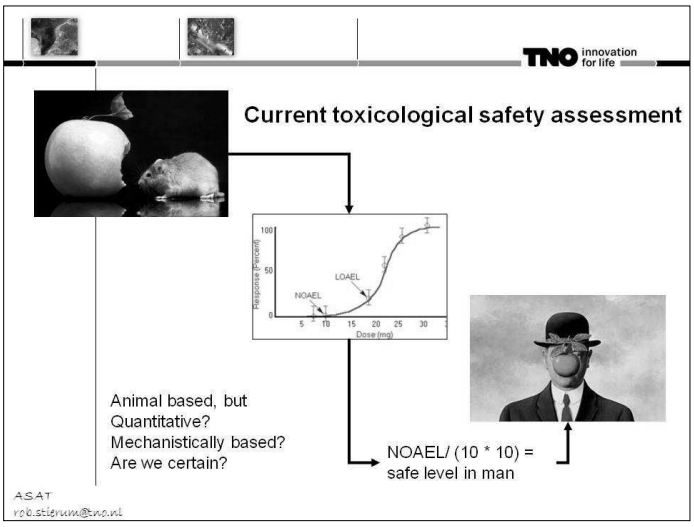
3. Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays .I . Choice of cell type – Paul Fowler et al., Mutation Research 742(2012) 11-25

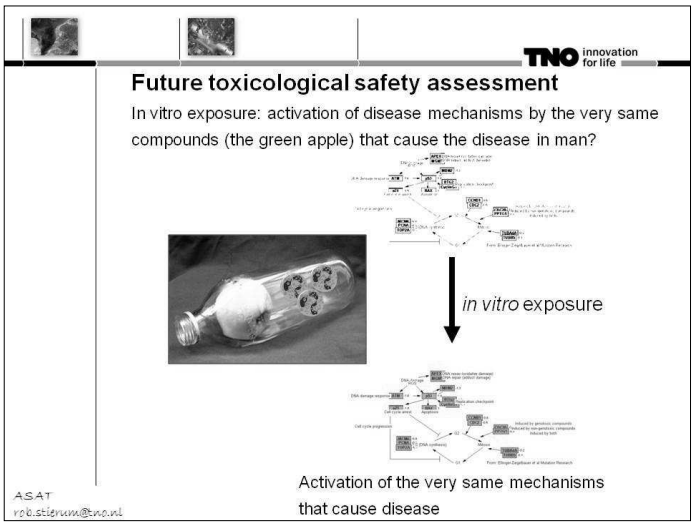


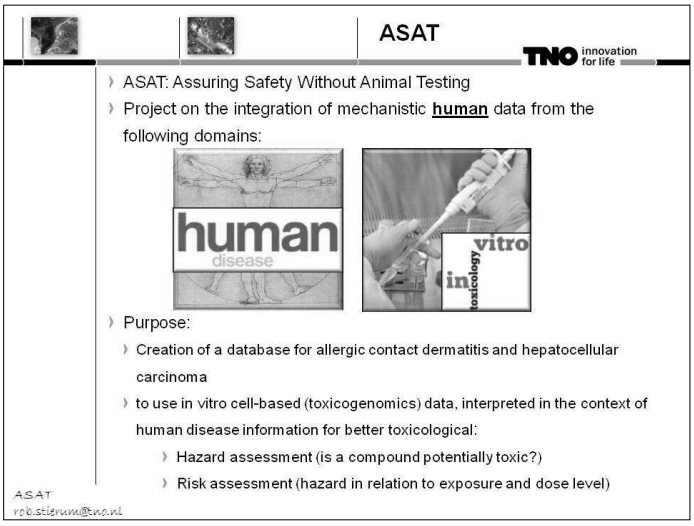
11:55 - 12:20

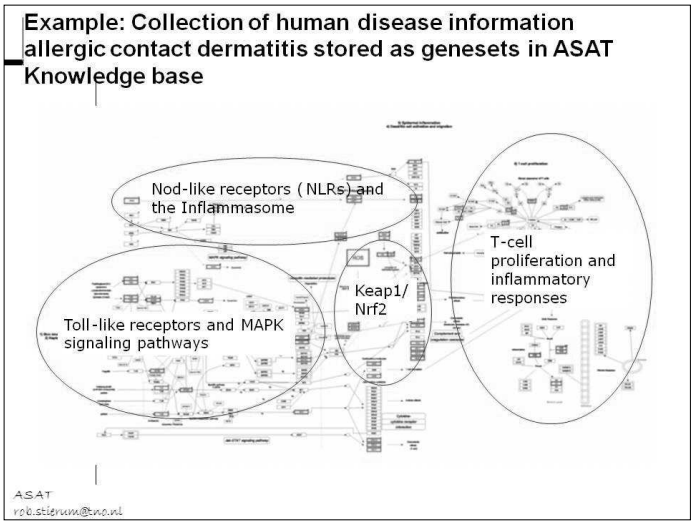
Assuring Safety Without Animal Testing

Rob H. Stierum, Systems Biology, TNO, Zeist, The Netherlands



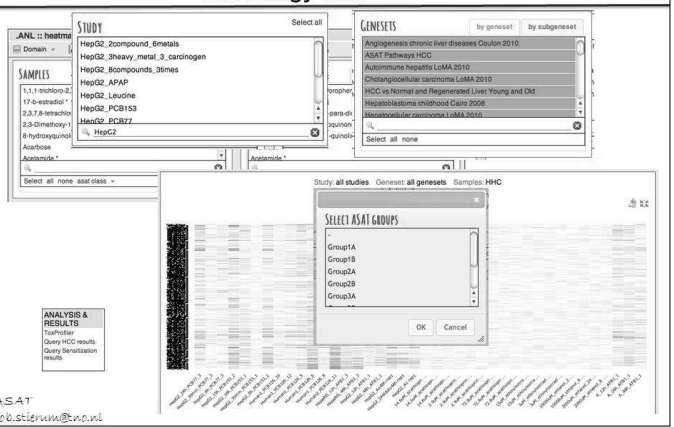






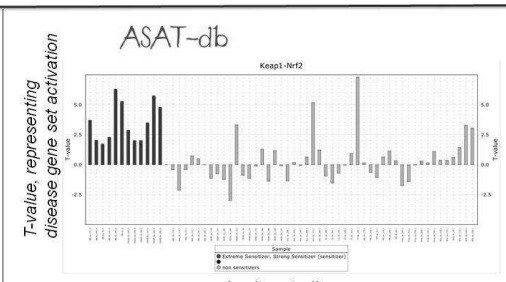
ASAT-db

ASAT Knowledge Base
database combining disease and in vitro toxicology data



ASAT
rob.stierum@tno.nl

ASAT Knowledge Base. Analysis & Results – Allergic Contact dermatitis (ACD, Sensitization)



In vitro studies

Possible proof of principle: disease specific mechanisms (in this case Keap1-Nrf2), are primarily activated in *in vitro* cell experiments with chemicals known to cause disease (ACD) in man (red bars) in comparison to chemicals not causing disease (green bars)

ASAT
rob.stierum@tno.nl

Funding by ZonMW is acknowledged (Project number 165600002).

For more information about funding of 3R relevant projects through the 3R Research Foundation Switzerland, please consult our website

(in German, French or English)

www.forschung3r.ch

or contact one of the members of the Evaluation Committee at the Meeting.

(distinguished by a dark blue badge)

The Evaluation Committee assesses applications for research grants and advises the Administrative Board as to which applications should be accepted, with a corresponding recommended amount, and which should be rejected. The Committee is made up of 11 members from academic circles, private industry, public administration and animal protection. As specialists in their different fields they ensure, as a body, that all applications are assessed in a professional and well balanced manner.

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Tuesday 20.11 2012

Room Basic (2nd Floor) and Video in Room Pascal (3rd Floor)

Session IV

	Support of the 3Rs Chair: Odile de Silva and Adrian Smith	ecopa
13:50 14:20	Directive 2010/63/EU on the protection of animals used for scientific purposes	S. Louhimies European Commission, Brussels
14:20 14:45	Implementation of the directive 2010/63/EU in Finland-how did the different stakeholders react	T. Heinonen University of Tampere, Finland
14:45 15:10	EPAA and Academic Research	K. D. Bremm EPAA, Platform of Science, Brussels
	COFFEE BREAK - INDUSTRIAL EXHIBITION - POSTER VIEWING - *InSphero* LAB TOUR	
15:30 15:50	Horizon 2020: Advancing safety science and health research with modern, non-animal tools	T. Seidle Humane Society International London, U.K.
15:50 16:10	Interrelationships between the National Science Foundation and the 3R Platform in the Netherlands	J. de Boer ZonMw, The Netherlands

13:50 - 14:20

Directive 2010/63/EU on the protection of animals used for scientific purposes

Susanna Louhimies, Unit Chemicals, Biocides & Nanomaterials, DG Environment, European Commission, Brussels, Belgium

Tools to promote uniform transposition

The Commission facilitates the process through

- Twice yearly National Contact Point (NCP) meetings
- Legal and technical questions
- NCP discussion
- ***Expert Working Groups*** on priority topics to arrive at a common approach
- Information portal at the Commission web-site

Topics of priority for discussion at Expert Working Groups

- Statistical reporting
- Genetically Altered (GA) animals
- Severity Assessment Framework
- Education and Training
- *Information on the Three Rs*
- *Project evaluation / Retrospective assessment*
- *Inspections and enforcement*

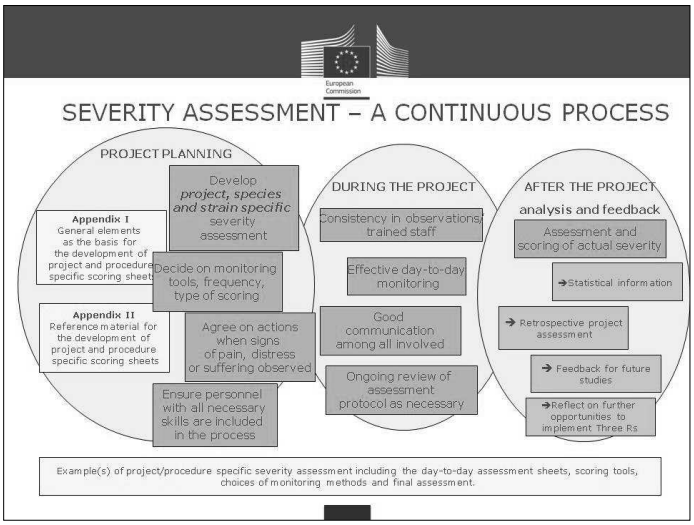
Statistical reporting - requirements

- *Total* number of *naïve animals*
- Use of *animals* in *procedures* with *details*
- Related *actual severity* for animal for procedure
- Genetically altered animals are reported either
  - *when used for the creation of a new line;*
  - *when used for the maintenance of an "established" line with an intended and exhibited "harmful phenotype"; or*
  - *when used in other (scientific) procedures (i.e. not for creation or for the maintenance of a line)*

Genetically altered animals

Developed and agreed general principles as to

- Terminology
- How GA animals are to be considered under project authorisation and statistical reporting
- The severity assessment of GA animals to determine what is considered a harmful phenotype



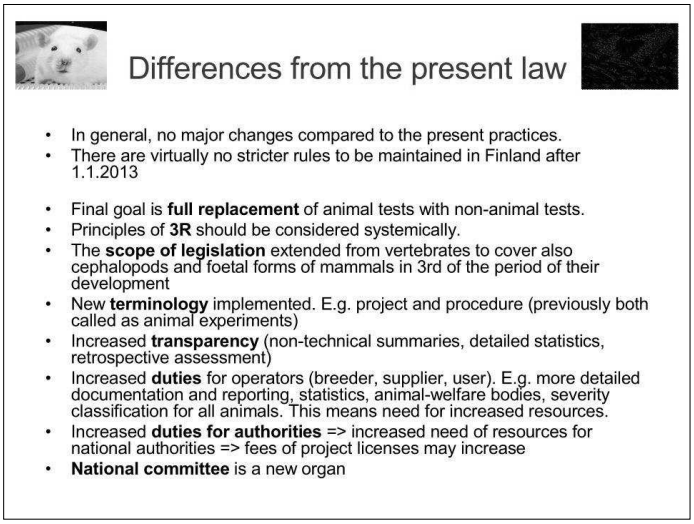
Link to the general web-site for the protection of animals used for scientific purposes: http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm

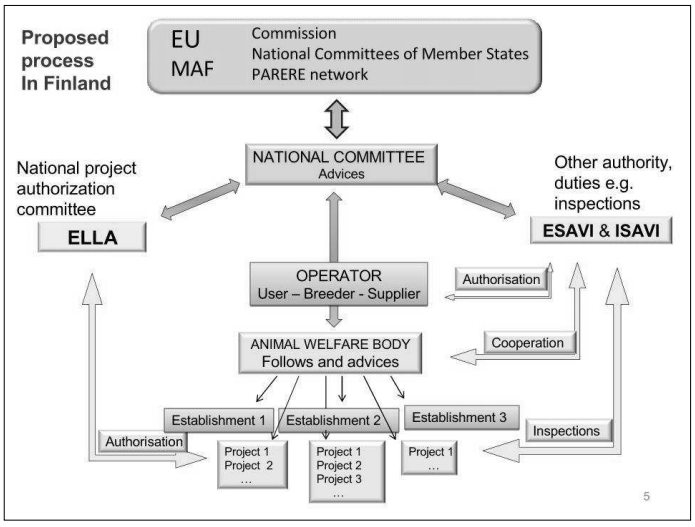
Link to the results of Expert Working Group work as well as other key documents concerning interpretation and legal question on the new Directive: http://ec.europa.eu/environment/chemicals/lab_animals/interpretation_en.htm

14:20 - 14:45

Implementation of the directive 210/63/EU in Finland - How did the different stakeholders react?

Tuula Heinonen, FICAM, University of Tampere, Eila Kaliste Regional State Administrative Agency of Southern Finland, Tiina Pullol, Ministry of Agriculture and Forestry, Finland.





Summary

- 3R aspect included in all activities: Positive
- More duties to researchers (documentation, statistics, retrospective evaluation): Too much – leads to time taken from animal welfare
- Retrospective evaluation: Positive or negative responses depending on the content of information needed (unclear now)
- More duties to operators: No negative or positive comments
- More duties to authorization bodies: More resources required
- Increased project authorization fees: Obstacles for research. May lead to large projects that may then be difficult to manage.

14:45 - 15:10

EPAA and Academic Research

Klaus-Dieter Bremm, EPAA Platform of Science, Brussel and Bayer Pharma AG, Wuppertal, Germany

Use of animals in pharmaceutical R&D

- As long as there are unmet medical needs Pharmaceutical industry's role is to develop and provide patients with innovative, safe and efficient medicines.
- For ethical, scientific and regulatory reasons and despite significant technological progress, currently this mission is dependent upon studies in animals.
- Whenever performing studies in animals pharmaceutical industry fully applies the 3Rs principle: refinement, reduction, replacement.

Replacement:

'Desirable as replacement is, it would be a mistake to put all our humanitarian eggs in this basket alone.'

(Russell & Burch, 1959)

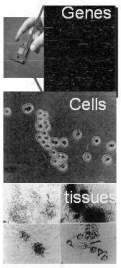
Platform on Science

Example: Toxicological Testing Strategy for Potential Drug Candidates During Early Phase of Drug Discovery

„in cerebro" and *„in silico"*

Are there structural alerts?
Can potential toxicities be predicted based on mode of action?

First Safety Assessment



Tox Profiling *„in vitro"*

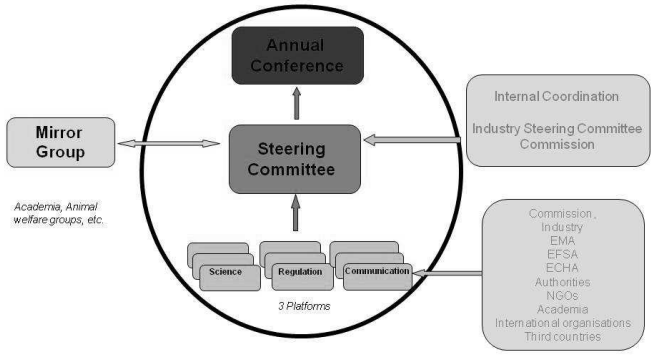
Genotoxicity: Ames, Cytogenetics *in vitro*,
Cardiotoxicity: hERG Assay *in vitro*,
General toxicity: cytotoxicity *in vitro*,
others (e.g. omics) if appropriate

Second Safety Assessment

Only compounds which have passed these hurdles are tested in animals

Platform on Science

EPAA structure



Platform on Science

Mission of PLATFORM ON SCIENCE

- Identify **scientific gaps** - recommend areas for further research
- Address the identified **research needs** through relevant programmes/calls for proposals from research funding institutions
- Seek **involvement of scientists** from diverse areas to increase knowledge and understanding of 3Rs
- Assess the feasibility of **technology transfer** across sectors

Platform on Science

Shifting from the traditional paradigm

Not looking for an 1:1 replacement of current animal tests but for new ways of doing science and safety assessment

Shifting from the traditional work of Replacing, Reducing or Refining to:
- a more science-based ambitious approach
- less method-specific approach

Allowing better **cooperation** for multidisciplinary research :
- between sectors
- between regions

Current initiatives:
USA: Toxicity Testing in the 21st Century (US National Academy of Sciences), FDA's Regulatory Science

EU: EPAA's New Perspectives on Safety/Computational chemistry, SEURAT-1, AXLR8, "New challenges for Risk Assessment" (EU Scientific Committees)

Platform on Science

Summary

- Continuous investment into science and technology contributes to a continuous replacement, reduction and refinement.
- Encourage the best minds to engage with the unique challenges that advancing the 3Rs brings to biology and chemistry
- Two additional challenges:
  - Regulatory acceptance of new technologies
  - International harmonisation of accepted methods is necessary to improve animal welfare on a world-wide basis
- Wide recognition among the international scientific community of the value and scientific challenges of research aligned with delivering animal welfare benefits

Platform on Science

Website of The European Partnership for Alternative Approaches to Animal Testing: http://ec.europa.eu/enterprise/epaa/

An unprecedented collaboration between European Commission and seven industrial sectors

Home | About EPAA | Activities | Events | News | Contact

EPAA: A joint initiative

The European Partnership for Alternative Approaches to Animal Testing

The European Partnership for Alternative Approaches to Animal Testing (EPAA) is an unprecedented voluntary collaboration between the European Commission, European trade associations, and companies from seven industry sectors. The partners are committed to pooling knowledge and resources to accelerate the development, validation and acceptance of alternative approaches to further the replacement, reduction and refinement (3Rs) of animal use in regulatory testing.

Three platforms organise the work on different projects and ensure their consistency.

About some ongoing activities:

1. The Science Platform projects ***Read more >***

- New perspectives on safety - computational chemistry and systems biology: An expert workshop was organised with the objective of developing a strategic plan to exploit recent advances in computational chemistry in alignment with state-of-the-art expertise in systems and synthetic biology and in biological modelling. The workshop took place on 5-7 July, Brussels. Read more >

2. The 3Rs in Regulation Platform projects ***Read more >***

- 3Rs in acute toxicity testing: A cross-sector review of the drivers for acute systemic toxicity testing and of the alternative approaches was carried out by an EPAA Task Force in collaboration with the HSI and the NC3Rs. The findings will be discussed at a workshop on 16 September 2010, in Brussels. Read more >
- Consistency approach in vaccines testing: A joint ECVAM/EPAA workshop discussed the applicability of the consistency approach for routine release of human and veterinary vaccines and how this could reduce animal use. The workshop was held on 11-12 January 2010, in Brussels. Read more >
- Extended one-generation reproductive toxicity study: Exploring the use of an extended one-generation study to replace the two-generation study required by REACH. Four companies are checking the feasibility of this approach, which has a

Highlights

NEW!
EPAA 8th Annual Conference - 2012: Register now
Programme

NEW!
DTaP vaccines workshop - Flash report

NEW!
EPAA Vaccines 3Rs Public Initiatives Survey
More information here

EPAA Newsletter
Newsletter May 2012
Previous newsletters here

Events

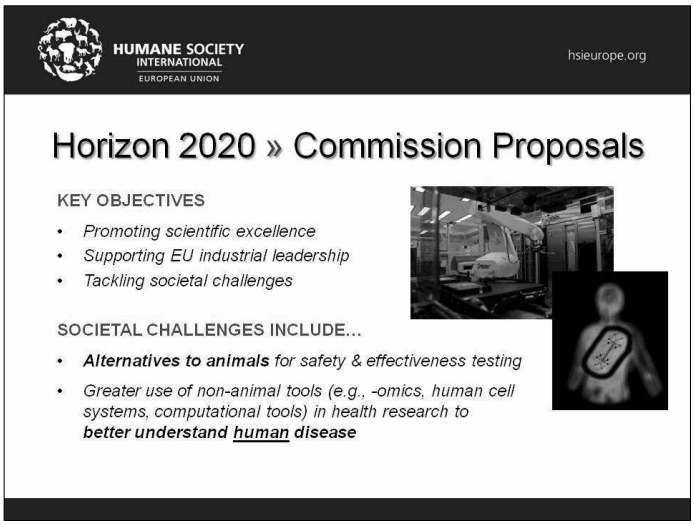
EPAA 8th Annual Conference - 2012
Brussels, Centre Borschette - 16 November 2012

The European Partnership for Alternative Approaches to Animal Testing

15:30 - 15:50

Horizon 2020: Advancing safety science and health research with modern, non-animal tools

Troy Seidle, Humane Society International London, UK



HUMANE SOCIETY INTERNATIONAL
EUROPEAN UNION

hsieurope.org

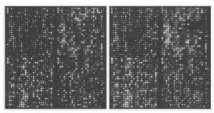
Horizon 2020 » Commission Proposals

INDUSTRIAL LEADERSHIP INCLUDES...

- **Key enabling technologies**
  - *Bioinformatics & systems biology*
  - *Genomics & molecular tools*
  - *Next-generation computing & robotics*

EXCELLENT SCIENCE INCLUDES...

- **Future & emerging technology "flagships"**
  - *Ambitious large-scale, science-driven*
  - *Alignment of EU & national agendas*
  - *Research infrastructures*



HUMANE SOCIETY INTERNATIONAL
EUROPEAN UNION

hsieurope.org

Horizon 2020 » Opportunities

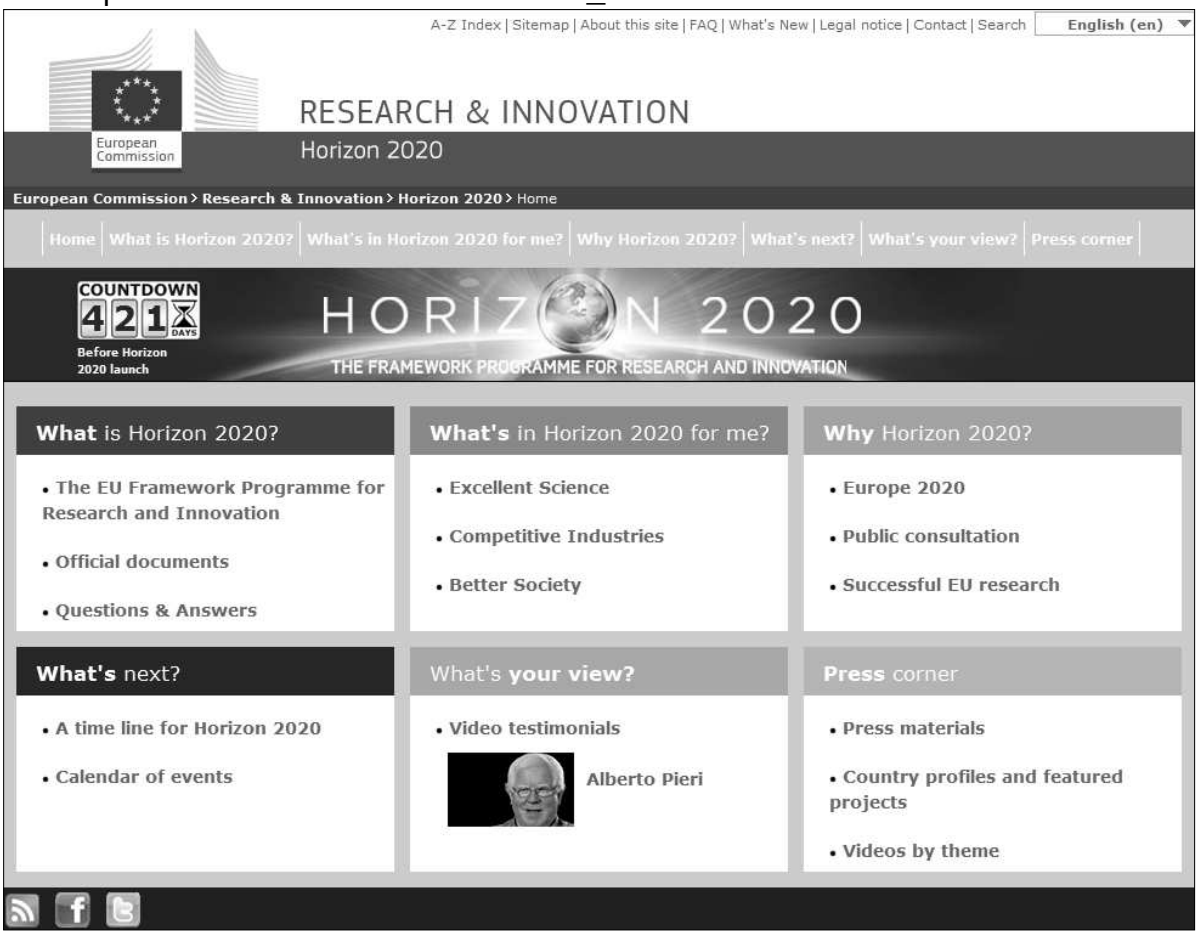
EUROPEAN "TOX21" FLAGSHIP RESEARCH INITIATIVE

- *Uncover how chemicals disrupt normal processes in the human body at the level of genes, proteins & cells ("**pathways of toxicity**")*
- *Develop human-relevant cell systems to test for disruptions*
- *Develop computer models to relate cell test results to real world*

KEY FEATURES

- *"Top-down" strategic focus*
- *Public-private partnership with industry*
- *International collaboration*

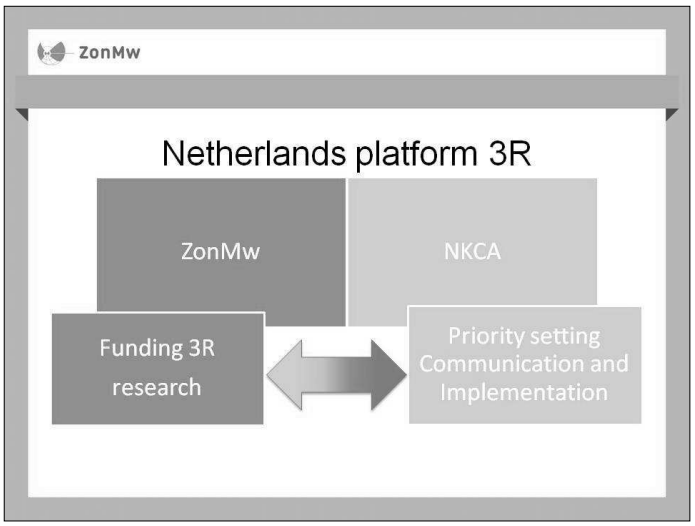
Website:
http://ec.europa.eu/research/horizon2020/index_en.cfm

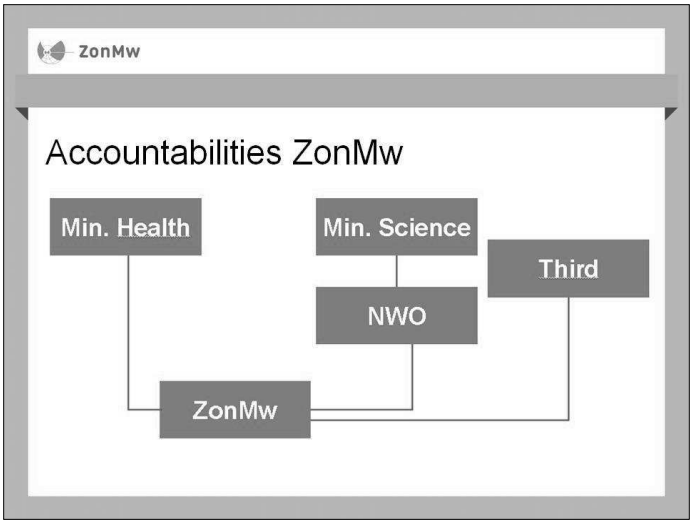


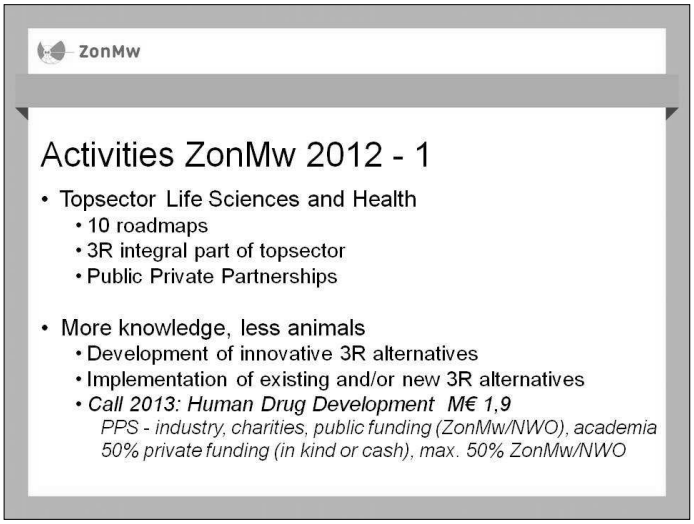
15:50 - 16:10

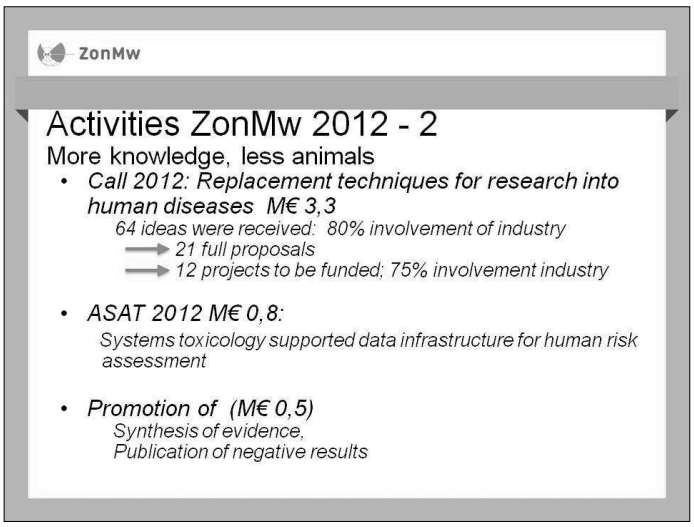
Interrelationships between the National Science Foundation and the 3R Platform in the Netherlands

Janna de Boer, Team Science and Innovation, The Netherlands Organisation for Health Research and Development (ZonMw), The Hague, The Netherlands








ZonMw
Activities ZonMw 2012 - 2
More knowledge, less animals
• Call 2012: Replacement techniques for research into human diseases M€ 3,3
64 ideas were received: 80% involvement of industry
21 full proposals
12 projects to be funded; 75% involvement industry
• ASAT 2012 M€ 0,8:
Systems toxicology supported data infrastructure for human risk assessment
• Promotion of (M€ 0,5)
Synthesis of evidence,
Publication of negative results

Tuesday 20.11 2012

Room Basic (2nd Floor) and Video in Room Pascal (3rd Floor)

Session V

	The 3Rs as a European consensus Chair: Lisbeth Knudsen and Thomas Hartung	
16:10 16:20	***ecopa***: Who, What, Where? Progress in Denmark	L. E. Knudsen University of Copenhagen, Denmark
16:20 16:30	Progress in Austria, *zet*	K. Sommer BioMed-zet Life Science, Linz, Austria
16:40 16:50	Progress in Finland, *Fincopa*	T. Heinonen FICAM, University of Tampere, Finland
16:30 16:40	Progress in France, FRANCOPA	Ph. Hubert INERIS, Verneuil-en-Halatte, France
16:50 17:00	Progress in Germany, Stiftung *set*	Ch. Buda Stiftung-set, Frankfurt, Germany
17:00 17:10	Progress in the Netherlands, NKCA	S. Deleu RIVM, The Netherlands
17:10 17:20	Progress Norway, *Norecopa*	A. Smith Norecopa, Oslo, Norway
17:20 17:30	Progress in Switzerland, SF3R	P. Maier University of Zürich, Switzerland
17:45 18:15	ecopa General Assembly 2012	L. E. Knudsen University of Copenhagen, Denmark

16:10- 16:20

Who, What, Where?

Lisbeth E. Knudsen, Faculty of Health Sciences, Department of Public Health, University of Copenhagen, Denmark

ecopa board from 2011

President: Lisbeth E. Knudsen, University of Copenhagen, Denmark , ACAD
Vice-President: Tuula Heinonen, FICAM, University of Tampere, Finland, ACAD
Treasurer: Philippe Vanparys , Altoxicon, Belgium, IND
NCP Delegates: Erwin Roggen, Novozymes, Denmark, IND
Marianne Norring, Finland, AW
Manfred Liebsch Federal Institute for Risk Assessment (BfR), Germany, GOV
Sophie Deleu, RIVM, The Netherlands, GOV
3R Experts Troy Seidle, Humane Society International London, U.K. AW
Thomas Hartung, CAAT Europe, Germany ACAD
Marianne Kuil , Dierenbeschwerming, The Netherlands, AW
Associated : Philippe Hubert INERIS, Verneuil-en-Halatte, France
Secretary : Mardas Daneshian CAAT Europe, Germany

National platforms continued in focus with emphasis on implementation of directive

Dissemination of information by website, newletters and altweb

The replacement R to be developed towards animal free tools in research and development

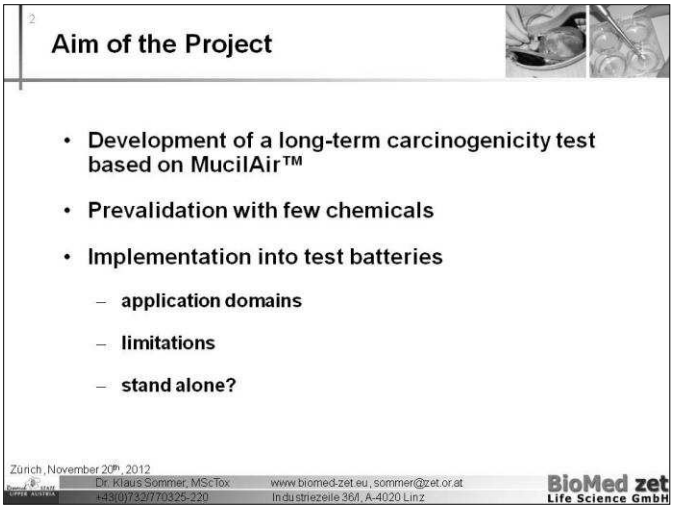
Coming meetings: 3R Switzerland 2012, Germany 2013 SET, World Congress in Prague evt Norway 2014

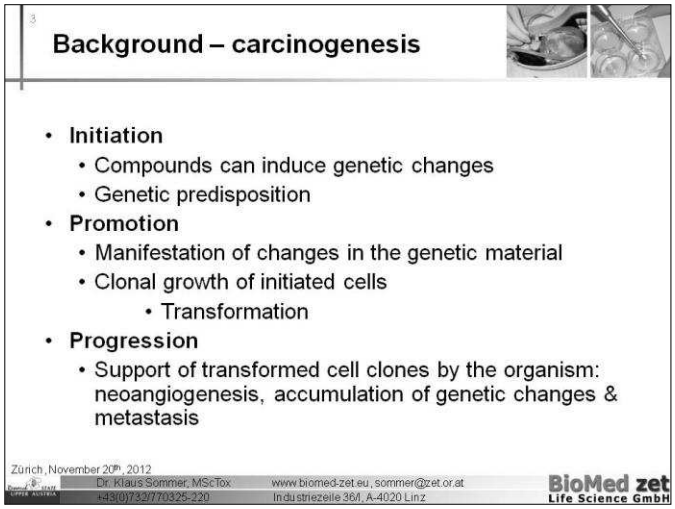
http://www.ecopa.eu/

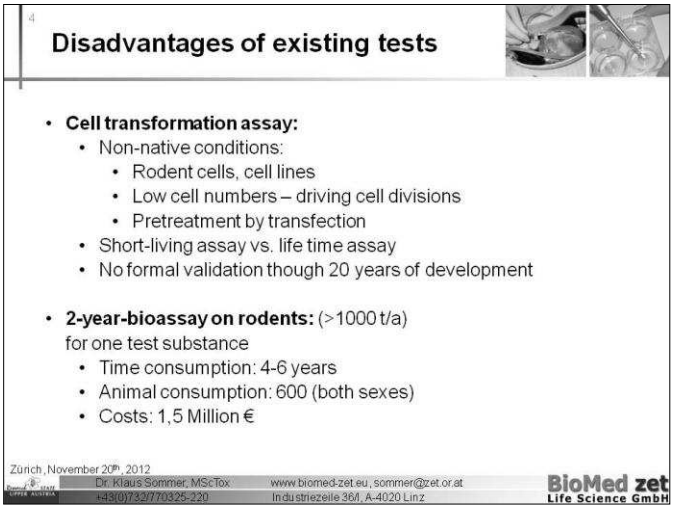
16:20-16:30

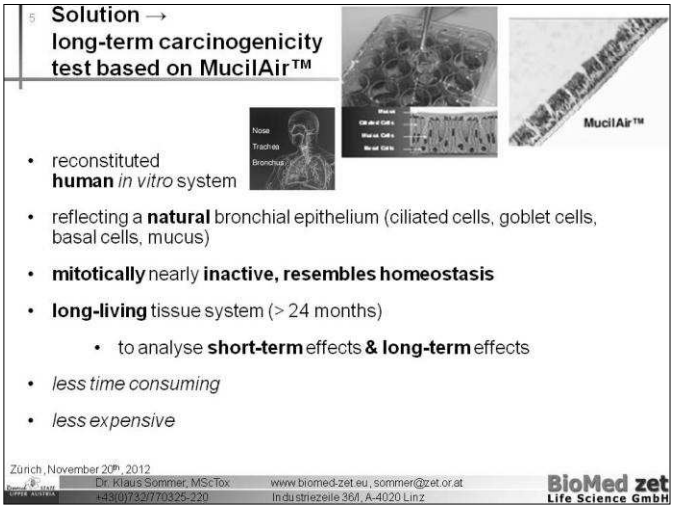
Austria: zet (Zentrum für Ersatz- und Ergänzungsmethoden zu Tierversuchen)

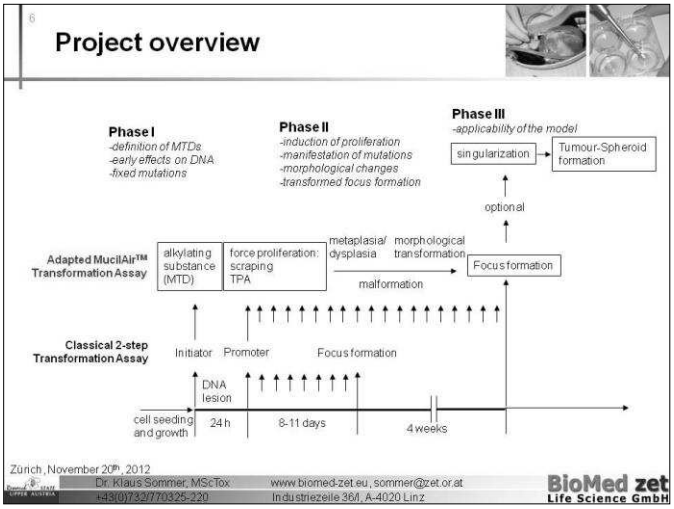
Klaus Sommer, BioMed-zet Life Science GmbH, Linz, Austria











Scientific Background:

- Gastroenterology
- Cancer Research
- Toxicology *in vitro*
- Metabolism
- Validation
- Teaching

Scientists:
Klaus Schröder CEO
Klaus Sommer
Christine Schäfer
Mohammad Reza
Lornejad Schäfer

Techniques:
Cell Culture Techniques
- Primary cells and cell lines
Reconstructed Organ Culture
- Hepatic, Epithelia, Mesothelia
Primary Organ Tissues
- Set up phase
Cell and Molecular Biology

16:10-16:20

Denmark: DACOPA (Danish Consensus Platform for Alternatives to Animal Experiments)

Lisbeth E. Knudsen, Faculty of Health Sciences, Department of Public Health, University of Copenhagen, Denmark

DACOPA

- Established by the Ministry of Justice June 2006 now under the Ministry of Food Safety
- The Chairman represents the Ministry
- Two members represent Industry
- Two members represent Research
- Two members represent Animal Protection Organisations
- Two members represent Authorities

DACOPA

DACOPA

- The Chairman: Dr Jann Hau
- The Industry: Dr JL Ottesen and Dr C Bülow
- The Research: Dr L Munksgaard and Dr LE Knudsen
- The Animal Protection Organisations: Dr. S Kent Pedersen and Mr. M. A. Larsen
- The Authorities: Dr C Nellemann and Dr E-M Vestergaard

DACOPA

DACOPA's remit is to

1. Serve as a forum to promote collaboration and discussion among the interested parties in laboratory animal experimentation
2. Promote knowledge about the 3R-concept
3. Support and motivate 3R implementation
4. Represent Denmark in ECOPA
5. Assist the Danish ESAC representative

DACOPA

DACOPA has decided to aim for

- Equal weight and focus on Refinement and Reduction as on Replacement
- The provision of an objective view on the societal effect of political and scientific 3R initiatives
- Collaboration with ECOPA and other relevant organisations/societies/platforms

DACOPA

Current activities

Implementation of directive in DK

Strategy for promotion of research, development and validation of alternative methods and dissemmination activities

Survey of ongoing activities

3R center under discussion to be established

16:30-16:40

Finland: Fincopa (Finnish Consensus Platform on 3R-Alternatives)

Tuula Heinonen, The Finnish Centre for Alternative Methods (FICAM), University of Tampere, Finland

Fincopa

- Established 2003
- The first president was prof.emerita Hanna Tähti
- Non-profit organisation
- Promote development of 3R-alternatives
  - Research
  - Policy
  - Education
  - Ethics

Nov 20 2012 ecopa Zurich

Board for 2012

Member	*Deputy member*	*Period*
Hallinto		
• Eila Kaliste	Pirkko Puranen	2012 – 2013
• Hannele Huuskonen	---	2012 – 2013
Research		
• Marika Mannerström	Kirsi Vähäkangas	2011 – 2012
• Heidi Diallo	Hanna Tähti	2011 – 2012
Industry		
• Tuula Heinonen (chair)	Paula Vesa	2012 – 2013
• Päivi Alajuuma (secr)	Markus Soimasuo	2011 – 2012
Animal welfare		
• Marianna Norring (treas)	Laura Uotila	2011 – 2012
• Paula Hirsjärvi	Helinä Ylisirniö	2012 – 2013

-Board meets 4 times per year
-Annual fee for members 25 eur, students 10 eur and for organisations 100 eur

Nov 20 2012 ecopa Zurich

Fincopa activities

- Disseminate 3-R information
  - Fincopa website: http://www.uta.fi/jarjestot/fincopa/
  - Organization of yearly workshops
- Has acted as an co-organiser of ecopa workshops (Refinement)
- Commenting actively directive 2010/63/Eu on EU-level and on national level

Nov 20 2012 ecopa Zurich

The 28th Workshop of SSCT and the FINCOPA Seminar
"Towards toxicity assessment without animals"
www.ficam.fi
Time: Wednesday 21st – Friday 23rd September 2011
Place: Tampere, Scandic Rosendahl, Tampere, Finland

For 2013

- Annual symposium workshop on Refinement
- Focus on laboratory animal personnel

16:40-16:50

France: FRANCOPA

Philippe Huber, **I**nstitut **N**ational de l'**E**nvi**R**onnement **I**ndustriel et des Risque**S** (INERIS), Verneuil-en-Halatte, France

FRANCOPA Plate-forme nationale pour le développement des méthodes alternatives en expérimentation animale

A contract association of 14 institutional members represented in a board.
Academics : 5; Authorities ; 4 Industry ; 3
Animal welfare ; 2
Some members are not easily classified :
Areas : chemicals, pharmaceuticals, cosmetics, biocides …medical research
An expert commission of 30 individual members
November 19th & 20th, 2012.

2011/ 2012 activities

- Sept 2011 workshop on « waiving ».
- End of 2011 Update of the « state of the art » report.
- Follow up of EU directive transposition, and involment of many members. .
- 2012 Expert Commision work on « read across and analogical inferences » and workshop preparation.
  - Goal : strenghtening bases for read-across.
  - 2012. Acquaintance with the concepts : skin and lung irritation case studies.
  - Preparation of an international workshop in 2013.

FRANCOPA

Infocentre : FRANCOPA info-center

- Opening mid 2012 : interactive website (www.francopa.fr)
  - Frequently asked questions
  - Newsletter
- Not limited to the research community, aiming to point out resources for users
- Early 2013 : launch of a FORUM : Which kind of validation ? Present limits of the animal model ? Good practice in 3R compliance ?

FRANCOPA

An Ethical challenge for FRANCOPA

- Increasing pressure on separation between indutrial and public expertise challenges the « platform » concept.
- A change in the association, an authority (pharmaceutical and health products) withdrawned from presidency., but stayed in the association.
- A question to ECOPA : What about other platforms ?

FRANCOPA

Members action : Kästler foundation award.

- An award from LFDA on alternative methods
- Late 2011 : To Dr V. Dangles-Marie for development –and qualification- of 3D cancer colorectal human cancer cells (colospheres) applied to « preclinical efficiency assessment » .
- A drastic reduction in nude mice, a faster assessment.
- *A proof that alternative methods are a key issue beyond regulations and toxicology.*

FRANCOPA

Members action ; OPAL d'OR award

- OPAL (Experimental reseach and Laboratory animal Protection) award dedicated to journalists;
- April 2012 : Award to Pierre-François Gaudry for a TV-movie (France 5) « Bye BYE cobaye »

FRANCOPA

16:50-17:00

Germany: set (Stiftung zur Förderung der Erforschung von Ersatz- und Ergänzungsmethoden zur Einschränkung von Tierversuchen)

Christiane Buda, Frankfurt am Main, Germany

.

Numbers

- Founded in 1986
- Initiative of German Federal Ministry of Food, Agriculture and Forestery
- Foundation capital 500.000 €
- Plus annual contributions from industry and ministry
- Budget: up to 320.000 € per year
- ~ 50 research projects in 26 years

www.stiftung-set.de

Structure

- Stiftungsrat (Foundation Board)
  - 4 members from animal welfare associations
  - 4 members from industry associations (chemical, pharmaceutical, cosmetics, agriculture)
- Wissenschaftlicher Beirat (Scientific Board)
  - 6 scientists from industry, university and public authorities
- Kuratorium (Board of Trustees)
  - Up to 16 members from ministries, scientific organisations, public authorities, animal welfare, churches…
- Geschäftsführung (Secretary)

www.stiftung-set.de

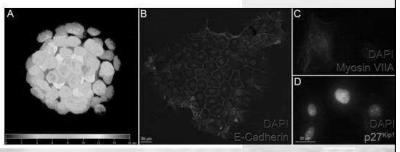
Member Organisations

- Animal Welfare Organisations
  - Deutscher Tierschutzbund (German Animal Welfare Association)
  - Bundesverband Tierschutz (Federal Association for Animal Welfare)
- Industrial Organisations
  - Verband der Chemischen Industrie (Chemical Industry)
  - Verband der forschenden Arzneimittelunternehme (Pharmaceutical Industry)
  - Industrieverband Agrar (Agricultural Industry)
  - Industrieverband Körperpflege und Waschmittel (Industry for Cosmetics and Detergents)

www.stiftung-set.de

Projects in 2012

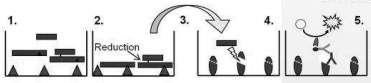
- Carcinogenicity Screening of Chemicals with High Throughput (Steinberg, Hannover)
- Otic Stem Cell Based Model of the Inner Ear (Löwenheim, Tübingen)
- Ex vivo Dry Eye Model (Schrage, Aachen)



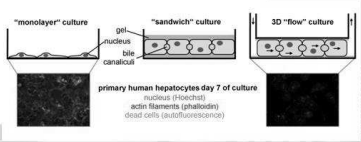
www.stiftung-set.de

Projects in 2012

- In vitro Method for the Determination of Tetanus Toxicity in Tetanus Vaccines (Weisser, Langen)



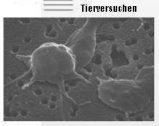
- 3D flow through model for long-term culture of polarized hepatocyte-like cells (Ehnert, Tübingen)



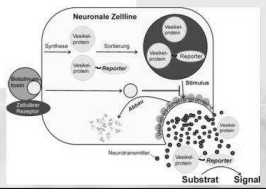
www.stiftung-set.de

Projects in 2012

- Human thrombocyte concentrates as substitutes for animal-derived serum in stem cell cultures (Gstraunthaler, Innsbruck)



- Analysis of EU-legislation regarding the implementation of the 3Rs in the data requirements (Kolar, Neubiberg)
- In vitro Method for the Determination of Botulinum Toxin Activity (Püschel, Potsdam)



www.stiftung-set.de

17:00-17:10

The Netherlands: NKCA (Nationaal Kenniscentrum Alternatieven)

Sophie Deleu, RIVM, The Netherlands

NKCA

- 3R activities in general:
  - knowledge management and education of professionals (lectures, expert meetings, workshops, website, social media etc.)
  - advice on 3R-policy and research programming (ZonMw)
  - Dutch 3R-platform: dialogue with scientists, animal welfare organizations, industry and government
  - communication with professionals and the public about animal use and promotion of the use of 3R-alternatives
- Organization: coöperation of the University of Utrecht (faculty of veterinary medicine) and the National Institute for Public Health and the environment (RIVM).
- Staff: about 5 full time equivalents
- Budget: € 700.000,-

4

3R activities 2012

- Workshops on barriers and drivers in regulatory acceptance chemicals and farmaceuticals

  Publication in Altex by Marie-Jeanne Schiffelers
  Regulatory acceptance and use of 3R models: a multilevel perspective

- Communication
  Improvement of the (Dutch) website "yellow pages" for 3R-professionals
  Experiments with social media like facebook, twitter and LinkedIn

5

3R activities in 2012

- Implementation of the new EU guideline
  - (permanent) education
  - registration and statistics
  - communication

- Inventarisation and evaluation of databases on animal use, animal experiments, the use of 3R-methods and the results of these
  - Prevention doubling of animal experiments
  - Stimulation of the use of 3R-methods
  - Improvement of experimental design (better science)
  - Registration and sharing of negative results
  - Lower administrative costs by using modern information management tools

6

3R activities in 2012

- Monitoring and evaluation of 3R-impact on animal use
  - signaling trends in the use of animals for policy makers and the national advisory committee on animal use
  - structural monitoring and evaluation, quantitatively and qualitatively

  Goals:
  - stimulate the use of 3R-methods,
  - better science,
  - openness and social accountability,
  - measuring the impact of 3R-policy,
  - changing professional attitude

7

Monitoring activities in 2012, 2013 and…

1. finding indicators for specific 3R-topics
   - mature impact
   - interim impact
   - signal
2. selection of the most relevant indicators by experts
3. pilotprojects to test (sets of) indicators
4. Implementation

8

Activities NKCA / Dutch platform 2013

- Communication with professionals and the public about animal use and 3R alternatives
- Information management
  Databases (registration, accountability and sharing data)
- Identifying and establishing indicators for monitoring and evaluation of 3R impact on the use of animals
- Support the implementation of the new EU guideline when de Dutch legislation is completed
  (delivering 3R-expertise, advice on registration and datamanagement, education, management of qualifications and competences of personnel, establishment of structures like new committees and authorities)
- Advice on 3R policy and research programming (ZonMw)
- Identification and sharing of international 3R-priorities

9

17:10-17:20

Norway: **Norecopa** (Norwegian Consensus Platform for Replacement, Reduction and Refinement of Animal Experiments)

Adrian Smith, Norwegian Veterinary Institute, Oslo, Norway

Consensus meetings where all 4 stakeholders meet:

Harmonisation of the Care and Use of:

- *Fish* *(May 2005)*
- *Wildlife* *(May 2008)*
- *Fish* *(September 2009)*
- *Farm animals* *(September 2012)*

These meetings aim to provide lasting resources and stimulate research:

- All presentations are available afterwards on the internet
- A consensus document is written by the participants, describing challenges and research needs
- Norecopa follows up the conclusions in the consensus document

Norecopa's 3R prize

(approx. 4,000 euros + diploma)

Awarded annually since 2010

- Replacement of mouse bioassay for shellfish toxins
- Replacement of live fish in toxicity testing
- Replacement of live fish in fish vaccine development

The potential to increase use of the 3Rs in the development and validation of fish vaccines

Photo: Marius Eidsvik

Kristian Ellingsen
Roar Gudding

15 recommendations, including greater use of *in vitro* methods and better statistical design of *in vivo* experiments

3R-CHART

An analysis of fish research facilities in Norway:

- *To what extent are the 3Rs already in use?*
- *How can we increase implementation?*

Questionnaire, followed by on-site interviews at most of the facilities.

A report, to be followed up by applications for research.

Sponsored in part by the Ministry of Fisheries and the Norwegian Animal Protection Alliance's fund.

Other projects

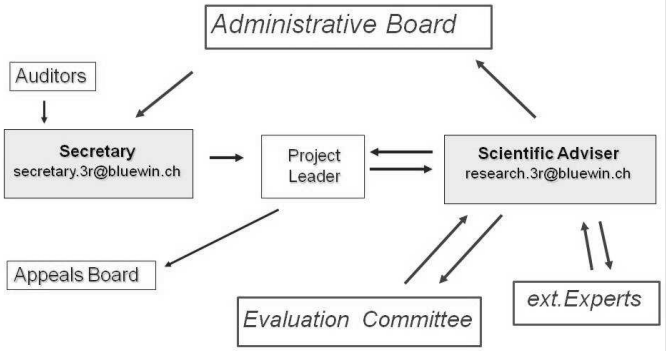
- Website with Guidelines and other 3R-resources
- Severity categories for experimental procedures on fish (published as Open Access in *Laboratory Animals*)
- Lectures about Norecopa and alternatives on courses in Laboratory Animal Science for researchers
- English translation and update of a compendium in Laboratory Animal Science for fish researchers
- Lobbying politicians and ministries
- Chronicles in national newspapers
- Member of the Steering Committee of the World Congress on Animal Use in the Life Sciences and Alternatives in Montreal 2011

17:20-17:30

Switzerland: SF3R (Stiftung Forschung 3R)

Peter Maier, 3R Research Foundation Switzerland and University of Zürich. Switzerland

Stiftung Forschung 3R
3R Research Foundation



http://www.forschung3r.ch

Instructions Concerning Project Outlines

Instructions for submitting Applications for Research Funding

Guidelines for Awarding Research Grants

Criteria for evaluating applications and projects

Principal areas for financial support of research projects

Funding for research is not provided in the following areas: .....

Area of Research :

The following types of projects are of particular interest to the 3R Research Foundation:

1. In which results from animal experiments are to be replicated using an alternative method
   (conclusion about the relevance of the in vitro testing or the validity of the in vivo procedure)
2. with 3R effects in connection with animal models for psychiatric illnesses, degenerative brain disease, diseases involving inflammation, cancer, infectious diseases, diseases of the respiratory tract, metabolic abnormalities and cardiovascular disease, among others;
   (animal experiments with high severity degree)
3. alternative methods for acute and chronic toxicity testing;
4. alternative methods for developing vaccines and batch testing as well as quality control.

2012: Four new projects funded

Cardiovascular simulator with autoregulation
Vandenberghe S,, Prof., ARTORG Center for Biomedical Research, University of Bern, Switzerland

Development of an in vitro system to grow and investigate vascular endothelial cells under physiological flow conditions
Rieben R., Prof., Department of Clinical Research, University of Bern, Switzerland

Identification of predictive in vitro markers of hematopoietic stem cell function
Lutolf M. P., Prof., Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

Antibody phage selection strategy for application in non-specialized laboratories
Heinis Ch., Prof., Ecole Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland

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Bacterial Meningitis: Investigating Injury and Regenerative Therapy *in vitro*

Bulletin 48 February 2012
A novel *ex vivo* mouse aorta perfusion model

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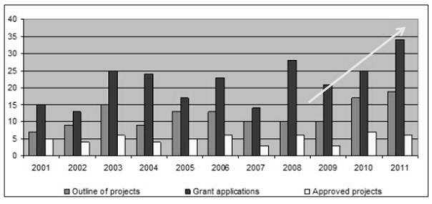

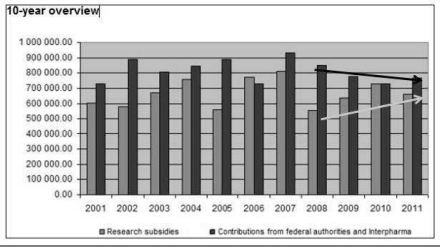

3R Posters 2012

Present status of ongoing projects funded by the 3R Research Foundation Switzerland

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3R-Project 113-08

Generic in-vitro evaluation assay for immunological correlates of protection to replace animal challenge infections

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Background and Aim

Vaccination against foot-and-mouth disease (FMD) represents an essential element in controlling and combating outbreaks, which would otherwise have disastrous consequences. This is pertinent to regions in large parts of the developing world in which the FMD virus (FMDV) is endemic, as well as during an epidemic in FMDV free areas such as Europe. Nevertheless, successful vaccination against FMDV requires selection of the appropriately matching vaccine strain providing protection against a particular circulating field virus. This problem originates from the existence of seven known serotypes of FMDV, with which high antigenic variation of virus is observed. In addition, subtypical antigenic variation within a serotype is under constant evolutionary change due to the high mutation rate of FMDV. For these reasons, continuous vaccine testing and modification in the light of recent antigenic changes to the virus is required. Currently, vaccines are tested and selected using vaccination-challenge experiments in cattle. Such procedures are not only extremely expensive, but are also environmentally and ethically problematic, considering the severe animal suffering associated with disease development, and the requirement that all animals be slaughtered at the end of the experimentation.

Accordingly, the proposed solution is to develop realisable and robust *in-vitro* alternatives to such challenge infection tests. To this end, the present project integrates with the EU project FMD-DISCONVAC, from which the necessary samples will be obtained. Through this integration with the FMD-DISCONVAC project, we will be fulfilling the objectives of the EU call KBBE-2008-1-3-02: To substitute vaccine potency tests in animals by assays enabling a correlation of in-vitro tests based on immunological principles of antibody-based effector immune responses operating in vivo.

Although vaccine-induced protection can be predicted when high levels of virus neutralising antibodies are induced, this does not apply for vaccinates with relatively low levels of antibodies. So far, no alternative tests applicable to such sera have been developed and systematically applied to vaccine testing. Thus, the approach selected will go beyond virus neutralization tests, and also enable the functional analysis of non-neutralizing antibodies with respect to FMDV destruction and the induction of antiviral activity. Developed tests will have the potential of serving as improved correlates of protection against homologous or closely related FMDV vaccine strains. In addition, the tests have the potential to identify correlates of heterologous protection induced by vaccination.

Method and Results

in progress (present status)

Based on immunological evidence obtained from previous work, we are developing *in-vitro* screening assays based on the relationship between protection and the avidity of specific antibody for binding to the pathogen in question. We will evaluate the relationship between protection and the capacity of a serum to promote Fc receptor-mediated phagocytosis of FMDV immune complexes by macrophages and dendritic cells in terms of virus destructionn, and Fc receptor-mediated IFN-alpha and cytokine responses in plasmacytoid dendritic cells (see Figure 1).

In various cellular system, to test the influence of immune complexes obtained from vaccinated cattle and pigs were established. These include an assay measuring enhanced infection of bovine monocyte-derived dendritic cells by immune complexed-FMDV and for the porcine system enhanced IFN-alpha responses by plasmacytoid DC.

Our results demonstrate a high sensitivity of both assays and show that opsonising antibodies possess a broad cross-reactivity against various FMDV isolates within one serotype and in some cases even across serotypes.

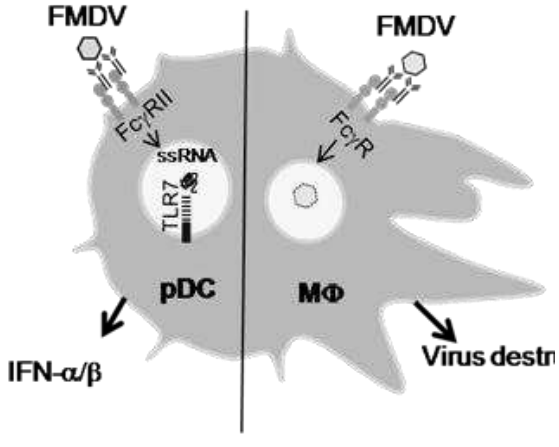

Figure 1: Immunological background: FcR-mediated anti-FMDV responses induced in plasmacytoid dendritic cells (pDC) and macrophages (MF) as examples of antibody-mediated effector functions independent of direct virus neutralization.

A main conclusion from was that opsonizing activity was clearly much broader than neutralizing activity suggesting that non-neutralizing IgG could also complex with FMDV to infect bovine MoDC or activate pDC.
To systematically investigate the relationship of opsonising antibodies with protection using a large panel of sera, we also have established a reporter system to measure opsonizing antibodies against FMDV. This is based on bovine FcRII (CD32) expressing murine RAW 264.7 macrophage cells. The principle of this test is that the RAW 264.7 cells are resistant to FMDV infection in the absence of antibodies but get infected and die when the virus is complexed with antibodies. Our results demonstrate that this test is highly sensitive, similar to the test using bovine MoDC, and has potential as an additional measurement to determine immunological correlates of protections.

Conclusions and Relevance for 3R
The experimental systems established now permits the quantification of opsonizing antibodies at a very high sensitivity. We are even able to detect reactivity across serotypes which was unexpected considering that there is no cross-protection between serotypes. We are now testing a larger collection of sera to determine the relationship of opsonizing antibodies to protection and to conclude on the applicability of this test to measure vaccine quality without challenge infection.

3R-Project 116-09

Organotypic brain slice cultures derived from regularly slaughtered animals as *in vitro* alternative for the investigation of neuroinfectious diseases in ruminants

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Background and Aim
Infectious disorders of the central nervous system in livestock may have severe economic and public health implications and are therefore of major concern. This was demonstrated dramatically in the mid 1990s, when it became evident during the upsurge of bovine spongiform encephalopathy (BSE) that the disease was transmissible from cattle to humans. Recently, atypical variants of transmissible spongiform encephalopathies (TSEs) have been detected in ruminants, whose potential to cross over to other species including humans is not known at present. Listeriosis, caused by *Listeria monocytogenes* (LM), is another infectious and zoonotic CNS disease of high impact on livestock and humans.
Despite intense research activities in the field of TSEs and listeriosis during the past decades, very few *in-vitro* models for their neuropathogenesis, host-pathogen interactions and strain-typing exist. Studies largely depend on bioassays either in laboratory rodents or in the natural ruminant host because they reflect best the intricate pathogenesis of CNS infections. Such experiments raise fundamental ethical concerns, considering the highly invasive inoculation routes and the resulting severe disease, because of which most of these experiments are classified into the highest severity degree (Schweregrad 3). In the case of TSEs, experiments may last up to several years until the animals are sacrificed at the end. In addition, is often not clear to what extent the results of rodent models can be extrapolated to the natural hosts' situation. On the other hand, pertinent cell models of ruminant neuroinfectious diseases do not exist. Our aim is, therefore, to develop an ethically sustainable, host specific, organotypic brain slice culture of regularly slaughtered ruminants as an *in-vitro* system for the identification and investigation of neuroinfectious diseases using the examples of prion diseases and listeric encephalitis.

Method and Results
in progress (present status)
The validation of bovine organotypic brain slice cultures derived from the slaugtherhouse has been accomplished.

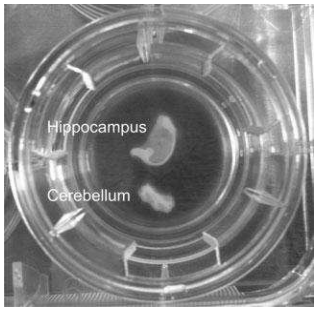

Figure 1: Hippocampal and cerebellar brain slice culture from a bovine. Anatomical architecture is maintained and at the edges cells grow out.
We assessed viability of brain slices by determining the difference between the number of dead cells and the total number of cells present in a slice (Figure 2).

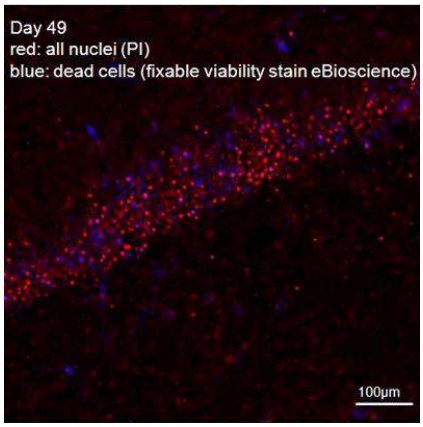

Figure 2:
Hippocampal slice, combined fixable-viability (blue, nuclei of

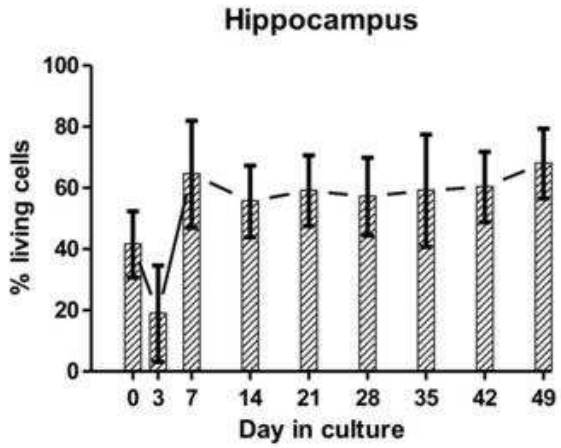

Figure 3:
The average proportion of viable cells as estimated from dead cell and total cell counts are indicated at different time points for hippocampal and cerebellar tissue-slice cultures from six different calves (error bars represent standard deviations).
and that all endogenous brain cell populations are present. Viability results of hippocampal slices (Figure 4) are consistently better than those of cerebellar slices.

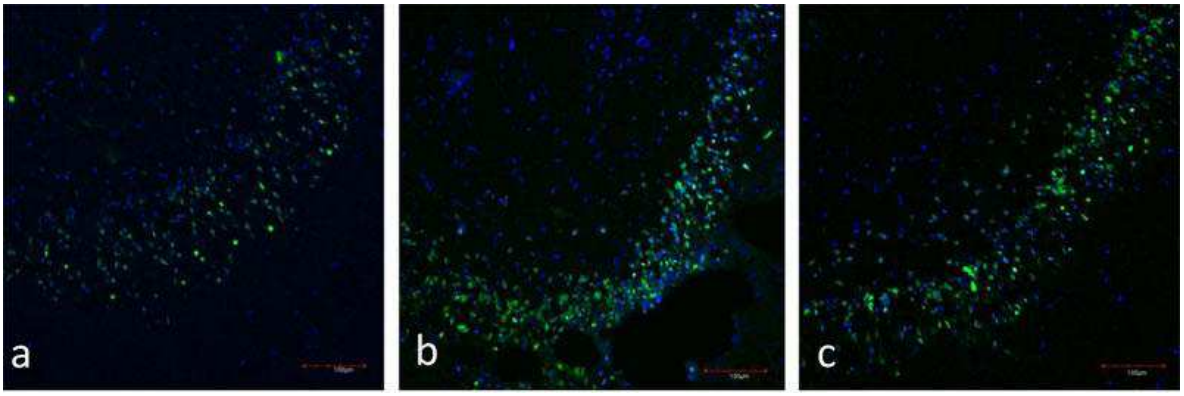

Figure 4:

a) Immunofluorescence of the hippocampal dentate gyrus (day 7 in vitro). Neurons are stained in green (NeuN), nuclei are stained in blue (TOTO-3).
b) Immunofluorescence of the hippocampal dentate gyrus (day 28 in vitro). Neurons are stained in green (NeuN), nuclei are stained in blue (TOTO-3).
c) Immunofluorescence of the hippocampal dentate gyrus (day 49 in vitro). Neurons are stained in green (NeuN), nuclei are stained in blue (TOTO-3).
Because viability of brainstem and cortical slices was low, experiments with these brain regions were discontinued. Future experiments will be performed with hippocampal slices. This in vitro system is susceptible to Listeria (L.) monocytogenes infection and replicates features of natural rhombencephalitis in ruminants (Figure 5; Guldimann et al., submitted).

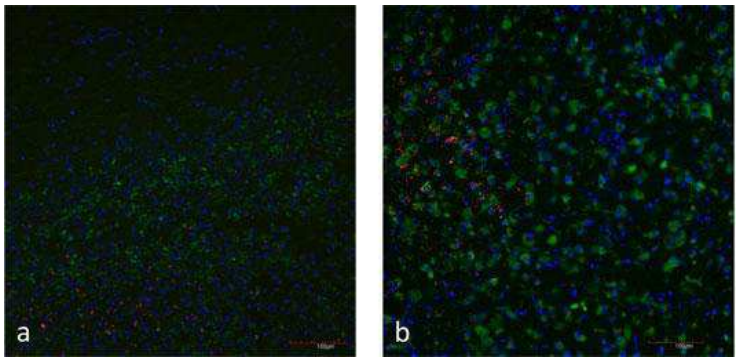

Figure 5:
a) Natural case of listeric rhomben-cephalitis, double-immunofluorescence for *L. monocytogenes* (red) and micro-glia cells (CD68 in green). Focal repl-ication of *L. monocytogenes*.

b) Brain slice inoculated with L. mono-cytogenes (red). Microglia cells are stained with CD68 (green). Focal re-plication of *L. monocytogenes*.

Conclusions and Relevance for 3R
We are confident that organotypic brain slices cultures derived from the slaughterhouse are an ethically sustainable and host specific in vitro system for the identification and investigation of neuroinfectious agents. In the future, ruminant brain-slices will offer the possibility to study agent-related determinants involved in neurovirulence and axonal spread of L. monocytogenes. Such cultures not only help to spare the life of animals, but also offer the combined advantages of reflecting the organospecific microarchitecture of the brain similar to the in vivo situation and at the same time of working under controlled conditions. The publication of a method paper on our *in vitro* model for ruminant CNS-infections in the International Journal of Experimental Pathology and oral presentations (at international congresses) might stimulate other scientists to consider our *in vitro* approach as an alternative to animal experiments for their own research. The suitability of the system for long culture times lends itself to the investigation of slow-growing agents like prions. Therefore, this system has the potential to significantly replace the use of animals in models of prion diseases and other neuroinfectious diseases with impact on veterinary and public health.

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3R-Project 117-09

Embryonic stem cell-derived in vitro model of tissue inflammation following confrontation with implant materials (INFLAPLANT)

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Background and Aim
Artificial implants are more and more common in clinical daily routine and are used in wide diversity. This includes, for instance, dental prosthetics, bone reconstruction materials, artificial joints, vascular prosthetics, and artificial heart valves.
In recent years biocompatible polymers and biodegradable polymers have been developed for a variety of clinical applications. They have enormous potential in the processing of artificial heart valves, artificial blood vessels, occluders etc. Biodegradable polymers support the healing process, i.e. they are able to degrade in a biological surrounding.
The immuno-compatibility of implant materials is currently tested in animal experiments by analysis of immuno-rejection. In these animal experiments typical signs of inflammation are analysed, e.g. diarrhea, loss of hair, hooked posture, lethargy, and increase of leukocyte numbers in the peripheral blood.
Alternatively conventional biocompatibility testing is performed *in vitro* with cell cultures of mostly one cell type (fibroblasts). The cells are plated on polymer materials and cell adhesion, proliferation, cyto-toxicity is assessed. The disadvantage of this conventional *in-vitro* testing is the absence of an inflammatory response induced by the interaction of tissue with the material which normally *occurs in vivo*.
In the current project we use embryonic stem cells to generate an immuno-competent vascularised tissue (INFLAPLANT-tissue) which displays cellular inflammatory response to specific materials, and therefore allows us to draw conclusions about the biocompatibility of the respective material. The aim of this project is to replace animal experiments which are presently performed to analyse the biocompatibility of newly developed implant materials.
Our INFLAPLANT-tissue allows the study of parameters associated with inflammation, e.g. neo-angiogenesis, the differentiation of inflammatory cells and their migration next to the polymer material, the secretion of inflammatory cytokines (TNF alpha, IL-1 beta, IL-6, CRP), the generation of reactive oxygen species as well as changes in intracellular pH and intracellular Ca^{2+}.

Method and Results
in progress (present status)
The interaction between tissues derived from embryonic stem cells (embryoid bodies) and polymer material particles is achieved in “hanging drops“ culture (Wartenberg et al., 2001). The polymer materials are developed and provided by Innovent Inc. (Jena). Following an adhesion time of 48 h, the adherent cells and the polymer particles are transferred to cell culture dishes. The following measurements are performed according to a detailed schedule.

1) Angiogenesis and inflammatory cell assay. Assessment of vascular areas and blood vessel sprouting. Determination of number and migration of leukocytes; fluorometric cytotoxicity testing: confocal laser scanning microscopy, FACS-analysis.
2) Determination of macrophage-specific mediators: ELISA-analysis.
3) Degradation of polymer materials in the co-culture with the INFLAPLANT tissue: spectroscopic determination of lactate.
4) Quantification of inflammatory markers in the supernatant: ELISA-analysis.
5) Microfluorometric determination of intracellular reactive oxygen species, pH and calcium using confocal laser scanning microscopy.
6) Type of polymers: we will use degradable polymers which are composed from two components: CLA 58 (L-lactid, caprolactone) and EP 37 (L-lactid, caprolacton methacrylate). A variation of material characteristics will be achieved by changing the mixing ratio or the sealing temperature (cooperation with INNOVENT e.V).

Conclusions and Relevance for 3R
The development of a 3-dimensional and multicellular *in-vitro* model for testing tissue inflammation and cellular retraction during exposition of the tissue to biodegradable polymers will reduce the number of animal experiments.

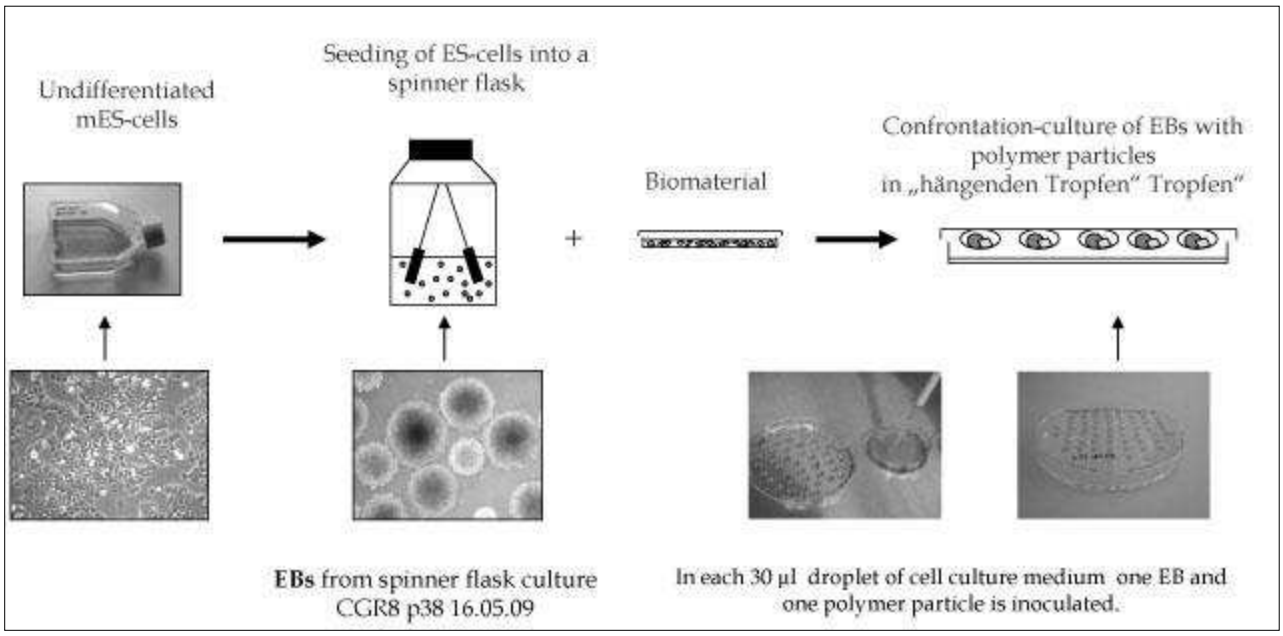

Figure 1:
Generation of INFLAPLANT Confrontation cultures derived from murine embryonic stem cells and polymer material particles. The embryonic stem cells were dissociated and seeded into a spinner flask culture. In this cell culture they build embryoid bodies (EBs). At day 10 the embryoid bodies were taken from the spinner flask and were inoculated in the "hanging drop" with a polymer particle of similar size. Following 24 h of adhesion the co-cultures were transferred to tissue culture dishes. They can stay there for 20 days, in this time the interaction of the INFLAPLANT tissue with the polymer will be studied.

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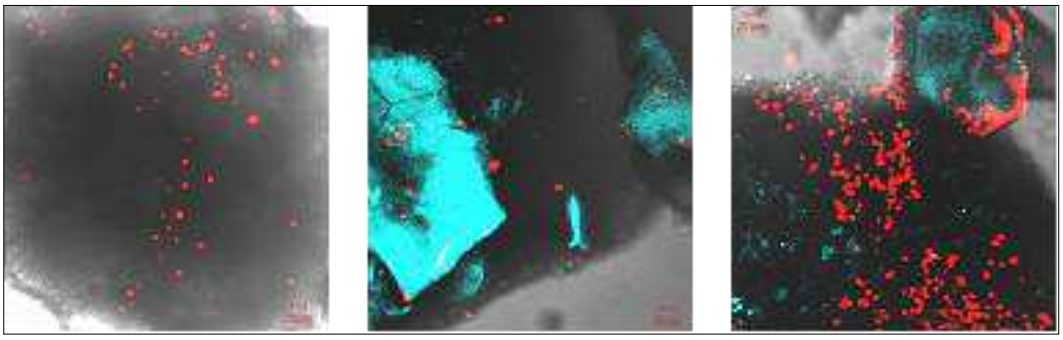
Figure 2:
Ethidiumhomodimer dead cell staining at day 16. *left*: staining of an embryoid body without co-culture, *middle*: the biocompatible polymer material (blue) is well embedded into the tissue, no increase of the number of dead cells (red) is observed. *right*: a badly compatible material (blue) induces a high number of dead cells (red).



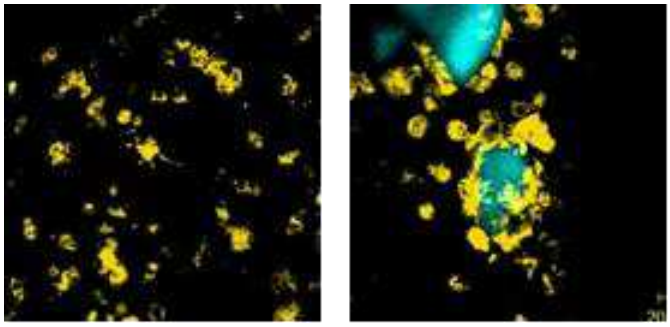


Figure 3:

CD68 expression (monocytes/macro-phages) in control samples (embryoid bodies) and in INFLAPLANT co-culture together with a polymer particle of CLA58 (blue) (provided by INNOVENT Inc.). Note that inflammatory cells are migrating towards the biomaterial.

3R-Project 118-10

Engineering of an in vitro hepatocyte tissue system for malaria liver infection research

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Background and Aim
Malaria continues to be an important global infectious disease for which mortality levels remain unacceptably high. The illness is caused by the Plasmodium parasite, most often *Plasmodium falciparum*, which is transmitted to humans in sporozoite form by infected Anopheles mosquitoes. Drug therapy represents an important intervention in controlling the disease. However the parasite has become increasingly resistant to treatment with the current line of available drugs. In certain regions tolerance is now exhibited even to artemisinin based therapy (Wongsrichanalai et al., 2008), which is widely considered to be the antimalarial of last defence. There is therefore an increasing urgency to develop new approaches and technologies that speed up the creation of effective new drugs and, ultimately, vaccines against the disease. Despite advances in our understanding of *P. falciparum* and its intra-erythrocytic development, significantly less is known about the parasite's development in hepatocytes. A major reason underlying this shortfall has been the unavailability of suitable *in vitro* models through which to study parasite-host hepatocyte development on a large-scale. Consequently, surrogate animal models based on related parasite species are widely employed for this purpose. Through joint collaboration between the CSIR Biosciences and Material Sciences and Manufacturing units,

work has been initiated to engineer a novel 3D polymer-based hepatocyte tissue culture system. This development is driven by a need to create new *in vitro* systems that correlate more closely with the *in vivo* liver state, providing a more reliable predictive model for research, in particular for investigating parasite-host interaction. Through the successful establishment of such a system, we envisage the possibility of perfomring detailed molecular investigations of *P. falciparum* hepatocyte development, leading to new opportunities in drug and vaccine discovery.

Method and Results

in progress (present status)

Through the use of conventional non-woven polymers and smart polymer technology, it has been possible to develop a proprietary three-dimensional scaffold system that is capable of supporting the growth of hepatocyte (and other anchorage dependent) cells in an enhanced manner. In particular, the system preserves proteins and glycans expressed on the extracellular matrix that are critical, together with other factors, to facilitating malaria sporozoite invasion and development. Primary hepatocyte cell lines (e.g. hNHEPS and HC04 (Sattabongkot et al., 2006)) will be employed to facilitate infection by *Plasmodium falciparum* sporozoites that have been isolated from Anopheles mosquito hosts. Conditions will be optimized throughout to achieve maximum rates of infection and development, culminating in the assessment of merozoite viability by exposing mature merozoites from the new system to conventional red blood cell cultures. Successful merozoite invasion of red blood cells, and subsequent intra-erythtrocytic development in these cells, will confirm the validity of the new *in vitro* model.

Conclusions and Relevance for 3R

This project aims to develop a 3D-based hepatocyte culture system that facilitates large-scale molecular analysis of the human form of malaria, *P. falciparum*, in this tissue. To date, this research has largely relied on the use of surrogate animal models, that utilize related species of this parasite. New opportunities in malaria drug and vaccine discovery research are anticipated from the new system.

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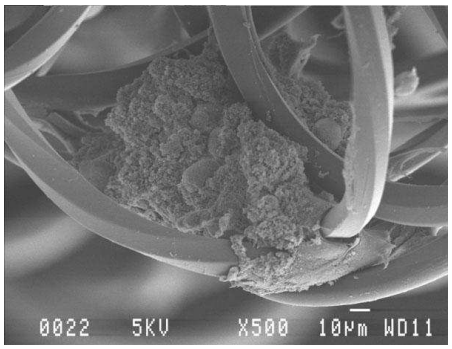

Figure 1: Electron micrograph of differentiated HepG2 hepatocytes grown on the 3D scaffold surrounded by extracellular matrix.

Figure 2: Fluorescence confocal image at 400X magnification illustrating differentiated HepG2 hepatocyte cell viability grown on the 3D scaffold following fluorescein diacetate staining.

Non invasive electrical monitoring of the population spiking activity in the central nervous system

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Background and Aim

Much of the knowledge about the dynamic functioning of the central nervous system (CNS) has come from invasive animal experiments in which parts of the nervous system are electrically monitored, stimulated or destroyed. For obvious ethical reasons, there is an increasing interest to replace the invasive approaches by less invasive ones.
While several non-invasive neuroimaging modalities have been developed in the last years, none of them is yet able to substitute invasive animal recordings aimed to monitor electrical activity at the single cell/population level. High spatial resolution techniques such as the fMRI lack temporal resolution and fail to provide a complete, clear account of spiking activity as measured in animals. The Electroencephalogram (EEG), likely to be the most direct correlate of neural activity that can be obtained non-invasively, lacks spatial resolution providing information limited to the scalp surface. The issue of the spatial resolution of the EEG can be circumvented by the use of physico-mathematical approaches capable of providing direct estimates of the neural intracerebral activity at the population level [1]. While this is yet far away from the information contained in the spiking activity recorded with neural implants, recent studies using simultaneous invasive recordings of spiking activity and scalp EEG suggest that there is a link between the phase of oscillations recorded at the macroscopic level and the spiking activity of cell populations. One goal of this project is to confirm this relationship in humans or in other structures different from the visual cortex in primates. This could serve to significantly enhance the spatial resolution that can be ultimately achieved with a method termed ELECTRA [2], which we have previously developed to estimate intracerebral activity from scalp EEG.

Method and Results

in progress (present status)

Recent technological advances are starting to allow simultaneous recordings of neural activity at different spatial scales in behaving animals. In one of this studies [3] published at the end of 2009 by Whittingstall and Logothetis, it has been shown that MUA in the primary visual cortex of monkeys can be predicted from the increases in the power of gamma band activity recorded at the scalp surface (i.e., EEG) occurring during the negative phase of the delta band oscillations. The fact that the phase of slow oscillations in the delta/theta band is a key element to understand spiking activity is indeed not new and has been locally observed in diverse structures in animals [4] and humans [5]. The main merit of the Whittingstall and Logothetis study is indeed to show that such links between the phases and power of the oscillations to spiking activity of populations still holds true for scalp EEG measurements. To combine this recent experimental evidence with the ELECTRA approach proposed in our group to estimate field potentials from scalp measurements (Figure 1), we need to verify that this relationship also holds for other brain structures in humans and primates.

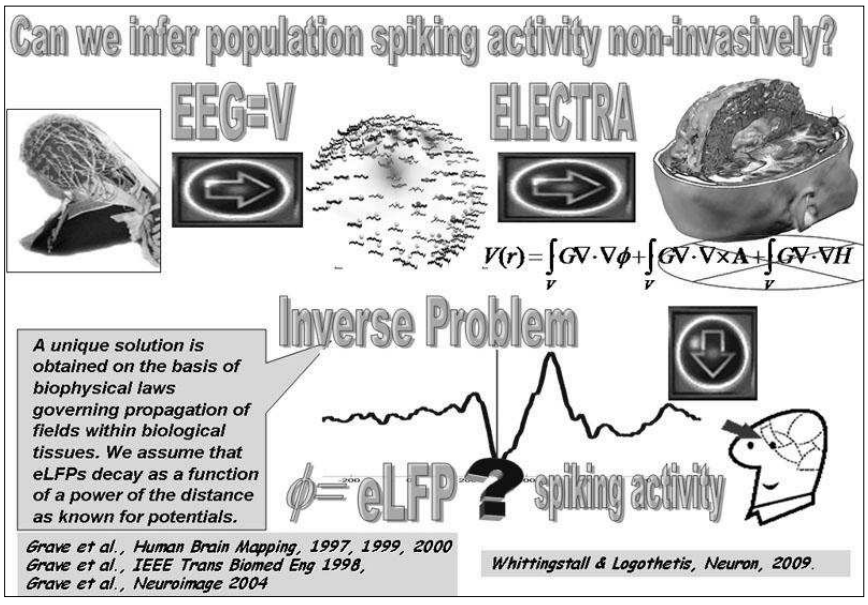

Figure 1: *Main schema of the project:*
The scalp recorded EEG provides the most direct correlate of neural activity that can be obtained non-invasively but lacks spatial resolution providing information limited to the scalp surface. However, it can be mathematically proven that scalp recorded potentials (V) relate to intracerebral field potentials (F) proportional to the invasively recorded Local Field Potentials (LFP) in animals. This relationship is at the basis of a source model called ELECTRA that can be combined with physical and mathematical constraints to solve an ill-posed inverse problem and estimate LFPs within the brain (eLFP). While this is yet far away from the information contained in the spiking activity recorded with neural implants, recent studies using simultaneous invasive recordings of spiking activity and scalp EEG suggest that there is a link between the phase of oscillations recorded at the macroscopic level (eLFP) and the spiking activity of cell populations. We would like to confirm this relationship in humans and in other structures different than the visual cortex in primates to evaluate whether and how well could we estimate population spiking activity non-invasively.

The analysis of simultaneous EEG/LFP and MUA recordings in rats and primates indicate that the relationship between delta phase changes, increases in gamma power and MUA activity is not ubiquitous. As shown in Figure 2, there can exist significant changes in the phase of delta oscillations (here shown for the central nuclei of the amygdala of awake primates) that bear no relationship with significant increases in gamma power but instead correlate with decreases in spiking (MUA) activity (within the basal nuclei).Modulations of the phase of delta oscillations at the time of stimulus (varied images of different emotional content) onset (zero time) are however significant and last for around 200 ms.

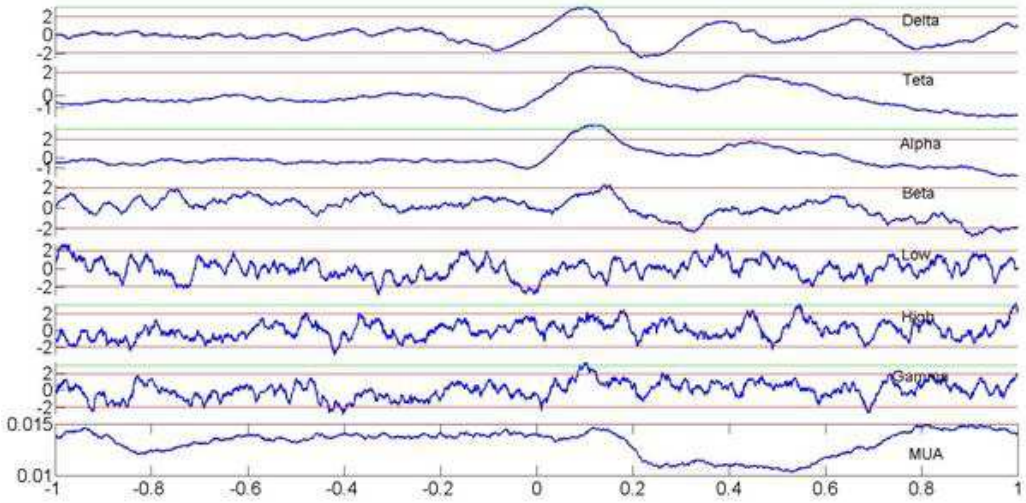

Figure 2: Relationships between delta phase (z-score) and amplitudes within different frequency bands recorded by an electrode in the central nuclei of the amygdala and MUA activity recorded in the basal nuclei of one awake primate (maccaque mulata). Note that modulations in the delta phase and gamma power in the central nuclei accompany MUA decreases in the basal nuclei. There is no clear increase in gamma power. This finding confirms a dissociation between delta phase and MUA.

A similar situation is observed in humans. As shown in Figure 3, recordings over the occipital cortex in a healthy volunteer performing a visual discrimination task (onset f the stimuli at zero) indicate that changes in the phase of delta oscillations are a systematic marker of evoked neural responses. However, no significant increases are seen in the power of other frequency bands.

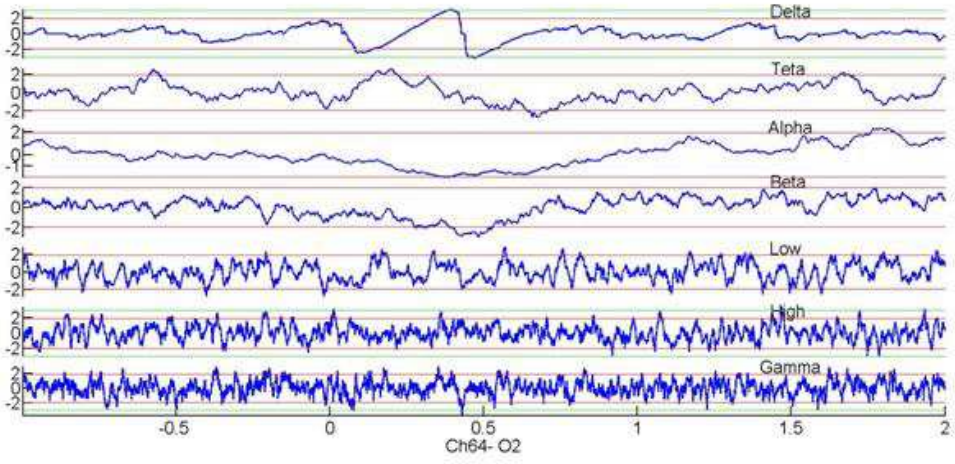

Figure 3: Relationships between delta phase (z-score) and amplitudes within different frequency bands recorded by an electrode over the occipital cortex in a healthy human subject performing a visual discrimination task. Note that changes in delta phase accompany stimulus onset but there are no significant increases in gamma power.

The main idea behind the project is to combine ELECTRA approach to determine intracerebral field potentials with the link between phase/power of neural oscillations and MUA disclosed in [3] to obtain non-invasive estimates, even if rough, of population spiking activity. This is certainly an ambitious goal and several steps are required before we can claim that this is indeed feasible. The first aspect that needs to be evaluated is whether the same or different relationships between frequency bands and MUA holds true in other neural structures (e.g., amygdala) or different species (humans instead of monkeys). Otherwise, we will need to identify the valid relationships through the analysis of concomitant invasive recordings of field potentials and MUA in different structures and species. To confirm the link phase-power of the oscillations with spiking activity we will rely on simultaneous LFP/MUA recordings in primates' amygdala and invasive recordings in epileptic patients. In parallel, we will compute intracranial field potentials from scalp EEG data using ELECTRA source model. The estimates of field potentials will be transformed into the time frequency domain using the S-transform to compute phase/power relationships over the gray matter (see [2] for details).

To investigate the resolution achieved in the non-invasive estimates, we will initially assume that the Whittingstall and Logothetis proposed relationship always holds true. We will therefore consider that a voxel carries substantial information about MUA when a significant increase in gamma power accompanies the negative-going phase of the delta oscillations. Resolution will be then estimated from the spatial dispersion around the voxel showing maximal activation for each area (most active voxel). The spatial dispersion is characterized by the spatial extent occupied by voxels fulfilling the Whittingstall and Logothetis condition. If novel relationships between LFP phase/power and MUA are disclosed by the analysis of intracranial data in monkeys and humans, we will repeat the resolution analysis previously exposed. This procedure will then allow us to assess whether and how well can we estimate population spiking activity non-invasively.

Conclusions and Relevance for 3R

The analyses of data from different animal species including humans indicate that there is a dissociation between delta phase-gamma power and MUA activity. To account for the observed experimental results we are currently developing a biophysical model to explain the generation of the macroscopic EEG/LFP signals and their relationship to action potentials. In the new formulation we drop the quasistatic approximation [6] and replace it by a dispersive model of neural tissue. The preliminary simulations indicate that this model is compatible with the electrophysiological recordings and explain the systematic phase locking of action potentials to ongoing oscillations in the short spatial scale as well as current experimental data. On the light of this model we will need to modify the whole physical-mathematical formalism behind the formulation of the EEG which serves to determine its sources via the solution of the inverse problem. However, according to the proposed formalism the EEG conveys more direct information about action potentials than currently thought. This observation could therefore significantly enhance the probability of imaging non-invasively the spiking activity in the CNS.

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A new in vitro model to study therapeutic approaches to improve spinal cord regeneration and repair after injury or neurodegenerative diseases

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Background and Aim
Spinal cord injury (SCI) or diseases such as multiple sclerosis (MS) are very debilitating and need the development of new therapeutic strategies. It is a topic of intense basic and applied research. Animal models have allowed a number of findings in understanding the mechanisms involved in SCI and MS, and in testing potential therapeutic strategies. However, such *in vivo* models are extremely distressful for animals. Moreover, the use of a wide variety of models, and their variability and complexity, limit the ability to identify new therapies. We present a new model based on organotypic cultures of mouse spinal cord longitudinal slices ("slice model") that we recently developed (Bonnici and Kapfhammer, 2008). This "slice model" allows to generate reproducible SCI-like and MS-like lesions and to study axonal regeneration.

Method and Results
We implemented a neuroinflammation/demyelination model on the slices by co-treating them with interferon gamma (IFNγ) and lipopolysaccharide (LPS) (Defaux et al. 2010). Using different readout including immunohistochemistry, Western blotting, qPCR, ELISA, and enzymatic assays, we characterized the features of this model and compared it to MS and SCI lesions. We observed significant demyelination with an early and progressive decrease in myelin gene expression and protein levels. Microglia was activated becoming macrophagic and proinflammatory cytokine expression and secretion were highly increased demonstrating neuroinflammation. Interestingly, astrogliosis seemed very limited. More likely as a consequence of the neuroinflammation, we also observed neurodegeneration indicated by axonal lesions, and quantitative (e.g. decrease of glutamate decarboxylase activity) and qualitative (e.g. change in phosphorylation state of neurofilament heavy chain) alterations in neuronal markers.

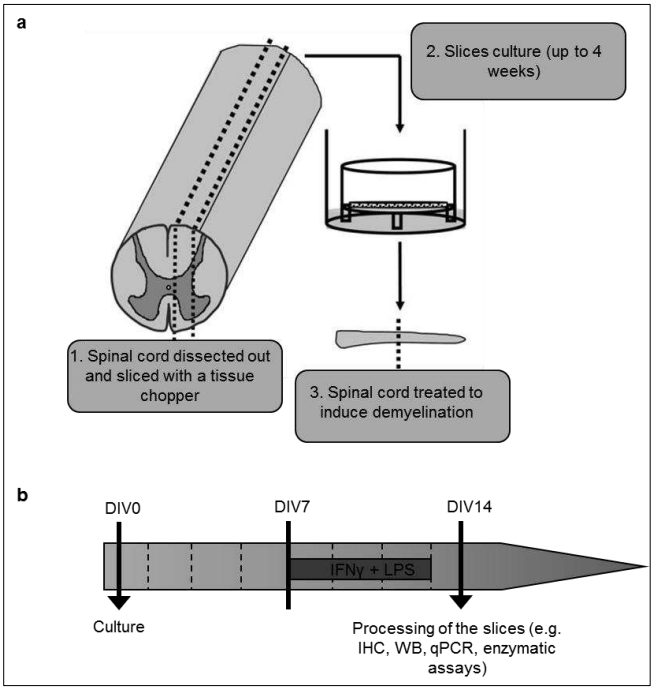

Figure 1: Induction of neuroinflammation/demyelination in our slice model.

a) schematic representation of organotypic cultures of mouse spinal cord sagittal longitudinal slices (modified from Bonnici and Kapfhammer).

b) timeline summarizing the process to induce neuroinflammation/demyeli -nation with IFNγ (100 U/ml) and LPS (10 µg/ml). Medium is changed every 2 days (dotted lines).

Conclusions and Relevance for 3R
The developed model allows in vitro reproduction of important features of both SCI and MS making it a relevant and multivalent model to study mechanisms or treatment for these diseases. Therefore, the "slice model" may substantially help to reduce animal experimentation in the field of SCI and MS where any *in-vivo* model is by definition extremely debilitating.

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3R-Project 122-10

Improved perioperative analgesia and reduced stress during recovery for the experimental animal: ultrasound-guided sciatic and femoral nerve block in sheep and quantitative assessment of block quality

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Background and Aim

Due to a lack of alternative methods, sheep are widely used for biomechanical research purposes, e.g. for studies concerning the knee joint. Until now, sheep have usually received a combination of analgesics at the time of induction of anaesthesia and again during the post-operative phase by intravenous or intramuscular injection. Based on knowledge of surgery in other species, the assumption may be made that such treatment might be insufficient and these sheep may still feel severe pain.

Regional anaesthesia as an adjunct to general anaesthesia may markedly improve the wellbeing of these experimental animals during the post-operative period [1, 2]. A possible side effect of neuraxial (spinal or epidural) anaesthesia is a bilateral blockade of motor fibres beside the blockade of sensitive fibres. Especially in flight animals, as sheep are, this bilateral paralysis may again cause severe distress. Treating only one side – the side of surgery – using peripheral perineural administration of local anaesthetics helps to prevent this source of stress. The reliability of peripheral perineural blockade can be increased by reducing the failure rate if performed using ultrasound guidance. [3].

Species-specific quantification of post-operative pain is mandatory to evaluate the efficacy of the analgesic technique used. In sheep, as in other flight animals, pain recognition and quantification are particularly difficult. The nociceptive withdrawal reflex (NWR) has been extensively used for the study of experimental nociception in animals and humans [4, 5]. A close relationship between pain threshold and NWR threshold has been identified [6, 7].

Behavioural pain assessment combined with the evaluation of mechanical and thermal nociceptive thresholds in the operated area and a quantification of the NWR will provide a complete way to assess the efficacy of the proposed local anaesthetic technique.

Method and Results

in progress (present status)

The technique of ultrasound-guided regional anaesthesia needs to be modified and adapted to the ovine anatomy. Ultrasound-guided blockade of the sciatic and femoral nerves will be introduced in sheep undergoing invasive hind limb surgery and ultrasound guidance will be used to accurately place catheters close to the sciatic nerve to facilitate repeated administration of local anaesthetics during the early post-operative phase. Furthermore, a newly developed pigtail-catheter will be evaluated to achieve a potentially better result.

Effects of the nerve blockade as well as eventual post-operative pain in the hind limb will be quantified. In the hind limb, NWR and temporal summation will be evaluated repeatedly to assess modulations of the reflex while reflexes in the front limb will be assessed to confirm the steadiness of pre-operatively measured reflexes.

Skin sensitivity of the surgical area will be measured using mechanical and thermal stimulation while degree of lameness, degree of pain after palpation of the wound, appetite, general condition, interaction with other sheep and observers, as well as clinical parameters will be evaluated using a multidimensional pain score. In case of signs of

pain, additional analgesic drugs will be administered alongside standard analgesia protocol. Durability of the catheter as well as modulation of the reflex will be re-evaluated one day after surgery.

Conclusions and Relevance for 3R

If we are successful in the description and optimization of ultrasound-guided single shot and catheter-based sciatic-femoral nerve blocks in sheep, these techniques may become standard treatment for hind limb surgeries in experimental sheep to provide efficacious intra and post-operative analgesia. Due to the remote location from the surgical area, regional anaesthesia techniques will not influence the surgical procedure.

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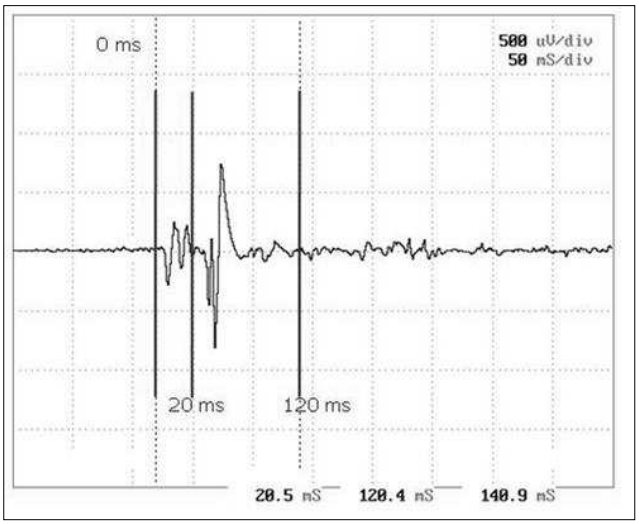

Figure 1: Quantification of pain by evaluation of the NWR in an experimental sheep after placement of a perineural catheter and consequent injection of local anaesthetic.

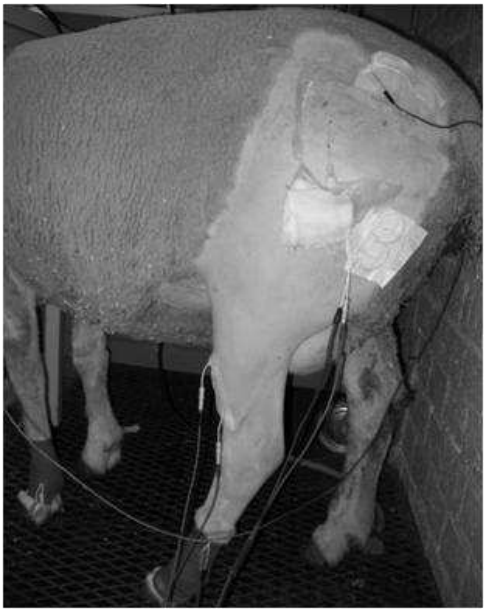

Figure 2: Electromyographic reading in a sheep following the stimulation of the lateral digital nerve with 8 mA. The latency describes the delay between end of stimulus and start of reflex. The extent of reflex is defined by measurement of the root-mean-square amplitude.

3R-Project 123-10

Use of "moribund" stage in the acute fish toxicity test according to OECD guideline 203 and its effect on LC50 values

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Background and Aim

In the acute fish toxicity test according to OECD guideline 203, LC50 is assessed in terms of the concentration of a test substance at which 50% of the fish die within an exposure period of 96h. 7 fish per sample are exposed to 5 different concentrations plus a control. The criterion for death is defined as (§ 19): "no visible movement, no response when touching the tail." A total of at least 6 x 7 = 42 fish are exposed to severity degrees ranging from 1 to 3. The duration of suffering could be reduced by using the criterion of ”moribund” commonly applied in rodent acute toxicity testing. Fish might be declared moribund if no visible movement is observed and are removed from the test as soon as this occurs. Dictionary definitions of moribund include words and phrases such as “dying”, ”at the point of death”, “in the state of dying”, or “approaching death” (there is no official definition for moribund stage in fish). The criterion has already been introduced to ecotoxicological testing in the UK, and is being used by some laboratories in Germany and Switzerland. It not only leads to a deviation from the OECD guideline but, and more importantly, also affects main study outcomes, in particular the LC50 values, which may be lower when moribund is applied. Accordingly the present study has several goals. 1) to find a suitable and commonly acceptable definition for the state moribund in fish, 2) to assess the magnitude of the potential decrease of the LC50 value, 3) to estimate the range of shortening the suffering of the fish, and 4) to provide information on how the test guideline should be adapted to reduce the subjectiveness introduced by the use of a the state moribund.

Method and Results

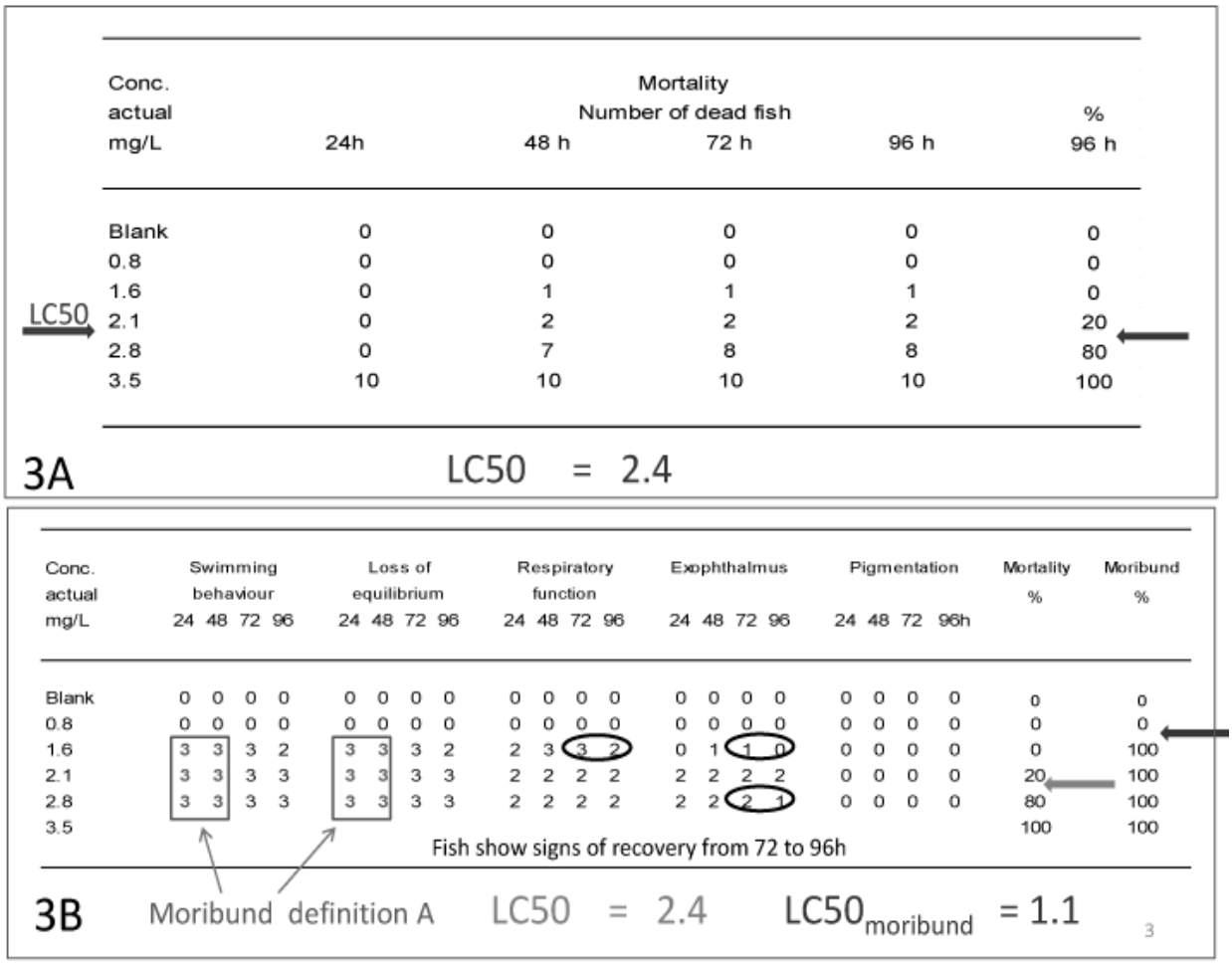
in progress (present status)

In a first step, moribund state was defined. A first definition (Definition A Table 2) arises from the reporting style of existing studies of an industry laboratory (observations of five symptoms, Swimming Behaviour, Loss of Equilibrium, Respiratory Function, Pigmentation and Exophthalmus as suggested in the OECD guideline)

Definition A:	swimming behaviour and loss of equilibrium over ≥48h
Definition B:	swimming behaviour over ≥48h, and at least single observation of complete loss of equilibrium or strong ventilation
Definition C:	swimming behaviour over ≥48h, and at least single observation of complete loss of equilibrium, strong ventilation, or strong discoloration
Definition D:	at least single observation of swimming behaviour over ≥24h, and complete loss of equilibrium or strong ventilation
Definition E:	at least single observation of swimming behaviour over ≥24h, and complete loss of equilibrium, strong ventilation or strong discoloration

Table 2: Definitions of “moribund” stage (all effects with severity degree 3)

Definition A is based on observations of at least two of the following sub-lethal symptoms Swimming Behaviour, Loss of Equilibrium, and Respiratory Function over 48h with severe symptoms (severity degree 3 in a range from 0 to 3).
The effect on the LD50 values was investigated by a retrospective analysis of 328 studies of an industry laboratory. The analysis was based on definition A of moribund. This allows determining the magnitude of the LC50 based on moribund fish, $LC50_{moribund}$, the frequency of a reduction of the $LC50_{moribund}$ compared to the conventional LC50, and the number of fish affected by a shortened duration of exposure and/or suffering.
A typical example of the symptoms and degrees of severity as reported by the industry laboratory is given in Table 3.



Conc. actual mg/L	Mortality Number of dead fish 24h	48 h	72 h	96 h	% 96 h
Blank	0	0	0	0	0
0.8	0	0	0	0	0
1.6	0	1	1	1	0
LC50 2.1	0	2	2	2	20
2.8	0	7	8	8	80
3.5	10	10	10	10	100

3A LC50 = 2.4

Conc. actual mg/L	Swimming behaviour 24 48 72 96	Loss of equilibrium 24 48 72 96	Respiratory function 24 48 72 96	Exophthalmus 24 48 72 96	Pigmentation 24 48 72 96h	Mortality %	Moribund %
Blank	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0	0
0.8	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0	0
1.6	3 3 3 2	3 3 3 2	2 3 3 2	0 1 1 0	0 0 0 0	0	100
2.1	3 3 3 3	3 3 3 3	2 2 2 2	2 2 2 2	0 0 0 0	20	100
2.8	3 3 3 3	3 3 3 3	2 2 2 2	2 2 2 1	0 0 0 0	80	100
3.5						100	100

Fish show signs of recovery from 72 to 96h

3B Moribund definition A LC50 = 2.4 $LC50_{moribund}$ = 1.1

Table 3: Comparison between LC50 and $LC_{moribund}$ in a real-life study:

3A: Mortaility reported, 7 fish per concentration, LC50 calculated.

3B: Sublethal symptoms observed

The severity degrees 3 of Swimming Behaviour and Loss of Equilibrium are marked within the blue frame as these indicate moribund status according to the definition A.

Explanation of symptoms: 0: none; 1: light; 2: moderate; 3: severe.

In this study, fish showed severe symptoms expressed in terms of altered swimming behaviour and loss of equilibrium from 24h to 96h (i.e. over a period of 72h). The fish would be declared moribund after 48h and removed from the test, thereby reducing degree 3 severity of suffering by 48h in concentrations of 0.88, 1.7, 3.3 and 6.5 mg/L (28 fish in total). Accordingly LC moribund would be 100% in the 4 concentrations whereas the "death" (lethal) criterion results in only 14, 14, 29 and 43% mortality within a 48h time span. Hence, the application of the moribund criterion results in a LC50 moribund of 0.66 compared to 4.4 mg/L using the conventional method (factor 6.7 lower), and to a classification of the chemical into the acute category 1 for hazardous to the aquatic environment (acute (short-term) aquatic hazard).

The procedure was repeated based on a second definition (Definiton B Table 2) of moribund deducted from the reporting style of other industry laboratories. Finally, the outcome of three additional definitions of moribund (Definiton C, D and E, Table 2) based on the reporting style of 10 different laboratories in Europe and the U.S. was evaluated both on the 328 fish acute toxicity tests of the industry laboratory and the 111 tests from the other laboratories.

In the studies evaluated, 10 to 23% of the fish were declared as moribund reducing the suffering of severity grade 3 (severe distress) by up to 92h. The median of the decrease of $LC50_{moribund}$ in relation to the LC50 was by a factor of about 2; the maximum factor observed was 15.7. While the reduction of the suffering is desirable, a decrease of the measured toxicity endpoint has consequences on the classification and on risk assessments.To produce comparable results between laboratories when moribund is used requires the following specifications in an updated test guideline: 1) A unique, generally accepted definition of the moribund state in fish, 2) specification on the type of visible abnormalities to be reported (symptoms), and 3) specifications on the degree of the effects (e.g. how many single observations per concentration).

Conclusions and Relevance for 3R
The potential of the project is in refinement. In 2009 and 2010, 46,000 and 36,000 fish were used for testing in Switzerland, about 13,000 and 10,000 of which in acute and chronic toxicity tests, respectively. REACh requires approx. 1.2 million fish each year, with a considerable proportion used in toxicity tests. The fish suffer degree 3 severity at least in the highest dose. A successful inclusion of the moribund criterion in the OECD guideline would mean that fish would be exposed to stress for a shorter period of time, and suffering would be reduced accordingly.

References
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3R-Project 125-11

Nerve-cell mimicking liposomes as in vitro alternative to potency-testing of toxins with multistep pathways, such as Botulinum neurotoxins

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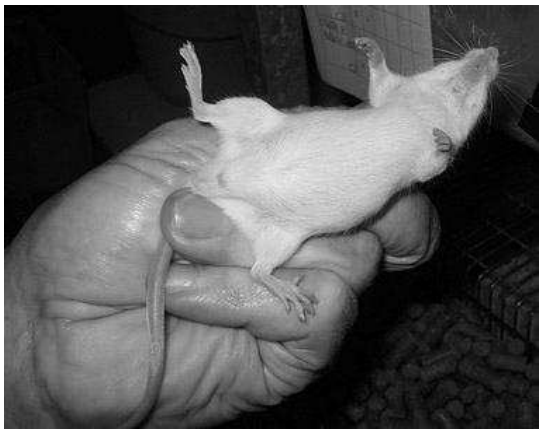
Background and Aim
Botulinum neurotoxins (BoNT), produced by the Gram-positive bacterium *Clostridium botulinum*, are the most lethal toxins known to man. Similar to other toxins with multiple active sites, BoNT exerts a complex function in the human body. In the latter case, the toxin binds via its binding domain (HC_C = heavy chain binding domain) to specific cell-surface receptors on motoneurons. The translocation domain (HC_N = heavy chain translocation domain) facilitates translocation of the endoproteinase domain (LC = light chain) into the nerve cell lumen, where the latter specifically cleaves distinct SNARE proteins. As a result, neurotransmitter release is inhibited and the adjacent muscle cells are paralysed. If untreated, this leads to cumulative paralysis, and eventually death by respiratory arrest. If applied in minute doses, however, the toxin exerts a locally constrained paralysing effect, which is employed in medical applications for the treatment of a wide range of diseases, disorders and in aesthetic surgery.
In the case of BoNT, the mouse LD_{50} test is so far the preferred method for batch control of BoNT-containing pharmaceutical products, such as BOTOX, Myobloc and others [1,2]. Hence, with an increasing number of medical applications, more than half a million mice are used each year for routine potency testing [3,4]. Owing to the often complex nature of multi-domain toxins such as BoNT, most existing cell-free *in vitro* methods fail to detect the entire toxic activity, instead measuring either merely part of their toxicity or only the toxins present, e.g. by immunological methods. Although cell culture assays have been established for use in toxicity testing, many of these assays still require expensive and difficult maintenance, qualified personnel and the appropriate cell culture facilities. In addition, if stem-cell derived cells are used, long differentiation times of up to four weeks can be necessary [5].



Here, we describe the groundwork for an *in vitro* alternative for testing the entire toxic activity of toxins with multiple active sites, taking BoNT type B (BoNT/B) as an example, using liposomes with integrated nerve cell receptors to imitate the motoneuron membrane. BoNT/B

Figure 1: Mouse LD50 test. For batch control of BoNT containing pharmaceutical products, different dilutions of the product are injected intraperitoneally into mice. The mice are observed for typical botulism symptoms. If the dose is high enough, mice die of suffocation within 96 hours.

binds via its HC_C to the presented receptors on the liposome surface. If the pH in the surrounding medium is lowered, the HC_N translocates the LC into the liposomal lumen, where the latter exerts its endoproteinase activity by cleaving a peptide substrate with specific cleavage sites and a quenched fluorophore pair. Upon cleavage, the fluorophore pair is unquenched and its fluorescent signal can be detected, allowing for quantification of the actual potency of BoNT in the system. With the use of well defined components, the liposomes may be produced under reproducible conditions. Moreover, liposomes can be stored long term at little expense and reconstituted on demand [6,7]. Furthermore, if the respective binding molecules (i.e. receptors) and substrates are available, the modular character of the presented system may also allow for testing of other toxins with multistep pathways which exert complex (chain reactions) functional changes, which are difficult to measure, in cells and the body.

Method and Results

in progress (present status)

So far, receptor liposomes have been successfully been produced with lipid compositions similar to those found in mammalian nerve cells. Furthermore, a peptide reporter assay was established to detect cleavage activity of BoNT/B at concentrations as low as 10 pM, even under liposome-compatible conditions. Successful encapsulation of a reporter peptide was confirmed and encapsulation efficiencies over 70% were established. Furthermore, first batches of fully assembled liposomes (FAL) have been produce. Latter contain ganglioside and protein receptor, as well as peptide reporter molecules encapsulated for the most part. Experiments are in progress to characterize the produced FAL and to test for the proposed proof-of-concept.

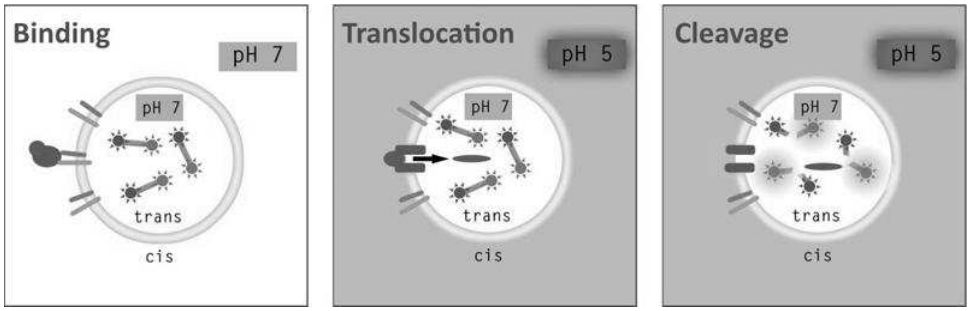


Figure 2: Proposed model of detection, using functionalised liposomes. BoNT (in red and blue) is bound to the outer side of the liposome via two nerve cell receptors. Inside the liposome a peptide reporter is located with a fluorophore and a quencher molecule on the opposing sides of the peptide chain. After a shift from physiological (app. pH 7) to an acidic pH that mimics the endosome-lysosome transition (app. pH 5) the BoNT/B Light Chain is translocated into the liposome lumen. There, it may cleave the appropriate peptide reporter, resulting in a quantifiable fluorescent signal.

Conclusions and Relevance for 3R

If the proof-of-principle is to be successful, then exact validation of the system's capability will be the following step. If this demonstrates that the assay is sensitive and robust enough it may be used to replace the mouse LD_{50} test for batch control of pharmaceutical products that contain BoNT. Although the proof-of-concept aims to test for BoNT/B (active compound e.g. in Myobloc), modification of the assay may provide the means of detecting additional relevant BoNT types, especially BoNT/A (the active compound in BOTOX and others). Also, if the necessary receptors and substrates are available, it may theoretically also be used to detect Tetanus toxin, and even other toxins with multiple active sites which act in a way similar to BoNT.

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3R-Project 126-11

Model development and validation to investigate myeloid cell homeostasis

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Background and Aim
Neutrophils develop in the bone marrow from hematopoietic stem cells and play a critical role in infection and inflammation. Understanding the molecular mechanisms that regulate neutrophil homeostasis is therefore crucial in designing new therapeutic strategies to modulate inflammatory and immune responses. Mature neutrophils are short-lived and thus poorly amenable to molecular manipulation. In the present project, we will validate the use of immortalized mouse neutrophil progenitors that can be conditionally induced to differentiate in vitro into unlimited numbers of neutrophils at different stages of differentiation (1). We propose the following specific aims to validate this methodology to study neutrophil death mechanisms regulated by serine proteases and serpinB1 (2, 3).
Specific Aim 1. To reproduce our in vivo findings that serpinB1 regulates neutrophil homeostasis and survival upon differentiation using an alternative method to primary cells isolated from experimental animals. *Specific Aim 2*. To investigate the role of specific serine proteases in neutrophil homeostasis.

Method and Results
in progress (present status)
We will first characterize immortalized wild-type and serpinB1-/- myeloid progenitors generated by estrogen receptor (ER)-mediated conditional expression of hoxb8 in the presence of stem cell factor (SCF). Markers for proliferation, differentiation, pattern of granule protein expression and cell death pathways will then be investigated at several points in time in differentiation induced by ER-signaling withdrawal.
We will then generate myeloid progenitor lines with deficiency in neutrophil serine proteases and investigate death pathways in neutrophils differentiated in vitro.

Conclusions and Relevance for 3R
By achieving these aims, we will extensively validate an alternative method for studying neutrophil development, homeostasis and cell death pathways using large numbers of cells at a defined differentiation stage in the neutrophil lineage generated *in vitro*.

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3R-Project 127-11

Establishing a novel system for quantitative production of murine basophils in vitro

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Background and Aim
Basophilic granulocytes (basophils) are a rare leukocyte population, constituting ~0.5% of peripheral blood leukocytes. Recently, several high impact studies have identified previously unrecognised roles for basophils in allergic responses as well as in immune regulation, indicating that the role of basophils may have been underestimated so far (1-5) The study of basophils is particularly challenging as no suitable cell culture model for human or mouse basophils exists to date. Moreover, *in vitro* differentiation of basophils from bone marrow is very inefficient. Therefore, large numbers of mice have to be sacrificed in order to isolate very limited numbers of primary basophils from blood. For those reasons, we have recently established a novel method to generate conditionally immortalised, basophil-

committed progenitor cell lines, which can be differentiated *in vitro* in near-unlimited amounts into mature basophils. The main aim of the 3R project is the full phenotypical and functional characterisation of this, to our knowledge, unique cellular mode for murine basophils.

Method and Results

in progress (present status)

Basophil-committed progenitor cell lines are generated from murine bone marrow (or foetal livers) using conditional Hoxb8 by a significantly modified protocol published for the quantitative production of mouse neutrophils/macrophages (6). The most important changes include the use of different cytokines and a novel lentiviral system for conditional Hoxb8 expression. (7) Once immortal cells lines are established, they can be easily handled and manipulated and maintained in culture for prolonged times (Figure 1A). Differentiation into mature basophils is achieved 'on demand' by shutdown of exogenous Hoxb8 expression (Figure 1B). Within 6 days, an enriched population of cells resembling mature basophils is obtained. Mature Hoxb8 basophils are end-differentiated (non-cycling), have lobulated nuclei and a granular appearance and display a surface expression profile of a number of membrane bound, cell type specific surface proteins ($Fc\varepsilon RI^{high}CD11b^{+}ckit^{neg}Gr1^{neg}IL5RnegCCR3^{neg}$, see Figure 1C, D and not shown). The high affinity receptor for IgE, FcεRI, is functional, as receptor crosslinking leads to degranulation and release of N-acetyl-β-D-hexosaminidase (Figure 1E).

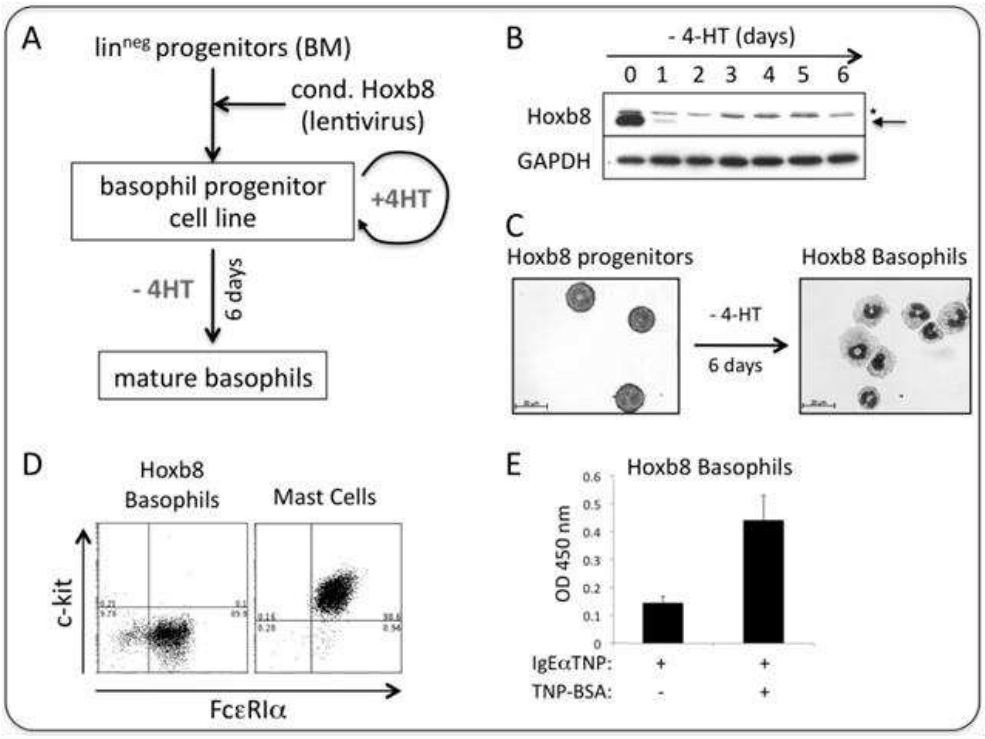

***Figure 1*:**

Generation of murine basophils in vitro using conditional Hoxb8
(A) Simplified scheme of generation of basophil committed progenitor cell lines from bone marrow.
(B) Western blot showing rapid shutdown of exogenous Hoxb8 upon removal of 4-hydroxytamoxifen (4HT).
(C)DiffQuik staining of immature and mature Hoxb8 basophils.
(D) c-kit (CD117) and FcεRI surface expression profile by FACS of mature Hoxb8 basophils and bone marrow derived mast cells.
(E) N-acetyl-ε-D-hexosaminidase release (as a measure for degranulation) upon FcεRI crosslinking.

The planned phenotypical and functional characterisation will be performed in immature progenitors as well as differentiated basophils. We plan to investigate the expansion and clonogenic potential of Hoxb8 basophils and extend the characterisation of basophil specific surface markers and enzymes (e.g. mast cell protease 8 and 11, see Ugajin et al.(8)). Functionality of Hoxb8 basophils will be tested by crosslinking FcεRI, (9, 10) followed by the analysis of induction and/or release of lipid mediators (e.g. leukotriene C4), histamine and cytokines (IL-4, IL-13). Treatment with the protein fragment C5a (released from complement component C5) will be used in parallel as an immunoglobulin-independent stimulus. Chemotaxis, transmigration behaviour and oxidative burst upon activation with various stimulants will be investigated.

Conclusions and Relevance for 3R

We have established a method to generate basophil-committed progenitor cell lines using conditional Hoxb8. These cells can be differentiated in quantitative amounts into mature basophils *in vitro*, making it, to our knowledge, a novel

and unique cellular model for murine basophils. The basophil lines described here are genetically stable, can be generated from any genetically modified mouse strain and can easily be manipulated (e.g. knockdown by short-hairpin RNA, introduction of transgene, etc.) and progenitors can be differentiated into mature basophils in near unlimited numbers. As large amounts of mice are required to isolate primary basophils, we would like to promote this cellular model as an alternative method to study basophil biology, in particular to answer questions requiring large numbers of basophils (e.g. biochemical work, study of signalling pathways, etc.). Additionally, Hoxb8 basophil lines can be generated form foetal livers (E12-E14) of mouse strains displaying severe phenotypes. The main relevance for 3R of this project therefore lies in its potential to reduce the number of sacrificed mice and the need to grow mice with severe phenotypes to adulthood.

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3R-Project 128-11

Genetic modification of the human airway epithelium – a paradigmatic system to study host responses to human respiratory viruses

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Background and Aim

The human airway epithelium represents the entry port of many human respiratory viruses, such as respiratory syncytial virus, rhinoviruses, influenza viruses, human parainfluenza viruses, adenoviruses, human metapneumoviruses, and human coronaviruses. Recent advances in the cultivation of primary human airway epithelia (HAE) have enabled studies of human respiratory virus infections in a culture system that morphologically and functionally resembles human airways *in vivo*. However, despite these recent technical achievements, the analysis of basic virus-host interactions on the molecular level is still preferentially done in animal models. This is particularly true for virus infections for which a murine model is available. The main reason for this preference is that a murine model of viral infection allows for the use of widely available transgenic or knock-out mouse strains. Thus, in contrast to primary human cells, individual genes can be genetically deleted or trans-complemented in the mouse model, which permits detailed analyses of virus-host interactions on the molecular level. In order to overcome this limitation, we aim to render the HAE culture system amenable to genetic modifications.

Method and Results

in progress (present status)
In order to render the HAE culture system amenable to genetic modification, we will use lentiviral vectors for transgene expression and to knock down the expression of specific genes.

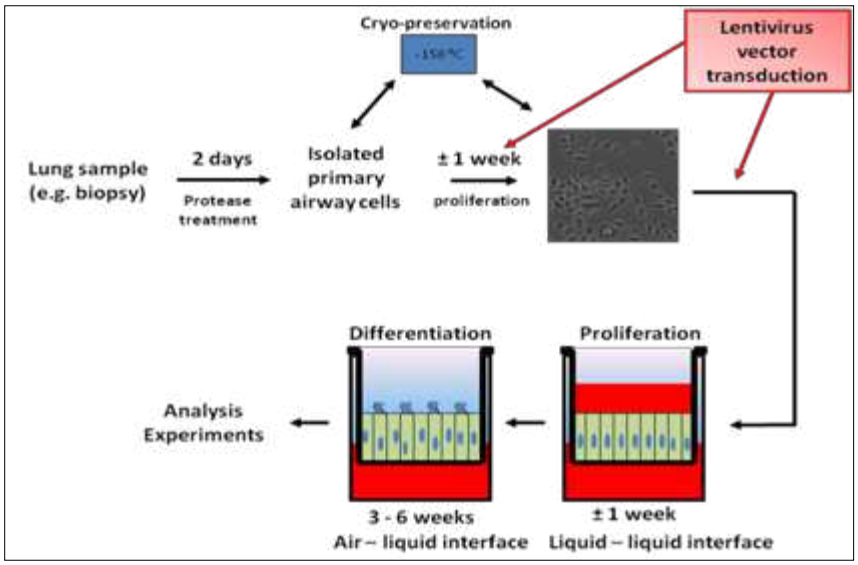

Figure 1:
Schematic representation of HAE culture generation and genetic manipulation using lentiviral vectors.

Therefore, the research objectives of the proposed project include the following specific aims:

- Generation of a set of lentiviral vectors encoding reporter proteins that are expressed upon induction.
- Optimization of transduction of primary HAE cultures and establishment of efficacious affinity selection methods to obtain HAE cultures with high lentivirus vector-based gene expression.

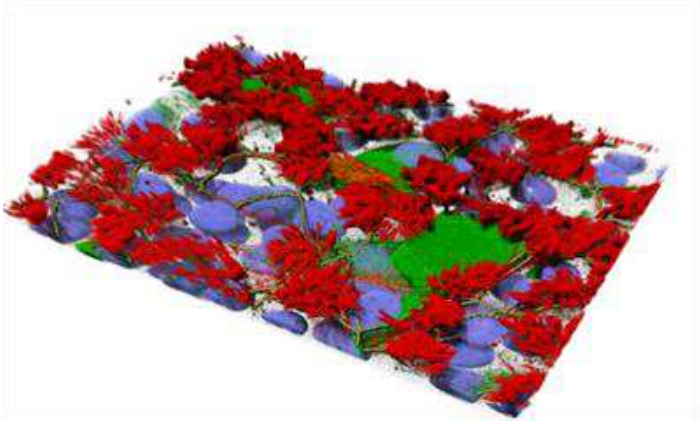

Figure 2: Human Coronavirus-infected HAE culture. Transparent 3D-rendered Z-stack image of an HCoV-229E-infected HAE cell culture is shown. The culture was stained with antibodies directed against Beta-tubulin IV (ciliated cells; red), anti-ZO1 (tight junctions; yellow) and with DAPI (cell nucleus; blue). HCoV-229E target cells express HCoV-encoded green fluorescent protein (green). The Z-stack was acquired with an EC Plan-Neofluar 40x/1.30 Oil DIC M27 objective on a Zeiss LSM 710 confocal microscope, and data were processed using Imaris (Bitplane Scientific Software).

Conclusions and Relevance for 3R

The possibility to efficiently inactivate the expression of particular genes and to express genes of interest *in trans* in an human airway epithelia culture system has the potential to replace many animal experiments that are based on the use of specific transgenic and knock-out mouse strains. Therefore, we expect that an HAE culture system amenable to the genetic modification of host gene expression will find wide application in the analysis of host-pathogen interactions and the molecular biology of many human respiratory pathogens.

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3R-Project 129-11

The use of microfluidic chambers to study axonal transport in PTEN and SOCS3 dependent axonal regeneration

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Background and Aim

Rodents are extensively used to study nerve injury. The mouse spinal cord injury model, widely used in nerve injury research, is extremely debilitating. *In vivo* studies have allowed major advancements in the comprehension of the incapacity of adult central nervous system axons to regenerate. Notably, *in vivo* studies have shown that axonal regeneration after nerve injury was possible in adult mice if PTEN or SOCS3 were deleted in knock-out mice (1,2,3). In order to test the hypothesis that would decipher the mechanism by which PTEN/SOCS3 deletion induces axonal regeneration, an *in vitro* system allowing straightforward manipulation and analysis is required. We propose to use microfluidic chambers (4) to mimic nerve injury and regeneration *in vitro*. This method permits (i) to injure axons without affecting the cell body, (ii) an easy manipulation of neuron cell bodies or axons specifically, and (iii) a single axonal analysis. In the present project, the method will be further evaluated in order to prove its suitability as a replacement method for specific *in vivo* studies.

Method and Results

in progress (present status)

Using freshly isolated cortical neurons from knock-out mice (PTEN, SOCS3 or PTEN/SOCS3) cultured in microfluidic chambers (Fig.1), we will test the regenerative capacity of these neurons after injury.

Neurons isolated from E18 embryos and from post natal pups will be used to assess the cellular response to axonal injury of the different genotypes as they develop. We will also use the unique feature of the microfluidic chambers to perform *in vitro* axonal injury in order to decipher the role played by axonal transport to support robust axonal regeneration induced by the deletion of PTEN and/or SOCS3. *In vitro* axonal injury will be inflicted on neurons after 6 days in culture. Because mitochondria appear to be of special importance in axonal physiology, we will track mitochondrial dynamics using live imaging techniques in regenerative axons 20 hour post injury (after 7 days in culture). The microfluidic chambers will allow us to follow mitochondrial transport in single axons minutes or hours post injury. Genetic manipulation or drug treatment specifically applied in the somal or axonal compartment of the chamber will be used to assess the mechanism by which the transport of mitochondria supports axonal regeneration. Finally, to fully validate the use of microfluidic chambers in the field of spinal cord injury/axonal regeneration, the regenerative capacity of knock-out axons injured *in vitro* will be compared with the results obtained in the lab using classical *in vivo* nerve injury models (optic nerve injury and spinal cord injury). Axonal transport rate of mitochondria after post injury and during regeneration will be compared with the rate of transport observed *in vivo* using a live imaging technique of the mouse optic nerve that we are currently developing in the lab.

Figure 1:

(A) First panel: Schematic view of a microfluidic chamber. Adapted from (4). Neurons plated in the somal side will project their axons through the 450 µm long microgrooves and reach the axonal side. Second and third panel: immunohistochemistry of E18 mouse cortical neurons culture (DIV7) in microfluidic chambers.

Second panel:
Red; anti-3-Tubulin (axonal marker), Green: anti-GFAP (glial marker), Blue: DAPI (nuclear marker).
Third panel:
Red; anti-3-Tubulin, Green: anti-MAP2 (dendrites marker), Blue: DAPI. 450 µm microgrooves allow a pure isolation of neurons.

(B) 3-Tubulin immunohistochemistry of E18 mouse cortical neurons culture (DIV7) in microfluidic chambers. No Injury (first panel), immediately after injury (second panel) and 3 days after injury (third panel).

(C) Regrowing axons can be observed 3 days after injury

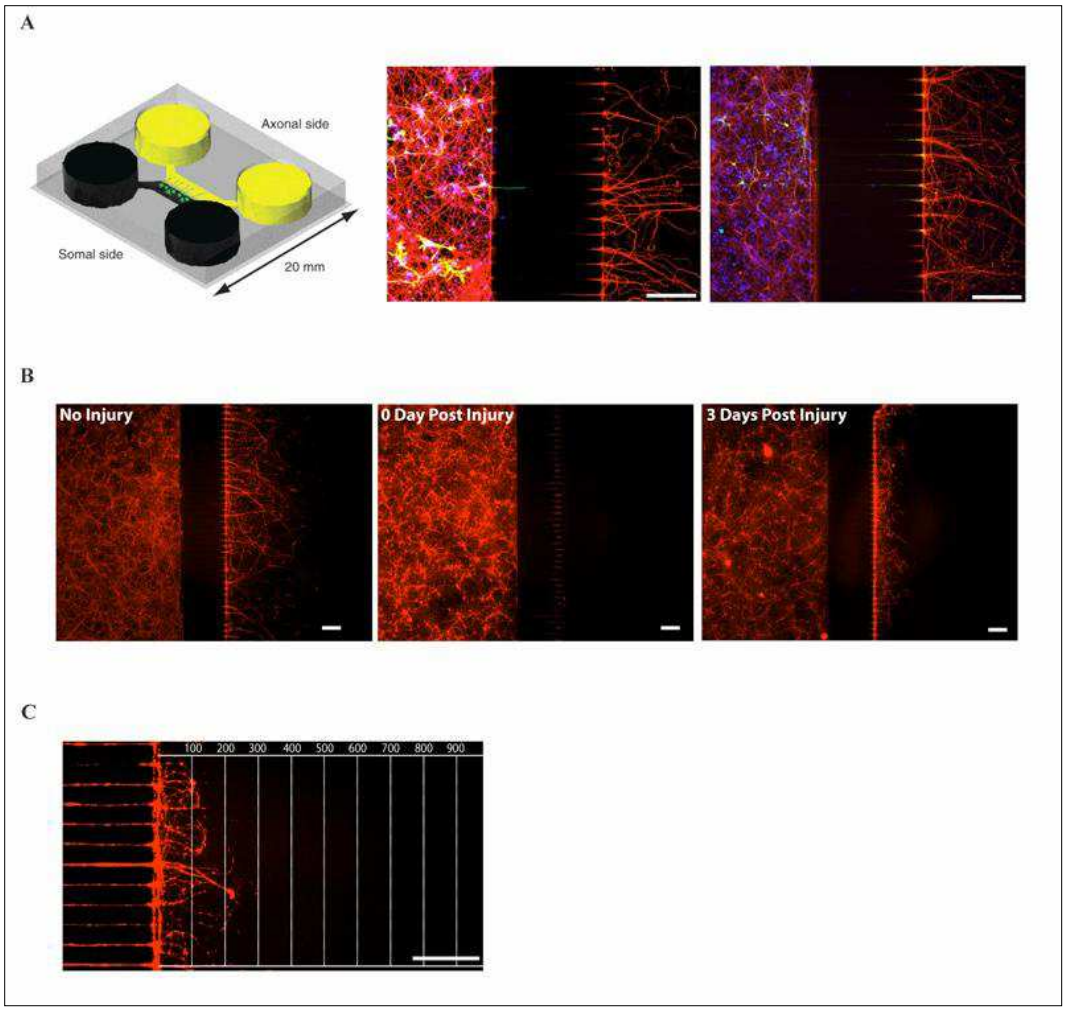

Figure 1

Conclusions and Relevance for 3R

A lab testing the regenerative capacity of axons using the spinal cord injury model (transgenic mice, drug treatment) will use roughly 5,000 mice per year. Some of these mice are used to test hypotheses that will not give any satisfactory results. To increase the chance of obtaining positive results *in vivo* while decreasing the number of mice used, we propose to validate the microfluidic chambers as an *in vitro* system that would be a primary test to establish promising hypotheses worth testing *in vivo*, if possible. We estimate that by first testing the hypotheses in a reliable *in vitro* would save one third of the mice used per year. We hope that our study will establish microfluiding chambers as a gold standard system in the field of the study of spinal cord injury/axonal regeneration.

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Establishment of an in-vitro organ-slice defect model for meniscal repair in orthopaedic research

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Background and Aim

The aim of this project is to develop a novel, simple and inexpensive organ-slice culture system to simulate the repair of meniscal lesions from synovial tissue *in vitro*. The model would be established using the menisci of bovine cows slaughtered at a local abattoir. In the field of orthopaedic research, appropriate *in-vitro* systems have been developed to simulate the repair of cartilage and bone, but not that of meniscal tissue. And even in the engineering of the former two tissue types, an *in-vitro* system is usually employed merely to establish the feasibility of the contemplated repair strategy, viz., to demonstrate that the selected growth factors can induce the targeted precursor cells to differentiate and elaborate a tissue-specific extracellular matrix.

The meniscus is an intra-articular fibrocartilaginous disc. By distributing the stresses to which a joint is normally exposed over a broad area of the cartilage surface, this organ exerts a chondroprotective effect [1, 2]. It is comprised of solid and fluid phases. When the meniscus is compressed, fluid is forced through the solid phase. Since frictional drag is exerted on the fluid by the low permeability of the solid phase, the fluid becomes pressurized, thereby assisting in the carriage of compressive loads. The bulk of the meniscal tissue is avascular; only the peripheral portion is vascularized. With respect to knee-joint pathology, the menisci, next to the ligaments, are the structures most frequently injured [3]. Meniscal injuries can occur secondary to overt trauma, and can also arise from the alterations in knee-joint function that are associated with aging, osteoarthritis, rheumatoid arthritis, disturbances in gait [4-8] and obesity. Sixty per cent of individuals over 50 years of age manifest some degree of meniscal pathology.

If an appropriate organ-slice culture system could be established to model the entire process of cell differentiation, tissue formation and defect healing *in vitro*, then it would be possible to develop and refine a therapeutic principle for the repair of meniscal tissue without having recourse to living animals except at an advanced stage, namely, when it has been established beyond reasonable doubt that the strategy is likely to be valuable in the clinical treatment of human patients. The availability of such an organ-slice culture model would permit a very significant reduction in the requisite number of living animals, and would also curtail the time expended on pre-clinical investigations as well as their expense. These substantial benefits would render the model highly attractive to academic institutions, industrial partners and contract-research organizations.

Method and Results

in progress (present status)

The medial meniscus will be harvested from the knee joints of bovine cows slaughtered at a local abattoir (Fig.1). Synovial tissue will be excised from the medial side of the same joints. Slices of meniscal tissue, approximately 3 mm in thickness, will be produced using a specially designed tool.

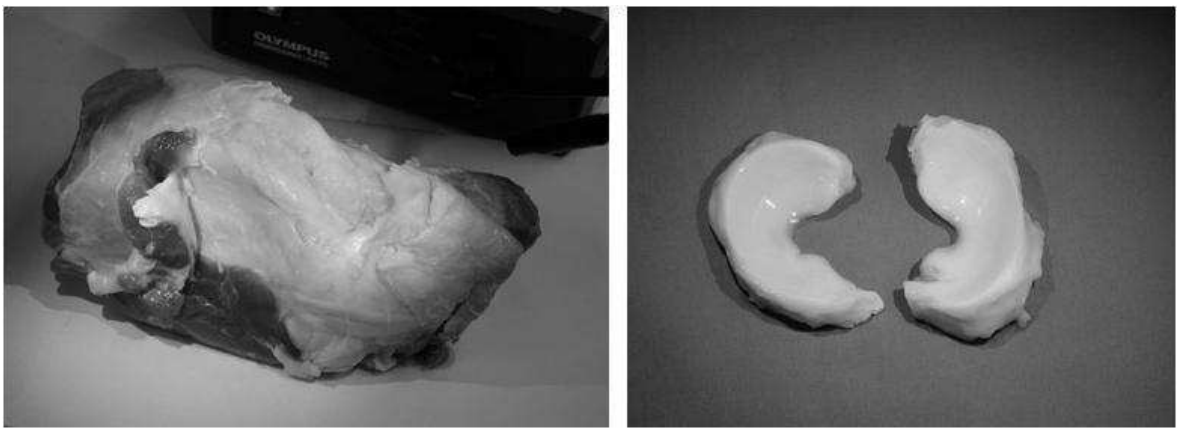

Figure 1: Bovine knee joint (left) together with the excised menisci (right).

Having successfully developed a suitable slicing tool and established that meniscal slices of reproducible thickness can be produced without inducing apoptotic cell death (and/or necrosis), we will then design and manufacture hollow cylindrical punchers with different diameters (satisfying the same cutting criteria as the slicing tool) to prepare discs of meniscal tissue from the slices and to produce an inner chamber that represents the defect (Fig.2).

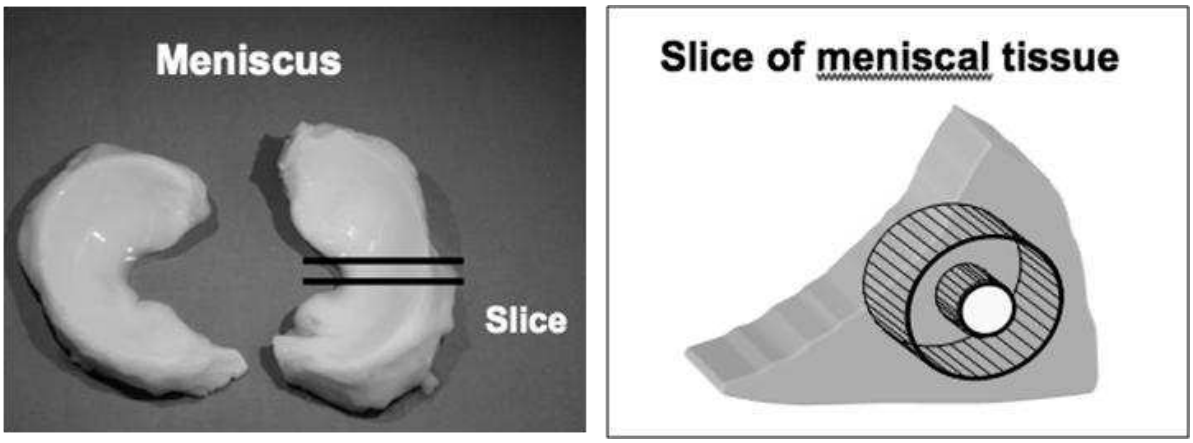

Figure 2: Preparation of the chamber (defect) model from a slice of meniscal tissue.

Two synovial discs with the same diameter as the meniscal defect will be punched out and respectively affixed by gluing with Histoacryl (medical grade) to the upper and the lower surfaces of the chamber (defect) to ensure that its dimensions are maintained during the course of culturing (6 weeks). Having successfully produced the "empty" constructs of meniscal and synovial tissue, we will then establish appropriate conditions for their culturing (Fig.3).

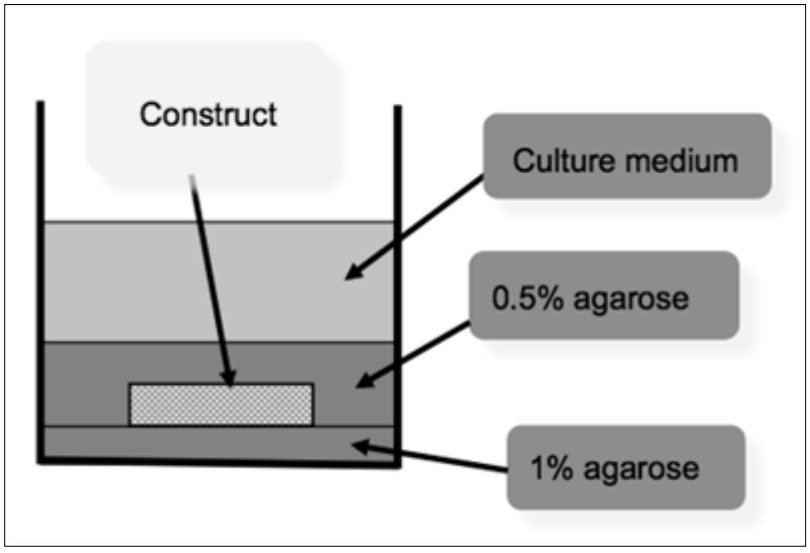

Figure 3: Culturing of the meniscal-chamber construct in agarose gel (cross-section).

Conclusions and Relevance for 3R

If our conceived model of a meniscal defect could be practically realized on a reproducible basis, then it would afford not only a means of establishing almost exclusively *in vitro* the stimulation conditions necessary for the lesion`s repair from synovial tissue, but also a welcome opportunity to cut down on the number of experiments with living animals, which, incidental to the important ethical benefit, would also be financially advantageous.

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3R-Project 131-12

Antibody phage selection strategy for application in non-specialized laboratories

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Background and Aim
Antibodies are widely used in research for the detection or purification of proteins. Although antibodies with tailored binding specificities can be generated by *in vitro* methods such as phage display (1, 2), most antibodies used in research are still produced by immunizing animals. *In vitro* techniques are not broadly applied to the generation of monoclonal antibodies for technical and economical reasons. Phage display involves many experimental and error-prone steps. Moreover, it is costly to establish the complex method in new laboratories. We have developed an inexpensive phage selection strategy that involves significantly fewer experimental steps (only one round of panning) and may be applied in non-specialized laboratories. A phage library will be provided along with a point-by-point protocol to laboratories who would like to develop antibodies that meet their protein targets.

Method and Results
in progress (present status)
We exploit newly available, powerful technologies such as high-throughput sequencing and DNA synthesis to develop a phage display selection strategy with significantly fewer experimental steps. In brief, a phage display library is subjected to a single round of affinity panning. DNA of isolated clones is sequenced using next generation sequencing technology that certain companies now provide at an affordable price. The DNA of many clones is then synthesized, expressed and the binding assessed by ELISA. Alternatively, the same approach is applied to generate ligands based on peptide macrocyles rather than antibodies (3). In this case, phage selected peptides can be chemically synthesized rather than recombinantly expressed.

Conclusions and Relevance for 3R
The proposed method should replace animal experiments that are commonly performed to develop polyclonal and monoclonal antibodies. Monoclonal antibodies are typically produced by repetitive injection of antigens into mice or other animals. The number of animal experiments that can be replaced depends on how broadly the method will be applied. A central characteristic of the proposed method is its simplicity, allowing its application by non-experts.

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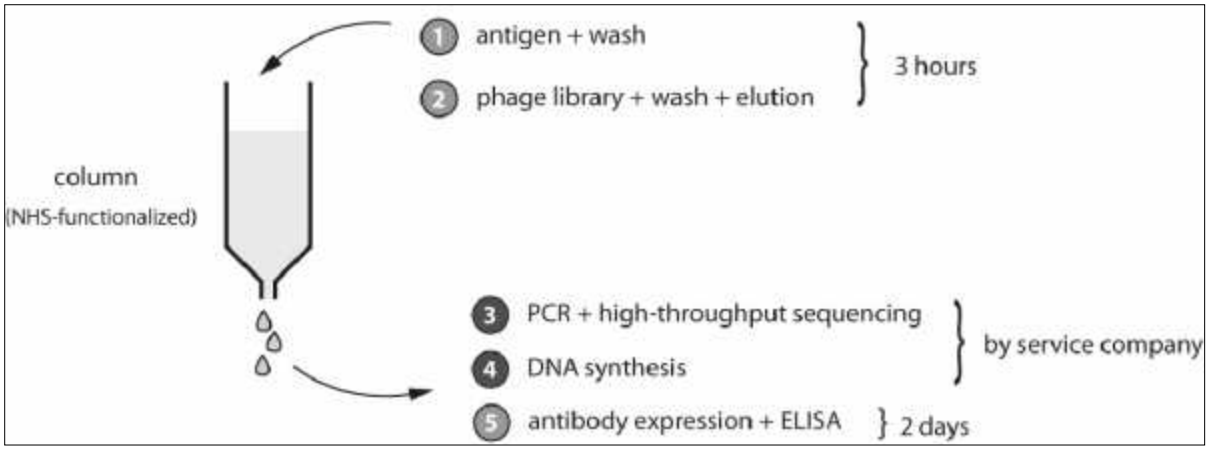
Figure 1:
Schematic representation of the proposed phage selection approach. Steps with green numbers are performed in house by research laboratories; steps in blue are outsourced to service companies.



3R-Project 132-12

Identification of predictive in vitro markers of hematopoietic stem cell function

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Background and Aim
Although hematopoietic stem cells (HSC) are the most successfully used clinical stem cells (1), their transplantation to treat hematological malignancies remains a risky procedure with major complications including infections and graft-versus-host disease. Moreover, the limited availability of human leukocyte antigen (HLA)-matched donors and the low numbers of transplantable HSCs that can be isolated from a donor are major issues that could potentially be overcome if efficient in vitro HSC expansion methods were available. Yet, as soon as HSCs are removed from their native microenvironmental niches in the bone marrow and put in standard cell culture, they lose their characteristic functions by undergoing rapid differentiation.
Progress to discover robust *in vitro* systems capable to expand HSCs has been hampered by a lack of prospective markers that predict reconstitution activity of cultured HSCs. Indeed, the only functional assay to probe long-term HSC multipotency remains transplantation, a method that is time-consuming, entails animal suffering and thus raises ethical concerns. Classical *in vitro* assays to assess hematopoiesis such as colony formation in a semi-solid matrix that contains cytokines are a powerful means to assess clonogenicity and differentiation potential of an unknown population of stem/progenitor cells. However, such assays are retrospective and fail to predict long-term reconstitution activity. Furthermore, it is well accepted in the field that immunostaining *in vitro*-cultured HSCs does not faithfully mark functional stem cells.
Given these problems, the main goal of this 1-year project is to identify predictive metabolic markers of the HSC state and to validate these markers on stem cells exposed *in vitro* to self-renewal and differentiation culture conditions, as well as different oxygen tensions. In contrast to the widely used 'immunophenotyping' of stem and progenitor cell populations via antibodies against specific receptors, we will focus on metabolic markers of the stem/progenitor cell state, since recent studies have shown that HSC quiescence and self-renewal are at least partially controlled through regulation of their metabolic state (e.g. (2)).

Method and Results
in progress (present status)

Long-term HSCs in mice are exclusively marked by low metabolic activity (preliminary data)
In a preliminary experiment, we tested whether progenitor/stem cells having lower metabolic activity represent long-term repopulating (i.e. functional) HSCs. To this end, Lineage^{negative} Sca-1^{positive} c-kit^{positive} (LKS) cells were purified by FACS based on mitochondria activity using tetramethylrhodamine methyl ester (TMRM), a cell-permeant fluorescent dye that is sequestered by active mitochondria (Figure 1). #Figure# Strikingly, upon transplantation of 1000 cells of

each population into lethally irradiated mice, we detected long-term reconstitution activity exclusively in the cell population with low mitochondria activity. This strongly suggests that mitochondria stainings might be predictive markers of the HSC state that could also be applied to *in vitro* cultured cells, for example to identify stem cell expansion conditions.

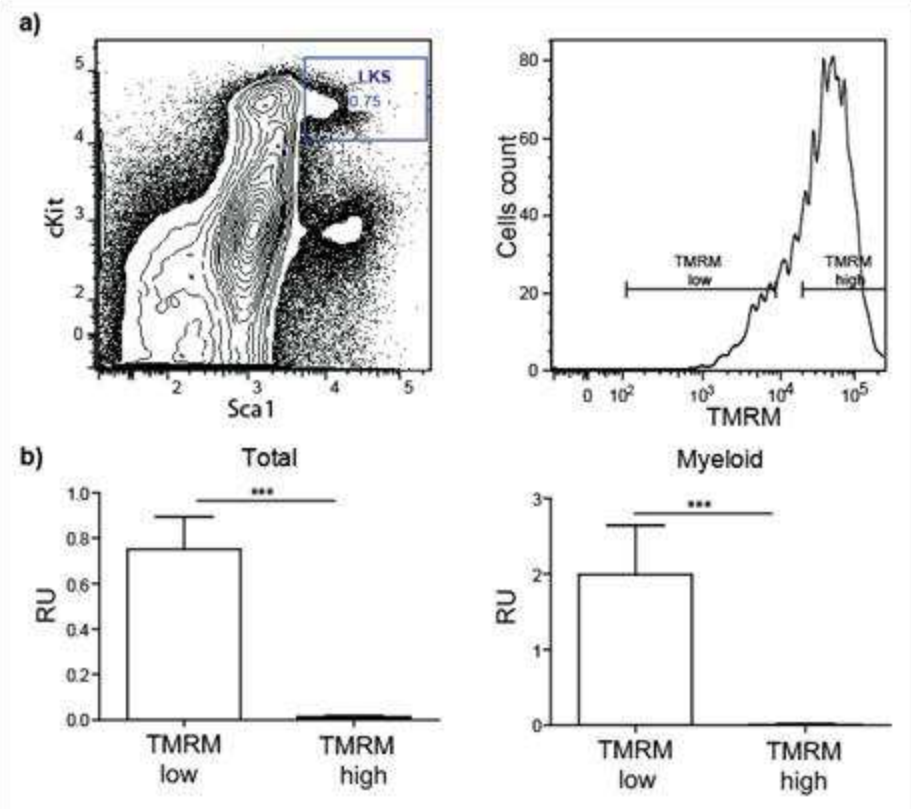

Figure 1: Transplantation of sorted stem/progenitor cells based on mitochondria activity. a) LKS are sorted in two populations based on their TMRM intensity (low and high). b) Total blood and myeloid compartment analysis of lethally irradiated transplanted mice demonstrate that only cells TMRM low (low mitochondrial activity) engraft successfully.

Probing mitochondria activity by microscopy-based techniques
Based on these encouraging data, we aim to establish a microscopy-based method to analyze and distinguish mitochondria in primary HSCs and progenitors *in vitro*. First, we will stain mitochondria by mitotracker and TMRM in freshly isolated, fixed HSC/MPPs and analyze them by confocal microscopy at high magnification. One read-out that we plan to use is the mitochondrial morphology that can be indicative of the mitochondrial activation status of stem cells (3). Mitochondria with low metabolic activity can thus be expected to have a punctated morphology, whereas highly metabolic mitochondria tend to fuse and form a network-like structure (4). We will develop image analysis tools to quantify individual mitochondria in single cells in order to extract information on the stemness. Furthermore, a measurement of the fluorescence intensity of the TMRM staining should provide a good read-out of mitochondrial activity. Finally, we will analyze mitochondrial morphology (by Mitotracker) and activity (by TMRM) in live single cells by fluorescent (confocal) microscopy, which could yield a potent *in vitro* read-out on the stem versus progenitor cell state.

Assessment of metabolic marker changes during in vitro culture
We will apply the above-mentioned read-outs to monitor how individual HSCs change their metabolic phenotype during *in vitro* culture conditions, inducing known self-renewal versus differentiation divisions, as well as under hypoxic culture conditions (1-5%, using premixed gas with defined oxygen composition). As the native HSC niche is an extremely hypoxic microenvironment that forces cells into low metabolism, we expect hypoxic conditions to contribute to the maintenance of the stem cell state *in vitro* as well. Oxygen tension will be measured using an oxygen-sensitive ruthenium probe (FOXY-slide, Ocean Optics, Inc.).

Conclusions and Relevance for 3R
HSC transplantations, currently the only functional means to identify and characterize mammalian HSCs, are radical and expensive procedures used in countless labs all over the world. Therefore, the identification of novel *in vitro* markers that are predictive of the functional HSC state would be highly significant. Among other things, it could open up new avenues to rapidly identify stem cells under conditions of *in vitro* expansion.

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3R-Project 133-12

Development of an in vitro system to grow and investigate vascular endothelial cells under physiological flow conditions

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Background and Aim

Activation and damage of vascular endothelial cells is an important mechanism in the pathophysiology of several diseases and clinical entities. In particular, it plays an important role in cardiovascular diseases. The activation of endothelial cells is essential in transplantation also, because they are the donor cells that make first contact with the recipient's immune system.

It is relatively easy to isolate and culture endothelial cells, at least from large blood vessels, but currently available *in vitro* systems offer only limited scope to replace animal experiments since the endothelial surface layer, the so-called glycocalyx, looks different when building up under normal, static culture conditions as compared to the *in vivo* situation with pulsatile blood flow [1],[2]. Available systems for endothelial cell culture under flow either provide only two-dimensional culture of the cells, for example on the bottom of micro channels, or three-dimensional culture in opaque, tubular carriers and technically demanding setups.

We want to develop a new, simple *in vitro* model to grow and investigate endothelial cells using physiological conditions of pressure and flow.

Method and Results

in progress (present status)

The new system will be based on application of nanotechnology to reach optimal conditions for attachment and growth of the cells. The use of transparent carriers will allow the observation of interactions between the cultured endothelium and blood cells using normal intravital microscopy. So far, endothelial cell adherence and growth on different nanostructure patterns has been tested (Fig. 1), and a method to coat glass tubes with defined nanostructures has been developed (Fig. 2).

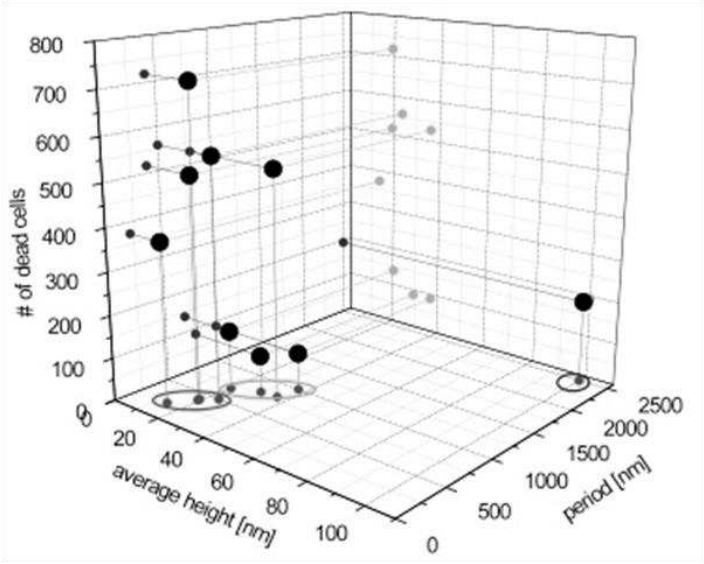

Figure 1: Comparison of 9 nano features coated on flat cover slips. When the number of dead cells was compared, it was lowest for nano surfaces of average height (20-40 nm) and average period (500-1000 nm; circled in green).

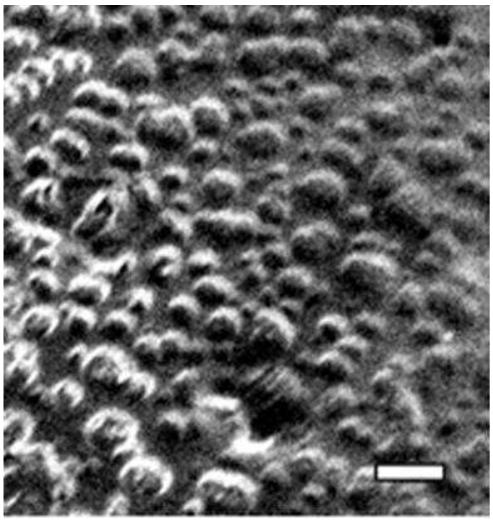

Figure 2 :
Scanning electron microscope that shows coating of nano surfaces on the inside of glass tubes. Scale = 3000 nm.

Conclusions and Relevance for 3R
Currently available, preliminary data suggest that the setup of the model is feasible. Once established, we hope that the number of animal experiments in which the investigation of endothelial function plays an important role can be reduced thanks to the novel *in vitro* system. This is particularly true for experiments on ischemia/reperfusion injury as well as transplantation, which usually are quite traumatic for the animals.

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3R-Project 134-12

Cardiovascular simulator with autoregulation

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Background and Aim
Regulatory approval of new cardiovascular devices depends on data obtained in in vitro and in vivo tests. Even though the in vivo tests can never be completely eliminated from the process, we believe that it is possible to enhance the importance and validity of *in vitro* simulators so they can replace part of the *in vivo* studies and thereby reduce the number of animal tests . Our proposed simulator will contain autoregulation mechanisms such as baroreflex and the Frank-Starling mechanism that will make it respond to hemodynamic interventions just as a patient would, and therefore will be more attractive for prospective tests.

Method and Results
in progress (present status)
An advanced simulator will be based on lumped parameter modeling of the cardiovascular system. Hereby properties of the circulation that are normally geometrically distributed will be compiled in one discrete component. For example, the hydraulic resistance built up in all the capillaries can be implemented as one resistor. Our lumped parameter model will be partially implemented in software and partially in hardware.

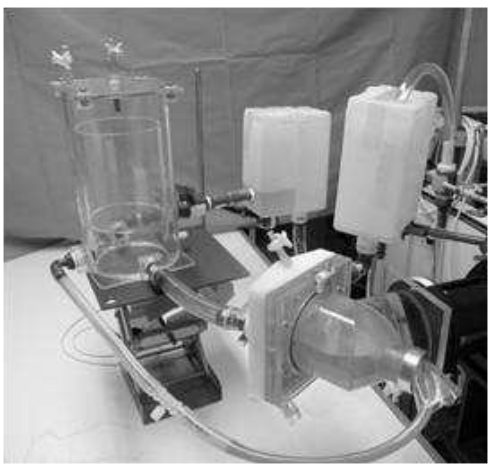

Figure 1: Hydraulic lumped parameter simulation of the systemic circulation with a pumping left ventricle in the foreground

For the hardware, an existing simulator of the left ventricle and systemic circulation will be expanded to include the pulmonary circulation. Also anatomically correct elastomeric models will be produced of heart chambers and blood vessels to enable the use of real cardiovascular interventional devices such as catheters, heart valves, and ventricular assist devices.

Components of the circulation that are not implemented in hardware can be programmed in a mathematical model (programmed in Matlab, The Math Works Inc.) where hydraulic and mechanical properties are captured by differential equations. In this way, the model becomes a hybrid between numerical and in vitro simulation that is modular and allows the user to choose only the hardware necessary for a specific test while the unneeded components are simulated on the computer. This yields for the tester the simplest hardware model possible, while no accuracy of the simulation is sacrificed. An additional advantage of the computer model is that it can interact with actuators in the hardware (pneumatics, motors) and that autoregulation functions such as baroreflex and Starling-responsiveness of the heart chambers can be implemented. This means that the hemodynamic results of cardiovascular interventions will be effectively represented and that for instance a valve repair will effectively result in a reduced stroke volume and lower heart rate, as it happens in real patients.

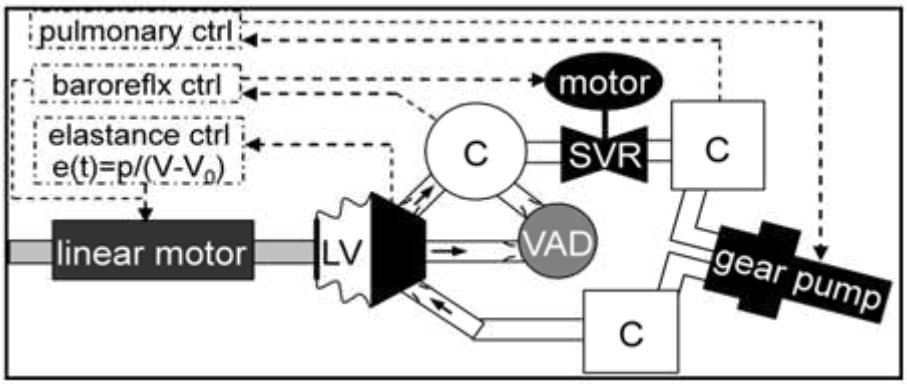

Figure 2: Schematic overview of a possible implementation of the hybrid simulator

Conclusions and Relevance for 3R

The final model can be used as a multifunctional testing tool for various cardiovascular devices that are under development. It can further be used as a training tool for the users of such devices. Thereby its main relevance lies in the reduction of the number of animals needed during the design and test phases of new devices. The advantages are repeatability, visibility, and a more controlled environment to come to findings with higher statistical significance. In addition, the simulator can be used as a training tool for students to get familiar with the physiology of the cardiovascular system and its responsiveness.

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