Protein biomarkers of embryotoxicity

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Biomarkers for pathways of toxicity

- **The biological reaction to toxic intervention is complex in molecular and kinetic terms**
- **Toxic exposure elicits immediate, intermediate and more long-term consequences: Necessity of kinetic control and synchronization**
- **Biomarker could be found on all molecular levels: DNA, RNA, proteins, metabolites**
- **Modern technologies applied in European projects: transcriptomics, epigenetics, proteomics and metabonomics**
- **Innovative toxicology testing clearly aims to avoid animal testing;**
- **Model systems submitted to the various "-omics"-technologies are preferentially human in vitro systems**

Overview about biophysical spaces of OMICS-technologies

- **Transcriptomics: # of species: a few millions?; abundance range: up to 5 o.m?**

  Arrays with e.g. 45,101 probe sets;

  Several amplification steps (RNA, cDNA, biotinylated cRNA); The detection limit is approx. 1.5 pmol

  The linear dynamic range is 1.5 to 100 pmol (2 orders of magnitude)

  Validation of interesting candidates by qRT-PCR or shRNA

- **Epigenetics: # of species less than a million? Abundance range: up to 5 o.m?**

  Microarrays, e.g. containing ~237 000 probes covering all known CpG islands (~27 800), and ~5 000 unmethylated regions;

  Detection limit (1 pmol) and linear dynamic range (1- 100 pmol)are comparable to transcriptomics;

The time frame of investigation after toxic exposure is rather in the range of many hours

Overview about biophysical spaces of OMICS-technologies

- **Proteomics: # of species: dozens of millions? Abundance range: up to 10 o.m.**

Methodology is heterogeneous: Quantification and identification are separated;
Separation of proteins by 2D-PAGE, of peptides from fragmented proteins by LC;

Radioisotopic labeling: detection limits of 1 amol and a dynamic range of 5 orders of magnitude;
Fluorescent labeling: d.l.: 1 -10 fmol, d.r.: 2-3- o.m.

Mass spectrometry for identification (MALDI-TOF, Q-TOF-MS-MS; iTRAQ);
Quantitative limit of MS methods is 1 fmol and the linear dynamic range is from 1 to 500 fmol

Cave: Prior fractionation steps necessary (e.g. acidic/phosphoproteome); these steps and the liquid chromatography need quantitative and quality control, SOP's

Strength: Proteomic methods can pick up very early events after toxic exposure

Overview about biophysical spaces of OMICS-technologies

- **Metabonomics: # of species: millions? Abundance range: up to 6 o.m.?**

NMR; ^{1}H-Nuclear magnetic resonance spectroscopy and spectral processing; multivariate pattern recognition
Detection limit is in nmolar range, linear dynamic range is one order of magnitude

Mass spectrometry; MS/MS; detection limit of both methods is 1 fmol and the linear dynamic range is from 1to 500 fmol; same considerations as for proteomics WP's

Cave: Still needs animals (or is cell culture metabonomics entering the stage?);
The liquid chromatography steps need quantitative and quality control, SOP's

Time frame: late after exposure

Synopsis of nucleic acid analysis

- Expression and epigenetic profiling produce quantitative fluorescent signals from hybridization of nucleic acids to arrays
- In one case immunoprecipitation precedes NA analysis (aceytlated histones)
- Affymetrix and Agilent technologies used (common output formats: Excel)
- Linear dynamic range is 2-3 orders of magnitude
- Detection limit is around 1 pmol
- Normalization, Reproducibility and Repeatability: Robust statistical methods established

Synopsis for Proteomics & Metabonomics

- Several different types of quantitative information:

Relative abundance ratios from radioactive signals of differentially labeled proteins: linear dynamic range, 5 orders of magnitude (PSY, 2-D PAGE) (detection limit 1 amol)

Mass spectrometry of proteins (2-D PAGE, LC-MSMS): linear dynamic range 2 orders of magnitude

Mass spectrometry of metabolites, small molecules: LC-MSMS: linear dynamic range 2 orders of magnitude;

- Detection limit for MS methods: 1 fmol, dynamic range 2-3 orders of magnitude

- Normalization? Reproducibility and Repeatability: Clear SOP‘s needed!

- NMR: linear dynamic range 1-2 orders of magnitude, detection limit 100 nmol;

Cell culture (is it always mixed cultures?): Biological stochasticity

Functional read-outs for dose-response curves dependent on cell compositions and time courses of cellular differentiation (or dedifferentiation)

- Hepatocytes assessed with readouts
  - basic hepatocellular functionality
  - impedance measurement (xCELLigence system)
  - phase I biotransformation capacity
  - phase II biotransformation capacity
  - drug transporter capacity
  - cell death
  - cholestasis
  - steatosis and phospholipidosis
- Cardiomyocytes
  - electrical activity by microelectrode arrays
  - impedance measurement (xCELLigence system)
  - release of cytoplasmic proteins (Troponin T, Creatine Kinase MB)
- Renal epithelial cells
  - impedance measurement (xCELLigence system)
  - lactate production
  - release of cytosolic enzymes (LDH)
- Neurons from hESC for DNT
  - various

Protein biomarkers

Differential proteomic approach

Protein biomarkers for in vitro testing of embryotoxicity www.reprotect.eu

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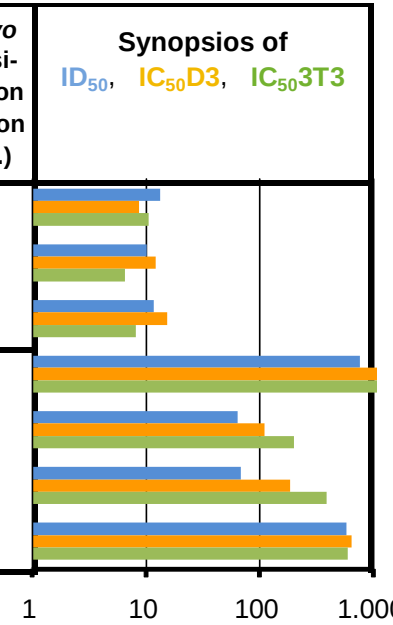
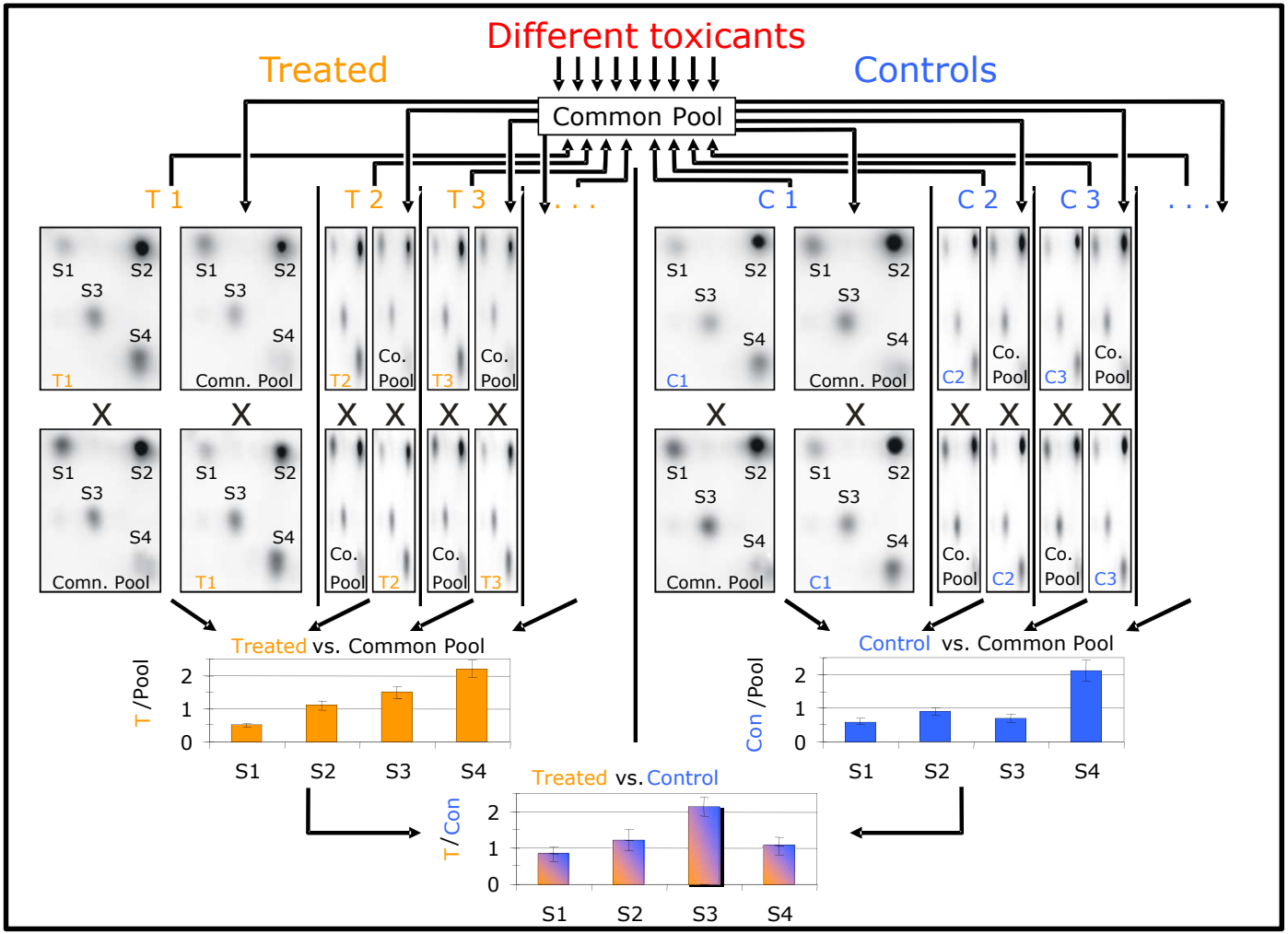
Results from Reprotect (www.reprotect.eu)

Groebe et al (2010) J. of Proteome Research, 9, 5727
Groebe et al. (2010) Reproductive Toxicology 30, 121

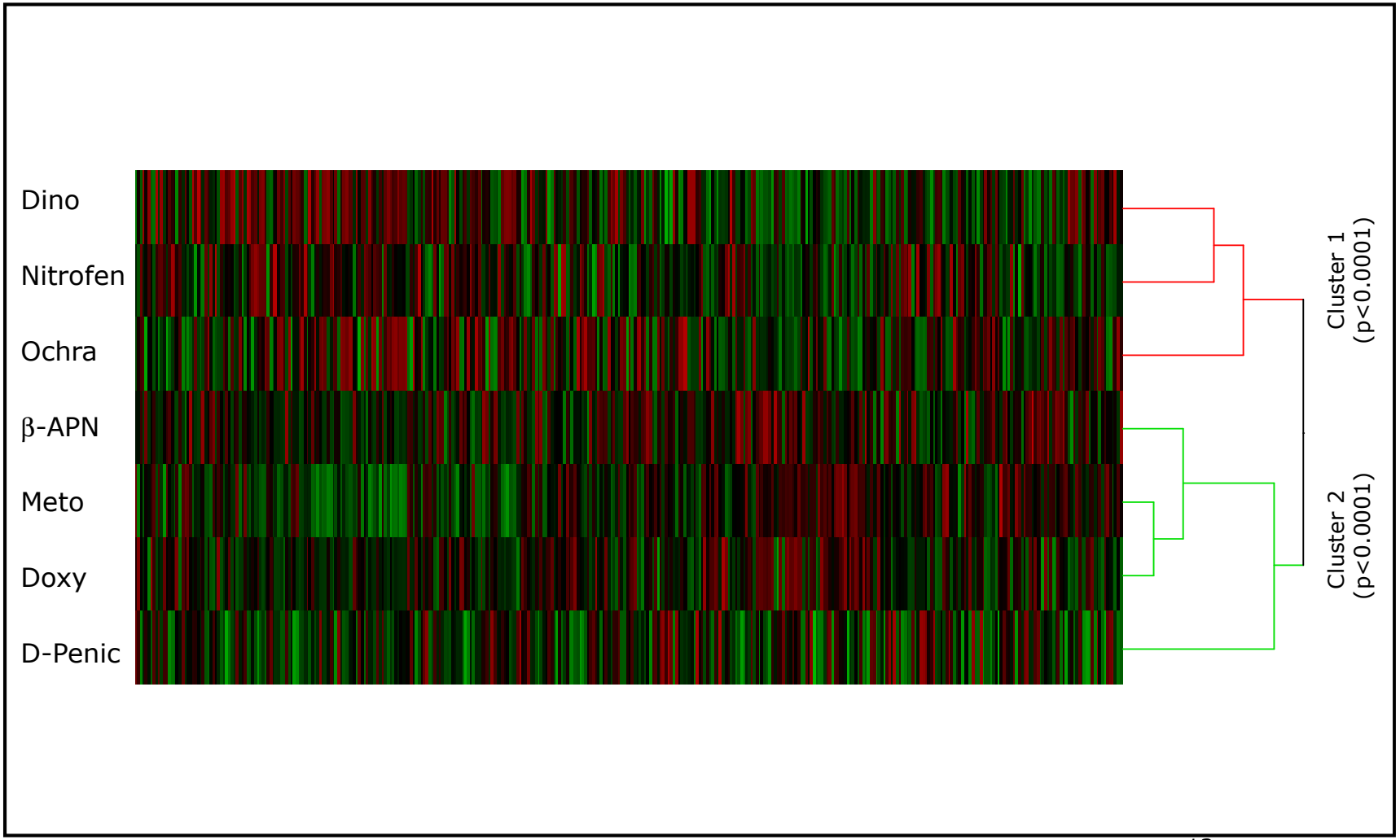
Functional end points of the embryonic stem cell test (EST) as substrate for differential proteomic profiling

Chemical	ID_{50} (myocardial differentiation)	confidence interval		$IC_{50}D3$ (Cytotoxic effects on ES cells)	confidence interval		$IC_{50}3T3$ (Cytotoxic effects on 3T3 fibroblasts)	confidence interval		*in vivo* classi-fication (Daston et al.)
		Lower bound	Upper bound		Lower bound	Upper bound		Lower bound	Upper bound	
Dino	**13.3**	9.12	19.3	**8.73**	6.21	12.3	**10.6**	10	11.2	3
Ochra	**10.2**	9.64	10.8	**12.1**	8.71	16.7	**6.54**	5.96	7.17	1
Nitrofen	**11.7**	11.5	11.9	**15.3**			**8.18**	7.48	8.95	1
β-APN	**765**	763	767	**1280**	995	1640	**1070**	846	1350	3
Meto	**64.4**	63.9	64.9	**109**	96.3	123	**200**	69.8	574	4
Doxy	**68.3**	65.3	71.4	**184**	66.8	509	**387**	121	1240	4
D-Penic	**581**	514	657	**634**	506	794	**589**	544	638	1

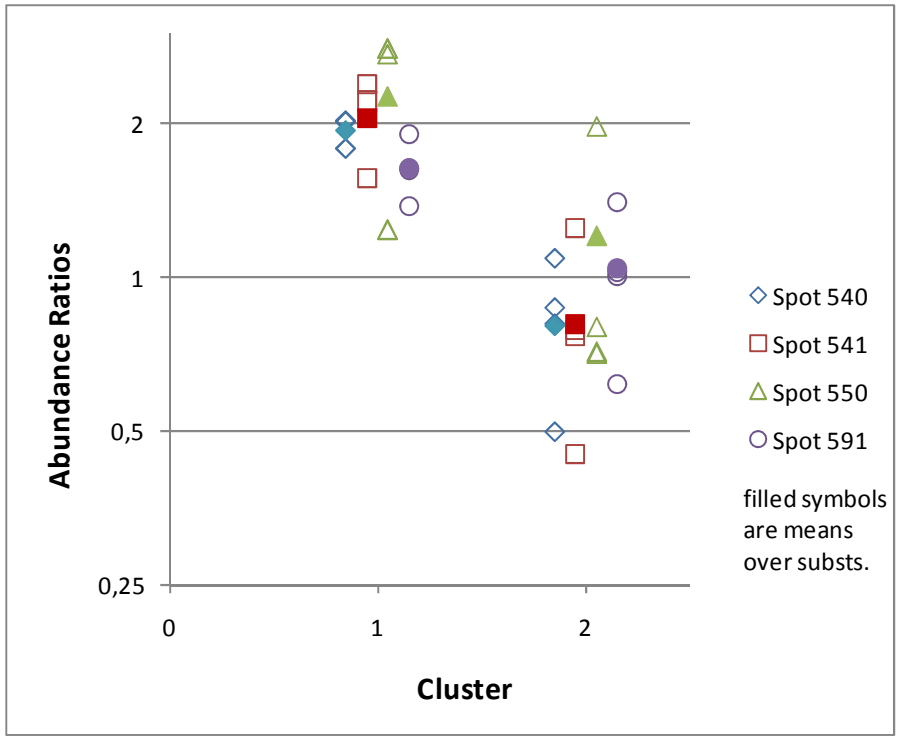



Different toxicants
Treated
Controls
Common Pool
T 1
T 2
T 3
. . .
C 1
C 2
C 3
. . .
S1
S2
S3
S4
T1
Comn. Pool
T2
Co. Pool
T3
C1
C2
C3
X
Treated vs. Common Pool
T /Pool
Control vs. Common Pool
Con /Pool
Treated vs. Control
T/Con
0
1
2

Proteomic changes separate embryotoxicity and cytotoxicity despite contributions from different cell types in mixed cultures

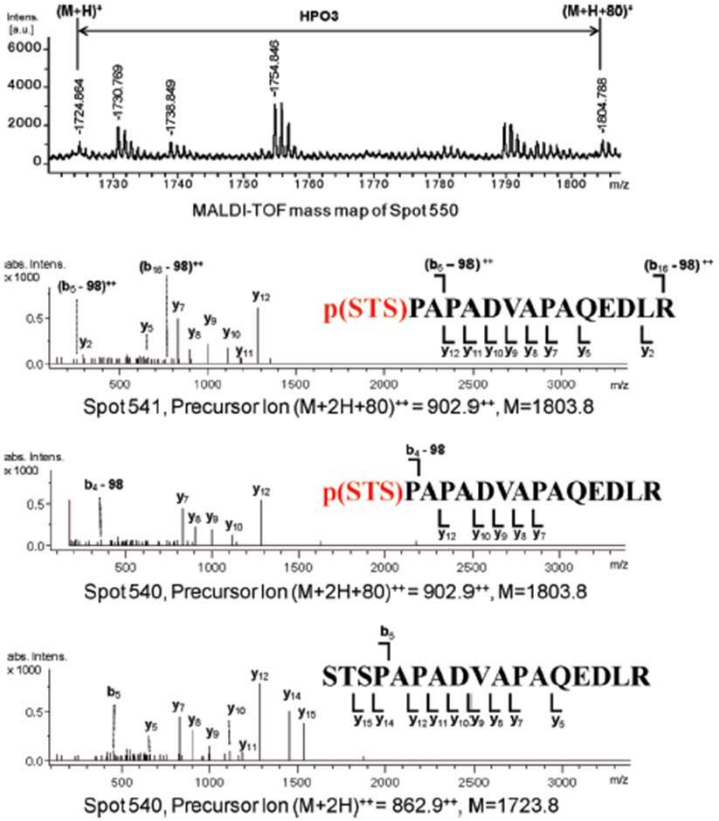


The few candidate biomarker proteins have patterns of redundant posttranslational isoforms, identifying nodal events in pathways derailing from normal function during toxic exposure



Candidate biomarker proteins analysed from the EST model are closely related to the “hot spots” in ageing, cancer and development

Some thoughts about the Ras pathway



The list of potential early signature protein biomarkers is short

Ras-GTPase-activating protein SH3-domain binding protein; 3 different isoforms (phosphorylation and fragment)

Nucleophosmin-1; 3 different isoforms
(phosphorylation and fragments)

HSP27; 3 different isoforms (phosphorylation and fragments)

Calreticulin(phosphorylation)

Dihydropyrimidinase-related protein 2 (phosphorylation and methylation)

One Protein is a marker for general cytotoxicity (an important one to have in case of mixed cell cultures) :

FSCN1 protein, fascin homologue 1, actin bundling protein;

Summary

- **Very specific posttranslational isoforms of ras-related proteins behave cluster-dependent and have potential as biomarkers for embryotoxicity**
- **Another set of posttranslational isoforms of structural proteins provide surrogates for general cytotoxicity form the very same set of samples**
- **Molecular integration in quantitatively and statistically controlled proteomic experiments provides high content information, even on a background of a huge degree of biological stochasticity**
- **Further validation in other models and with other methods under way**
- **Translation to higher throughput tests (antibodies, ELISA, targeted MS) under way**

Learning from clinical experience? Example M. Alzheimer

- **Large scale genomic screening for susceptability factors (mutations, SNP's and alleles):**

  ApoE4, APP, presenilins, NRG-1,…….

- **Pathology:**

  Protein aggregates (ß-amyloid, hyperphosphorylated τ-protein)
  Mitochondrial dysfunction

- **Functional level (memory):**

  NMDA- and acetylcholine receptors

- **Prediction:**

  HTS of the whole target space with huge industrial chemical libraries

→ **Success?**

Learning from clinical experience? Clinical diagnostics

- **Multiplexed diagnostic procedures (we talk about dozens of parameters) did not make it. The few key biomarkers are needed, not complex lists with hundreds of parameters**
- **The latest innovative trends , personalized medicine and compagnion dignostics, are based on the insight that human populations are heterogenous**
- **Certain risk groups are profiled on the genetic level (next generation sequencing)**
- **Treatments and disease progression are in turn controlled by more acute biomarkers, mostly proteins**

Innovative toxicity testing: personal view

- **On the background of clincial experiences, like the one above:**

  The key for novel predictive models will be the integration of OMICS-data, and the de novo development of mathematical procedures comforting the molecular and kinetic complexity of biological systems

- **Prerequisite #1:**

  High quality, statistically robust data from OMICS-experiments (2R's: reproducibility and repeatability)

- **Prerequisite #2:**

  High quality, statistically controlled and robust cell culture end points (providing the material submitted to OMICS- anlyses) (2R's again!)

Discussion (like in SEURAT):
Aren't the next important steps all about quality control?

How to deal with kinetics and dose responses of mixed cell cultures?

How to move towards integration? Common procedures of data acquisition, storage, validation and statistics