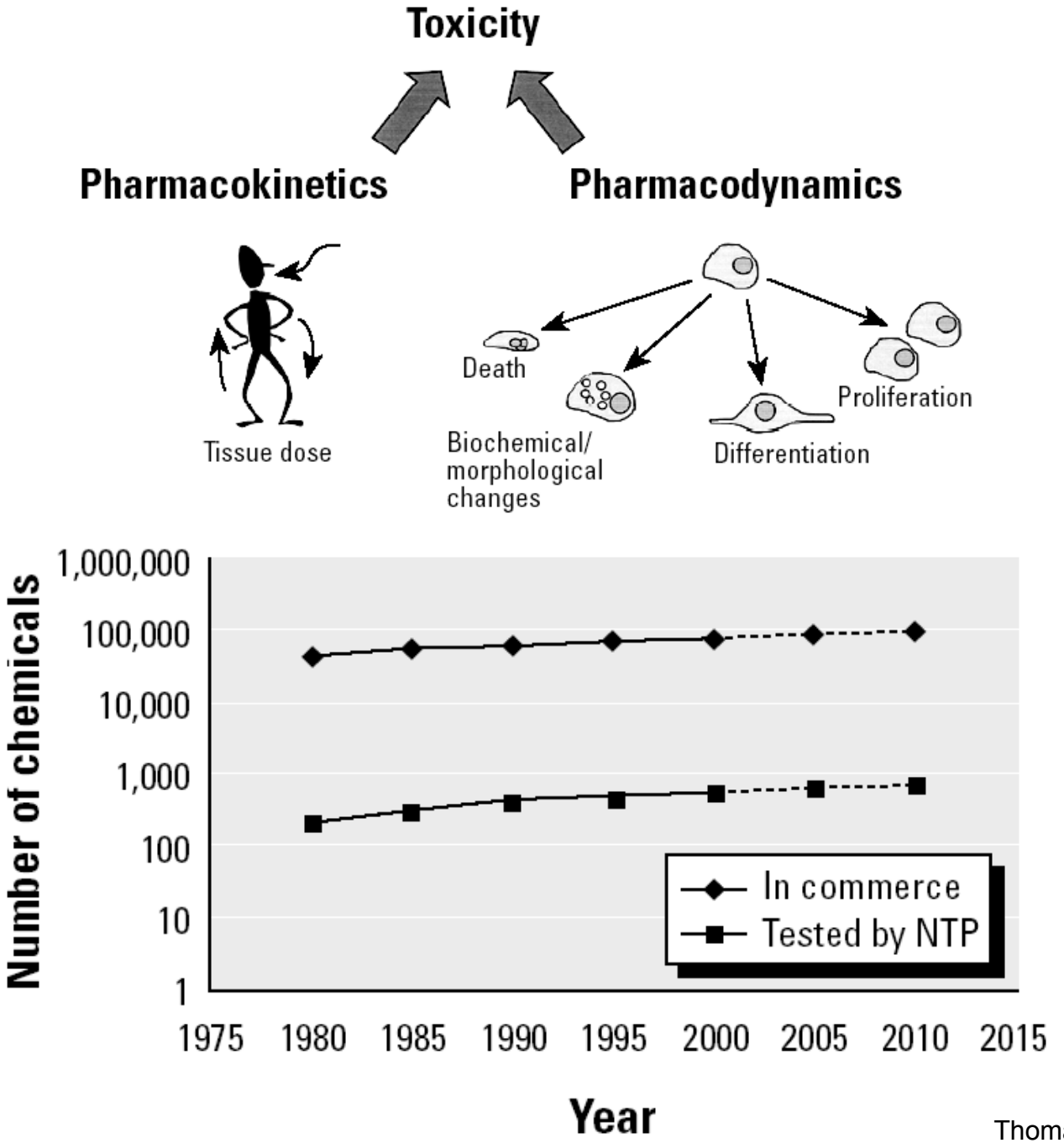
Model Systems and Organisms in Toxicology



Thomas et al. *EHP* (2002)

Parallelogram Approach to Characterize Toxicity

in vivo
characterise toxic effects in animal experiments
(e.g. determine LOEL)

take exposure situation into account

assess hazards for the human

extrapolation from in vitro to in vivo

in vitro
analyse sensitivities of animal cells, tissues
(e.g. from target tissue) to test compound

interspecies extrapolation

in vitro
analyse sensitivities of human cells, tissues
(e.g. from biopsies) to test compounds

Why Use Models?

Very limited number of studies can be done on humans

Allows for controlled experiments

Environmental variables can be controlled

Dosage/route of exposures can be controlled/varied

Experiments can be replicated

Physiology/anatomy can be matched to humans

Commonly Used Models

Unicellular organisms:

- e.g., bacteria, yeast
- basic cellular questions
- cannot use for multi-cellular interactions

Multicellular non-vertebrates:

- e.g., flies (Drosophila), worms (C. elegans)
- pathway analysis
- only rudimentary physiology, very different from humans

Commonly Used Models

Non-mammalian vertebrates:

- e.g., fish (Zebrafish), frogs (Xenopus)
- pathway and developmental analysis
- differ significantly in physiology from humans

Non-primate mammals:

- e.g., mouse (Mus), rat (Rattus)
- more physiological
- physiology similar, but not identical to humans
- can be engineered to match humans

Commonly Used Models

Non-human primates:

- e.g., baboons
- primate-specific analysis
- very similar in physiology to humans
- very expensive and raises ethical issues

***In vitro* models:**

- e.g., cells (primary/cell lines), organ slices, organelles, proteins, etc.
- basic mechanistic (cellular?) questions
- some degree of cell-cell interactions
- less expensive than animal models
- limited number of ethical concerns
- **THE FUTURE OF TOXICITY TESTING**

Use of Laboratory Animals in Research

- Veterinarians are toxicologists' best friends!
- Understanding of laboratory animals' biology, physiology is far from complete
- There is a wide variety of species from which the toxicologist may choose (background data, biological characteristics, cost, sensitivity, etc.)
- Quality of published information
- Public interest to animal research (PETA)
- Ethical and moral responsibility for the lives of animals used in research
- Responsible use of animals:
  3 "R"s (Refinement, Reduction, Replacement)

Use of Laboratory Animals in Research

- All research using animals must adhere to scientific, institutional, and governmental principles, policies, laws, regulations and guidelines
- Ethical and moral responsibility of each researcher for the lives of animals
- Researchers are responsible for:
    - Quality of animal care
    - Appropriateness of animal use
    - Minimization or relief of pain and distress

Regulations, Laws, Policies and Guidelines

- **Guidelines and Recommendations:**

  Developed by independent groups (e.g., Association for Assessment and Accreditation of Laboratory Animal Care [AAALAC]), are not regulated by law, but can (and are!) be included in an overall policy that governs institutional activities, or eligibility to receive funding

- **Laws and Regulations:**

  require mandatory compliance, failure to comply is enforced by legal actions (fines, revocation of rights to use animals, imprisonment)

USDA administers laws and regulations on use of animals: registration, inspections, control, prevention

USDA → Animal, Plant and Health Inspection Service (APHIS) → Regulatory Enforcement and Animal Care (REAC)

Regulations, Laws, Policies and Guidelines

- **The Animal Welfare Act (1966)**

- **The Guide for the Care and Use of Laboratory Animals**

  is a guideline, not a law, was developed by NAS, used as a reference for voluntary assurance and accrediting bodies such as AAALAC and NIH's Office for Laboratory Animal Welfare (OLAW)

- **Institutional Animal Care and Use Committee**

  is responsible for evaluation and oversight of the institution's animal care and use program and all related issues set forth in *The Guide*:

  Inspects animal facilities and laboratories where animals are used;
  Carries out programmatic reviews of individual research programs;
  Recommends actions to be taken by investigators and/or officials;
  Reviews and approves protocols for animal use in research.

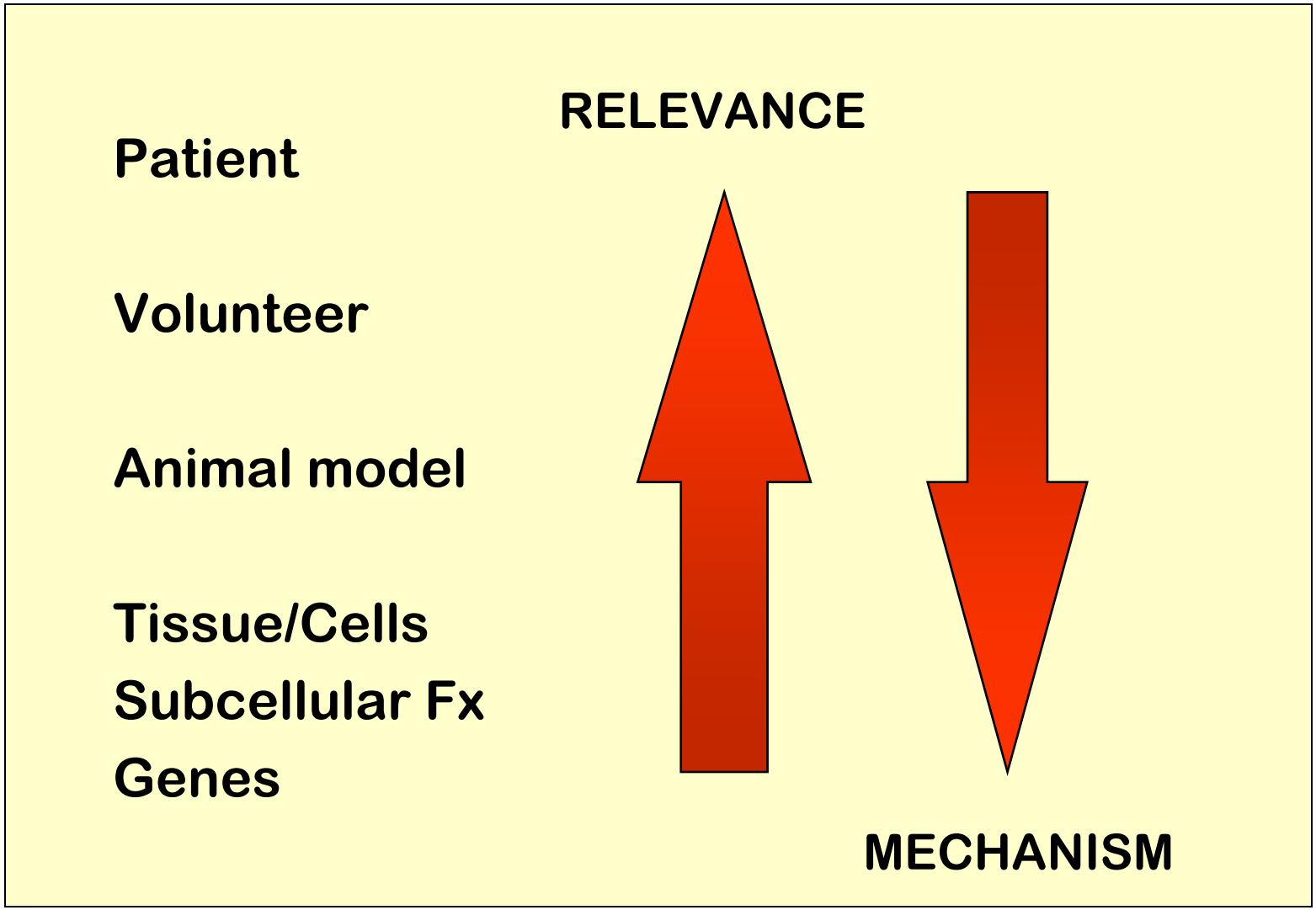
Alternatives to Animal Use in Research

- *In Vitro* models
- Computer-simulated models
- Computer structure-activity analysis

A suitable replacement for animals should:

- Reliably predict biological phenomena
- Be at least as good (or better!) and a consistent model for risk assessment in humans as well as in animals
- Be extensively validated, tested and accepted by regulatory agencies as suitable substitutes

Human Relevance of *In Vitro* Screens



Advantages of *In Vitro* Model Systems

- Relatively inexpensive, reproducible, and efficient ways to investigate toxicity at the cellular and molecular level.
- Facilitate the study of mechanisms of toxicity in specific cell types.
- Allow for rigorous control and easy manipulation of the experimental conditions.
- Significantly reduce the number of animals required for research.
- Provide a means for predicting the interaction and toxicity of xenobiotics with human-relevant systems.
- Provide rapid and effective means of screening and ranking chemicals.
- Are essential for bridging between experimental animals and humans, and for detailed understanding of the bases of species differences.
- Provide well-defined systems for studying structure-activity relationships.

Limitations of *In Vitro* Model Systems

- Not the "real" thing.
- Typically a "static" or "non-native" model system.
- Systematic loss of phenotypic properties or functions of original tissue and cells.
- Similar liabilities with immortalized cell lines.
- *In vivo* relevance often decreases with the simplicity of the model system.

In Vitro Systems for Studying Hepatotoxicity

- Isolated perfused liver
- Liver slices
- Hepatocytes (suspensions, cultures)
- Microsomes/S9 sub-fractions
- Plasma membrane vesicles
- Expressed hepatic proteins - P450's, transporters, receptors

Systems for Studying Liver Enzyme Induction

- *In vivo* animal studies, *ex vivo* analysis
- Liver slices
- Primary cultures of hepatocytes
- Cell lines (HepG2, Huh7)
- Nuclear receptor assays - cell lines stably or transiently expressing appropriate transcription factors and reporter genes
- Binding assays with specific nuclear receptors

In Vitro Procedures for Testing Chemicals as P450 Enzyme Inducers

LIVER SLICES

- In precision-cut liver slices, the extracellular matrix and cell-cell communications are preserved.
- Cells remain viable for several days and are excellent systems for studying phase I and II biotransformation for up to 12 hours, but P450 activity declines rapidly after the first 24-48 hours.
- There are limited data on hepatocellular morphology and function (*i.e.,* liver-specific gene expression) in long-term cultures.
- Liver slices are initially refractory to P450 inducers, and are not very responsive compared with cultured hepatocytes and the situation *in vivo*.

In Vitro Procedures for Testing Chemicals as P450 Enzyme Inducers

CELL LINES (HepG2, Huh7)

- Are transformed cells, so do not express liver-specific genes.
- Resemble extrahepatic tissues in terms of limited responsiveness to enzyme inducers.
- CYP1A1 (CYP3A7?) is inducible, most others are not.

REPORTER GENES

- May need to be transfected into primary cultures of hepatocytes.
- Cell lines may not express all required transcription factors.
- XRE-reporter gene construct is an exception because most cells respond to CYP1A1 inducers.
- Recent development: nuclear receptor reporter assays

Cultures of Primary Hepatocytes

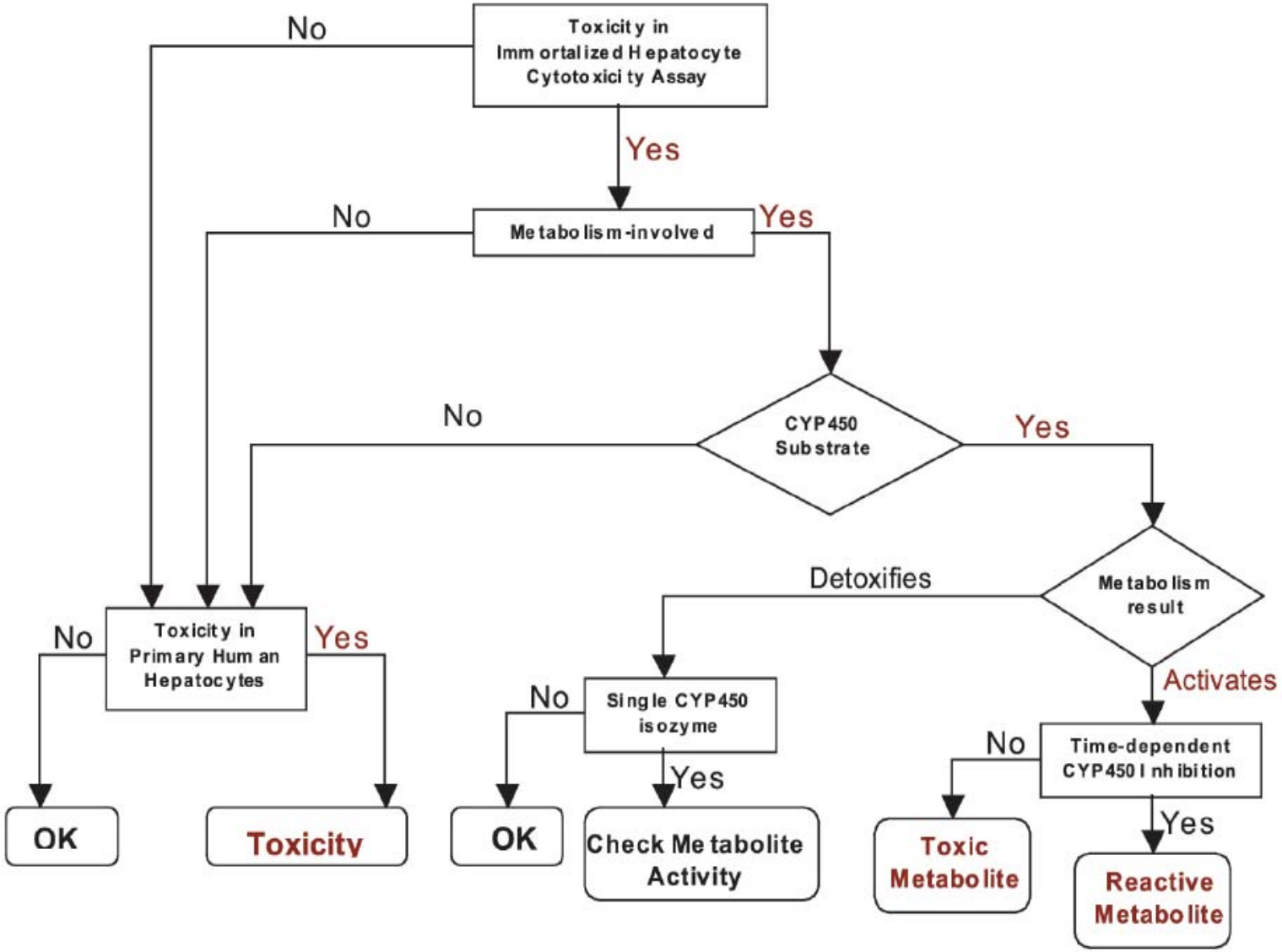
- Intact cells are the *in vitro* system of choice to study metabolism of xenobiotics and to predict chemically-induced hepatotoxicity *in vivo*.
- Intact cell systems retain requisite biochemical and molecular machinery.
- Long-term viability is achievable under appropriate culture conditions.
- Retain *in vivo* sensitivity and selectivity to inducers.
- Exhibit species-specific response to chemical inducers and hepatotoxins.

Factors involved in optimal gene expression in hepatocytes *in vitro*

- Cell-to-cell contacts (high cell density)
- Extracellular matrix not as critical but helps maintain cell contacts
- Composition of culture medium not as critical (WEM, DMEM, L-15)
- Supplements (insulin, glucocorticoids), important for long-term cultures

Principle culture systems for hepatocytes:

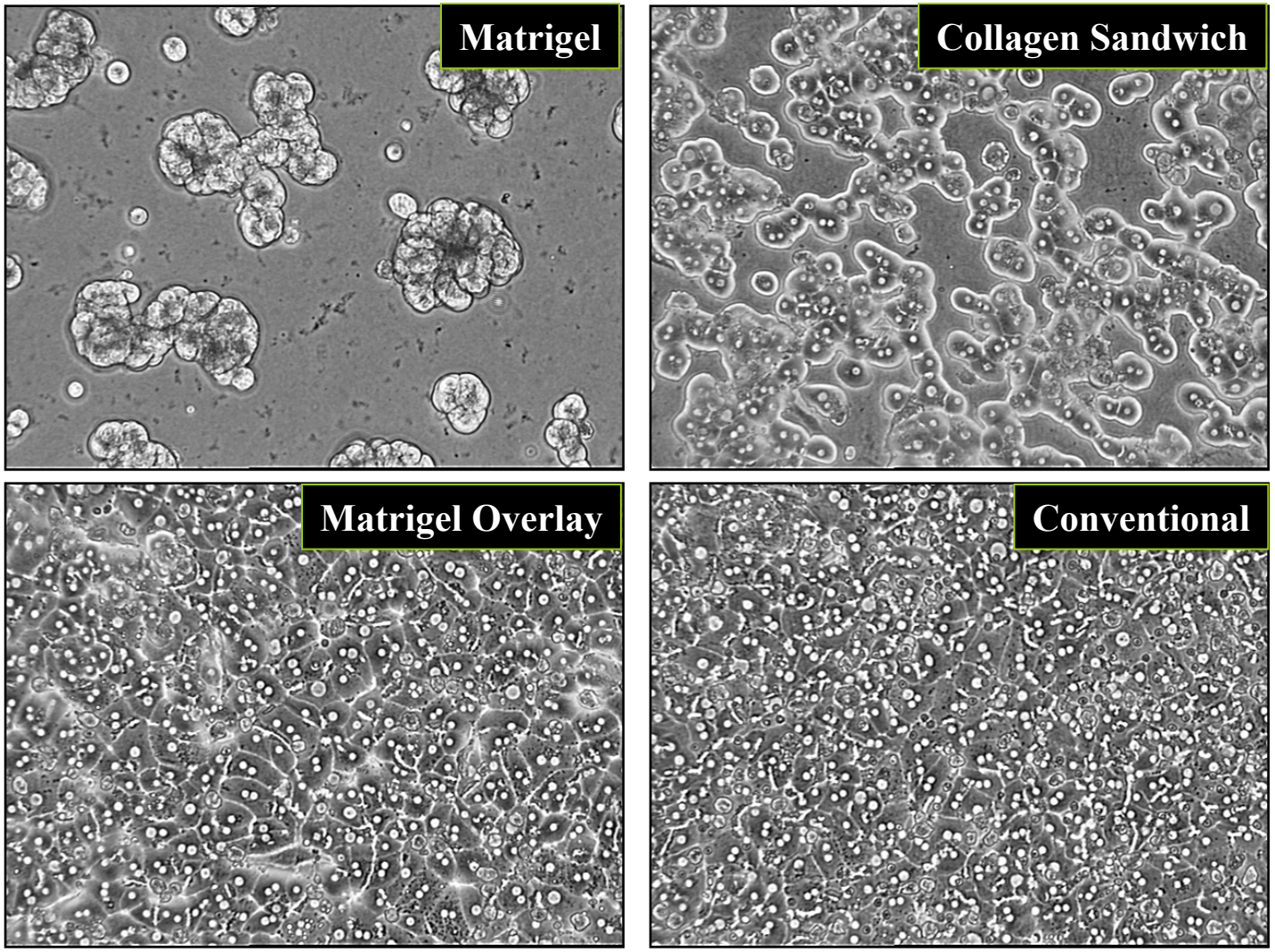
- Conventional monolayer culture
- Matrigel® substratum
- Collagen or Matrigel® sandwich
- Co-culture with other cell types
- Spheroid culture



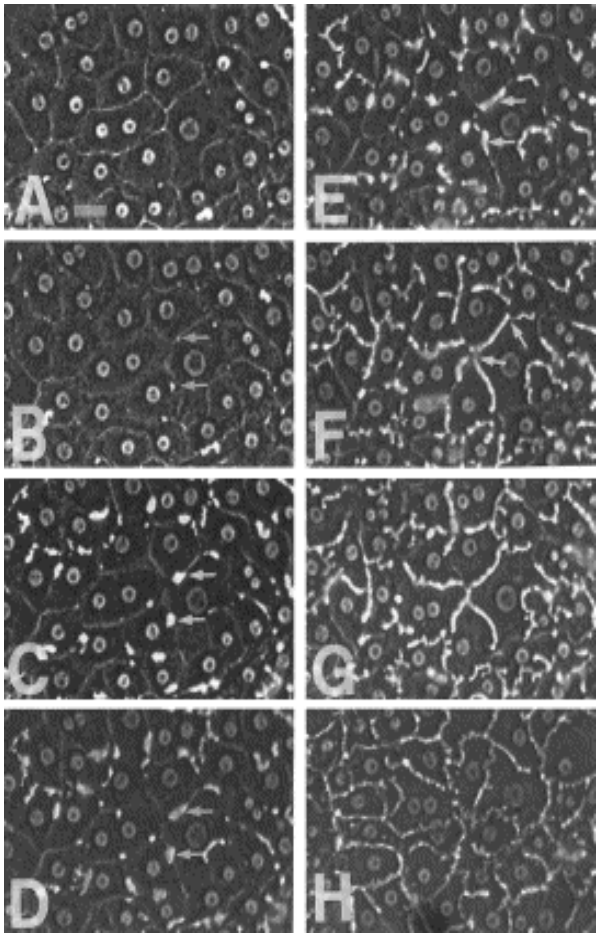
In vitro multi-tier hepatotoxicity screening paradigm illustrating the use of an immortalized human hepatocyte cell line assay system followed by assessment in primary human hepatocytes and evaluation of potential metabolites.

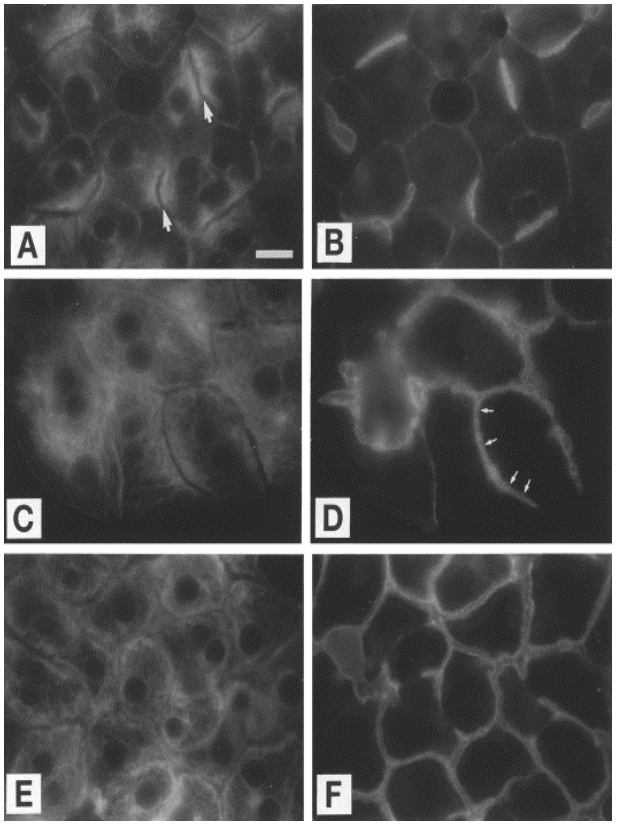
From Dambach et al 2005

Human Hepatocytes Cultured under Different Matrix Conditions



Hepatocyte Culture Conditions



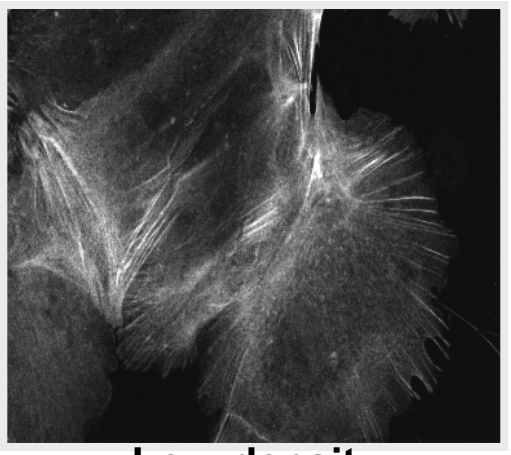


Microtubules **Microfilaments**

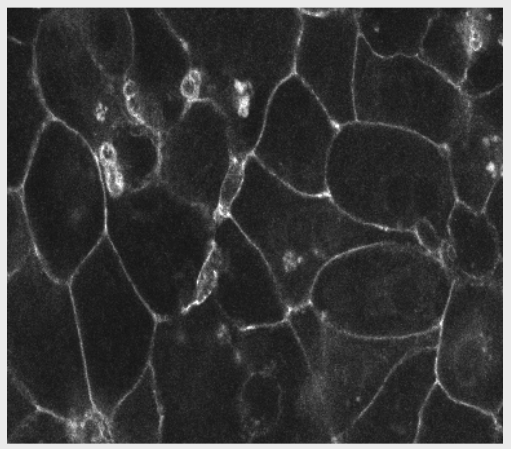
Time course of BC development in a culture (high density) of rat hepatocytes from 4 to 48 hr after collagen overlay illustrating the pattern of network formation

LeCluyse *et al.*, *Tox In Vitro* (2000)

Microfilaments

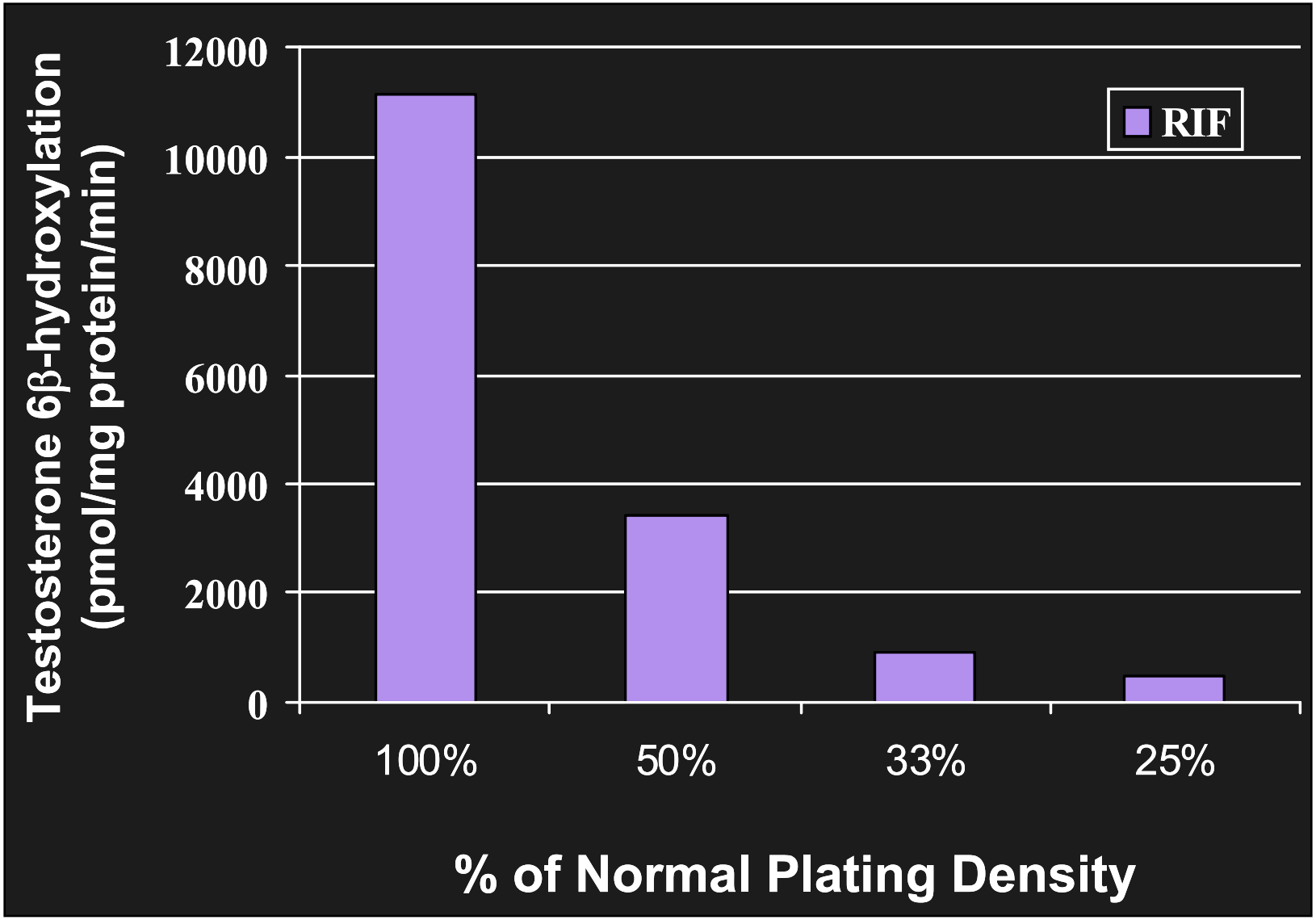


Low-density



High-density

Cell density effect on P450 expression



How is enzyme induction compared between treatments?

- **EC_{50}:**
  - Effective concentration for 50% maximal induction
- **Potency Index:**
  - Percent induction by test compound compared to that of a “gold standard” (e.g., RIF = 100%)
- **Induction Index:**
  - Fold induction or % of control activity
- **K_d:**
  - requires specialized binding assay
  - antagonist or agonist?

P450 Enzyme Induction: *In Vitro* Considerations

- Exposure time important (# of days)
- Relevant concentration range and endpoint important
- mRNA levels may not reflect P450 enzyme activities
- Imperative to compare response with positive (and negative?) controls
- Major species differences exist (*e.g.,* RIF, PCN, DEX)
- EC_{50}'s may be more relevant than any other endpoint (*e.g.,* "potency index"), but may not tell the whole story

In Vitro Procedures for Testing Compounds as P450 Enzyme Inducers

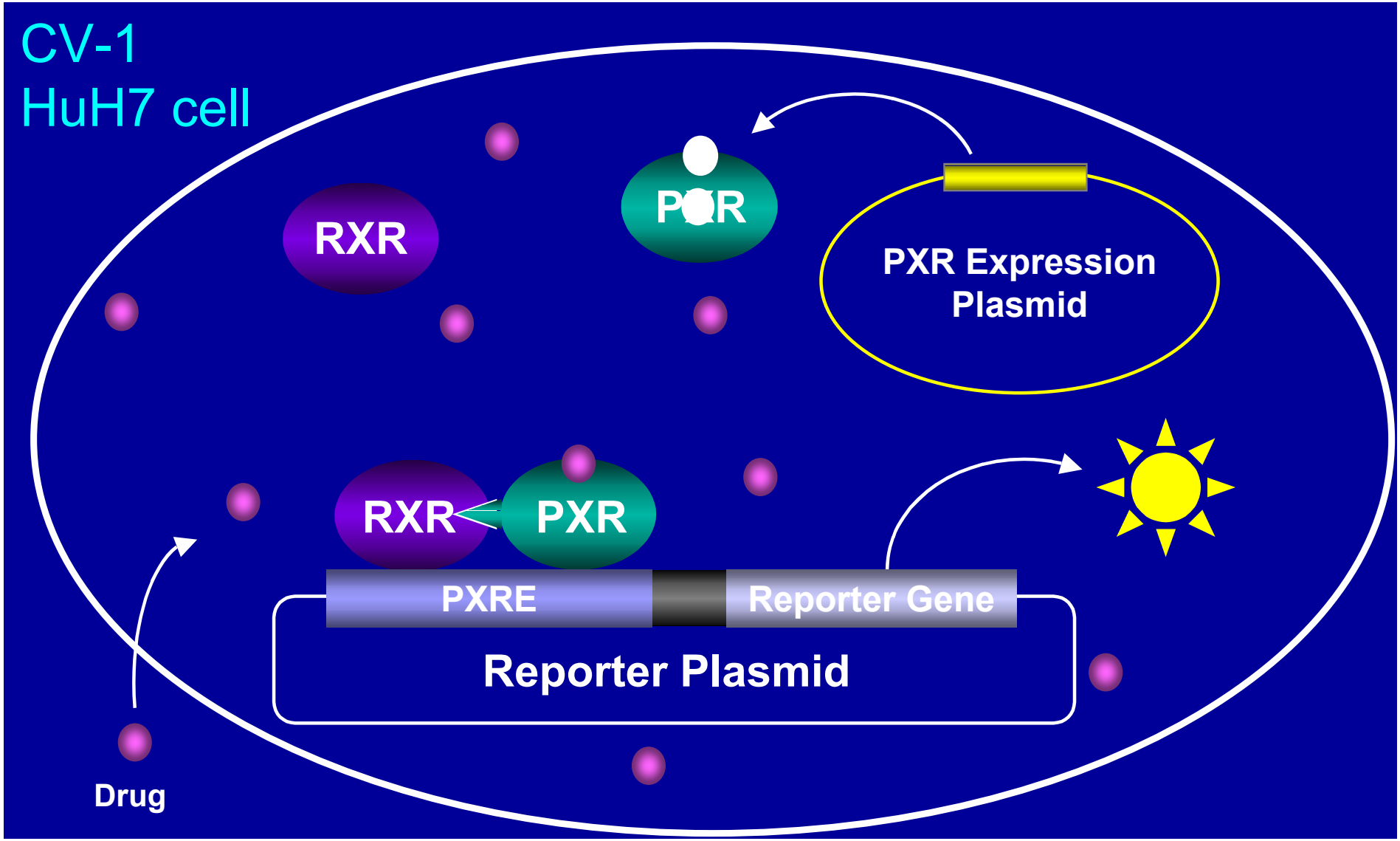
FALSE NEGATIVES

- Inducer is a metabolite (musk xylene, cyclophosphamide).
- Chemical concentration is too low.
- Induction is not primarily dependent on transcriptional activation.

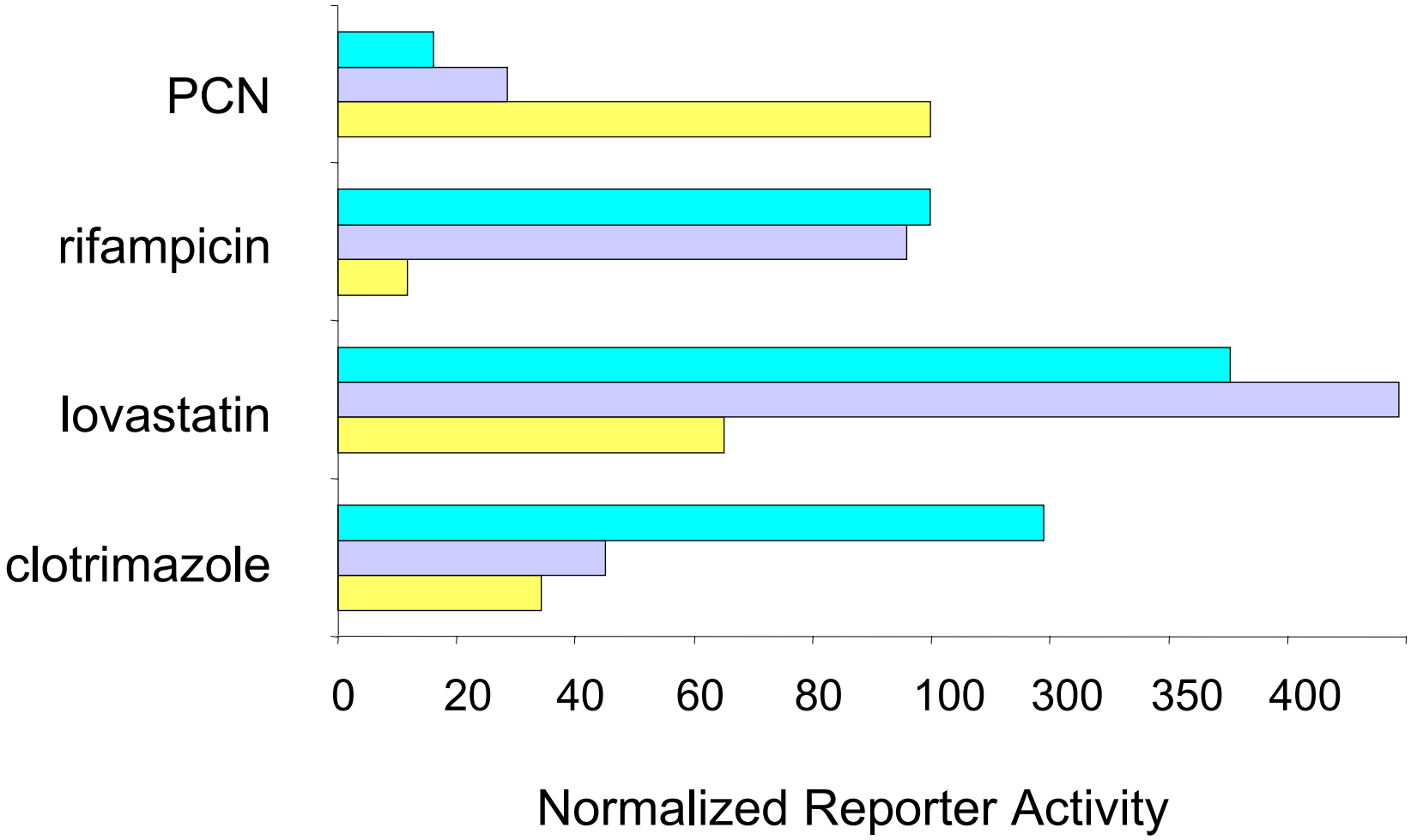
FALSE POSITIVES

- Chemical concentration is too high (clinically irrelevant).
- Parent compound is extensively metabolized/degraded *in vivo*.

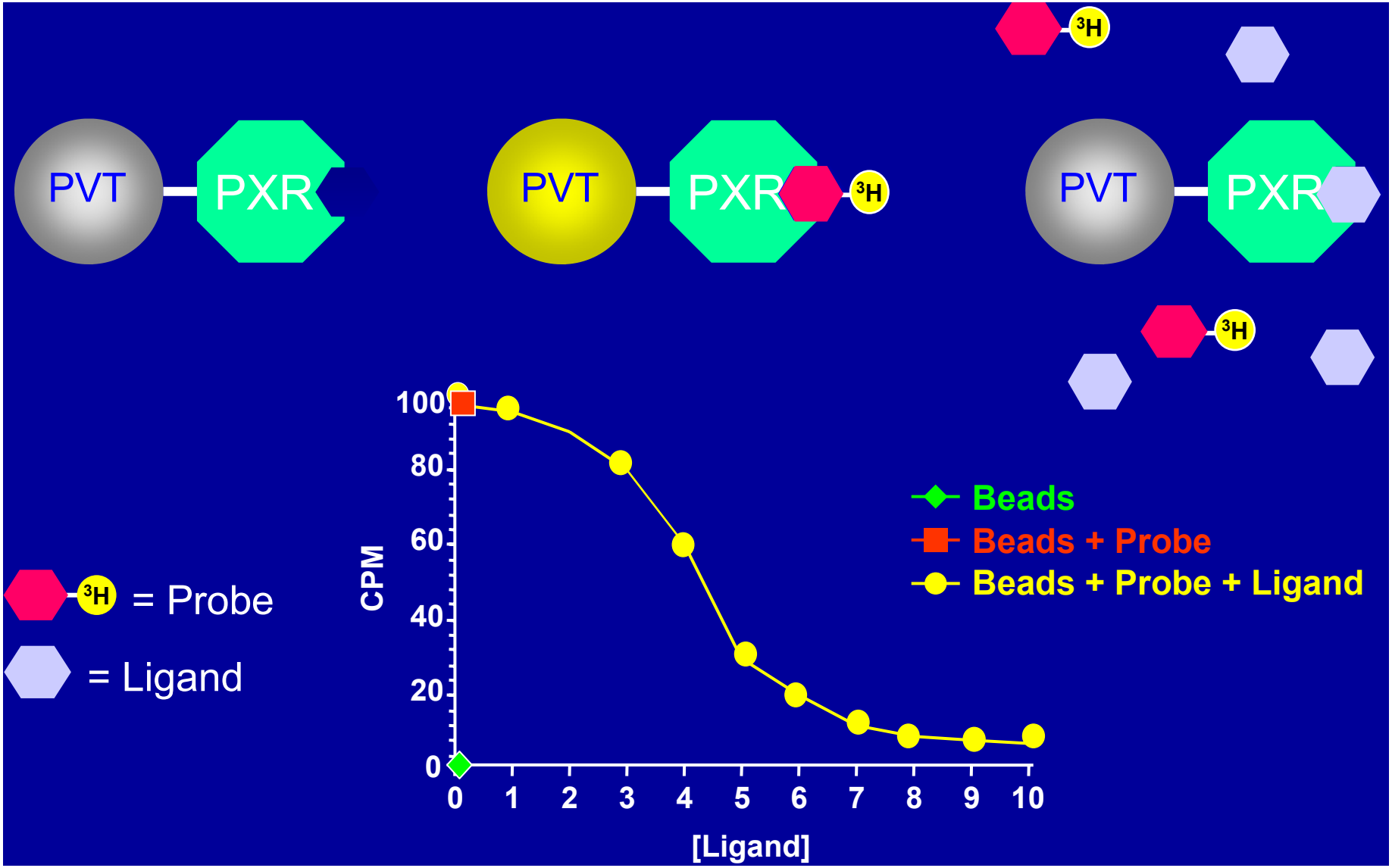
NR Transient Transfection Assay



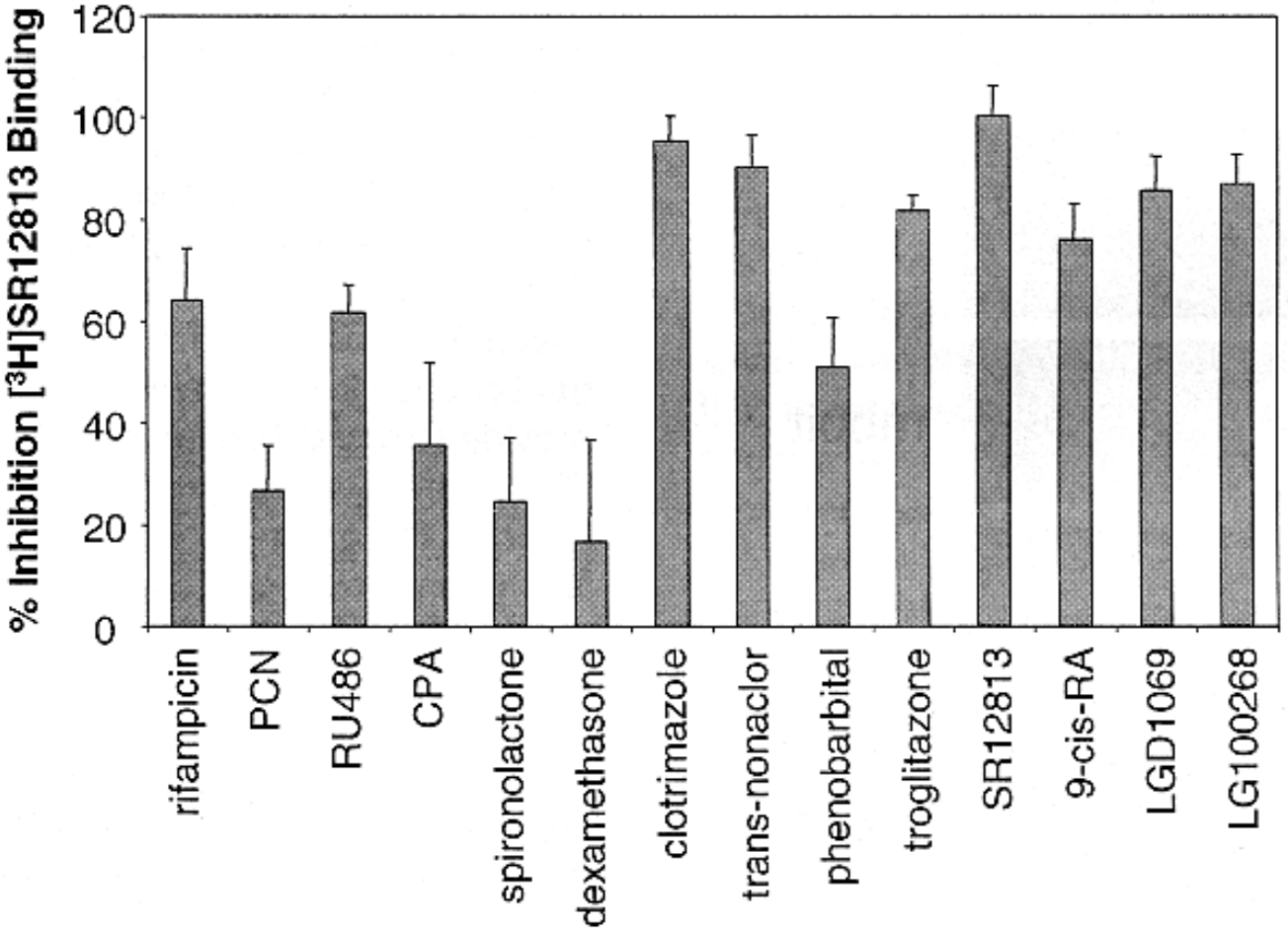
CYP3A Inducers Activate Human, Rabbit, and Rat PXR



PXR Scintillation Proximity Assay



Screening of Chemical Binding to hPXR by SPA

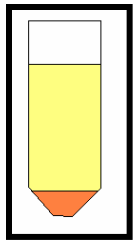


In Vitro Assays for P450 Induction

Assay	Turnaround Time	Compound Capacity per week	Caveats
Hepatocytes	4-7 days	**5-10**	• Individual variability • Tissue availability
NR Functional Assay	2 days	**60**	• Only looks at 1 P450? • Very artificial system
NR Binding Assay	0.5 day	**320**	• Assumes activation of PXR • Radioactive assay

Tissue Engineering: Liver

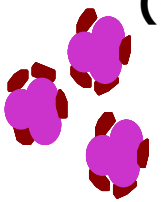
(day 0)



Isolation: Hepatocyte fraction contains ~5% non-parenchymal cells

Spinner flask

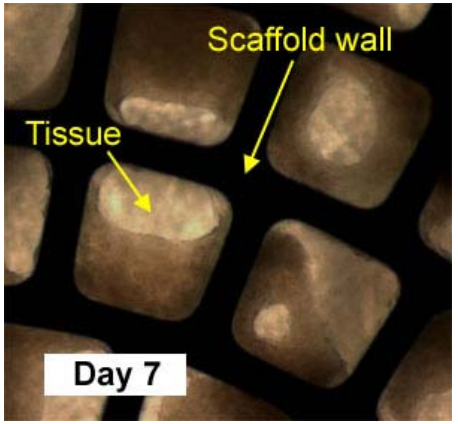
(day 3)

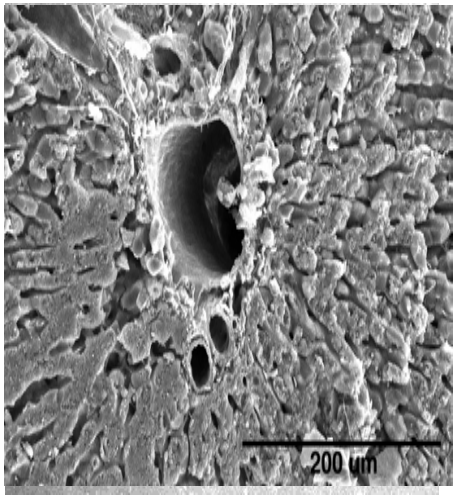


spheroid formation

Static cultures (control)

Bioreactor seeding



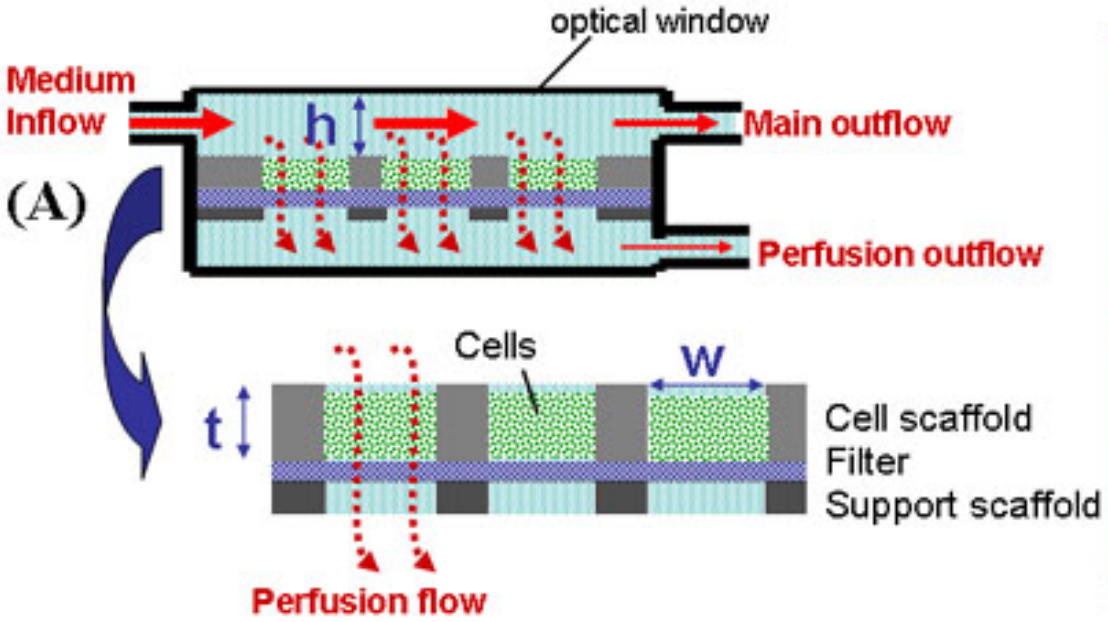


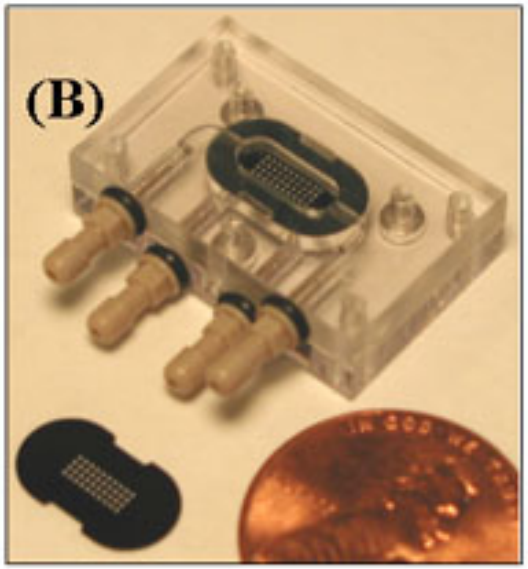
Morphogenesis
Within 1-2 days

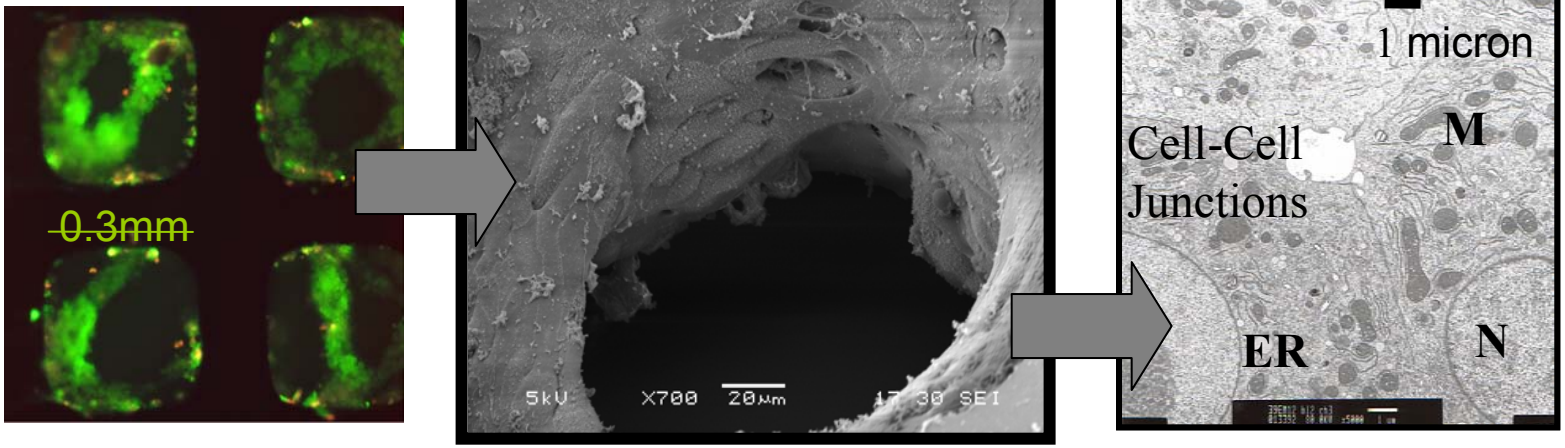
Hepatic functions monitored
Albumin, CYP450

Slide courtesy of Linda Griffith, MIT

Tissue Engineering: Liver



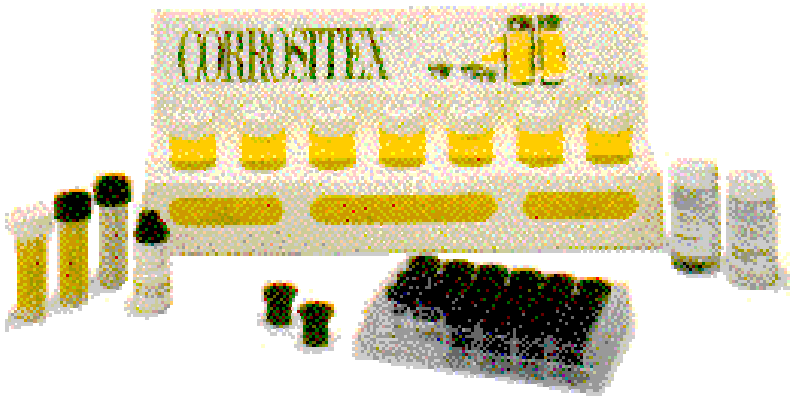




Slide courtesy of Linda Griffith, MIT

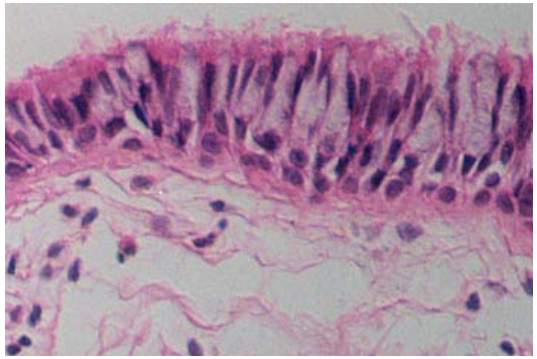
Tissue Engineering: Skin

"**Corrositex** is an ***in vitro*** test that determines chemical corrosivity and permits assignment of Packing Group classification for Class 8 corrosives. This test replaces the rabbit test of dermal corrosivity by providing a reliable means of mimicking this test. The proprietary core technology of the **Corrositex** test is based upon a biomembrane and chemical detection system, which becomes colored when exposed to potentially corrosive substances."

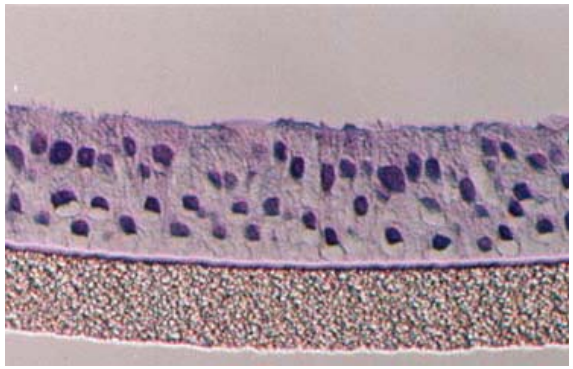


"The **Corrositex** testing system consists of a glass vial filled with a chemical detection fluid capped by a proprietary bio-barrier membrane, which is designed to mimic the effect of corrosives on living skin. As soon as the corrosive sample destroys this bio-barrier, the fluid below changes color or texture. Users simply record the time it takes for the sample to break through the membrane. Then, depending on their needs, they can assign the proper U.N. Packing Group classification for U.S. DOT or EPA compliance, or use the data as a ranking tool or to substantiate marketing claims."

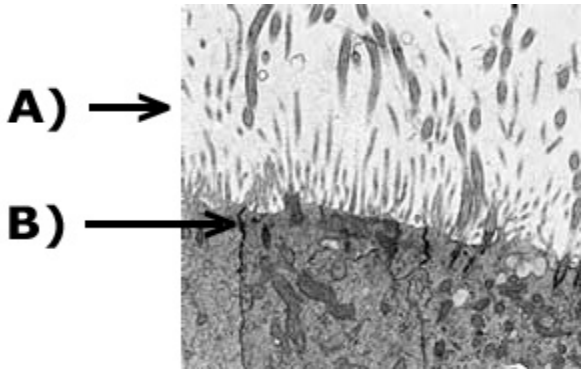
Tissue Engineering: Lung



Normal human bronchiole (10X)



EpiAirway tissue (10X)



EM micrograph of EpiAirway tissue

EpiAirway® cultures (grown on cell culture inserts at the air-liquid interface):

- airway inflammation and irritancy studies (gas phase exposure to volatile materials)
- inhalation toxicity studies
- inhaled drug delivery studies (measurements of trans-epithelial permeability)
- mechanisms of bacterial infections of the respiratory tract
- pharmaceutical prevention of bacterial infections of the respiratory tract
- mechanisms of asthma, cytokine responses, or various airway disorders

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

- Established by NIEHS in 1997;
- Coordinates the interagency technical review of new, revised, and alternative test methods of interagency interest;
- Coordinates cross-agency issues relating to the validation, acceptance, and national/international harmonization of toxicological testing methods;
- Is composed of representatives from 15 Federal regulatory and research agencies that use or generate toxicological information;
- Promotes the scientific validation and regulatory acceptance of toxicological test methods that will improve agencies' ability to accurately assess the safety or hazards of chemicals and various types of products, while refining (less pain and distress), reducing, and replacing animal use wherever possible.
- Additional information about ICCVAM can be found at: *http://iccvam.niehs.nih.gov*

Alternative Methods for Toxicity Assessment

LD_{50} Test: finds a single lethal dose of a substance that kills 50% of the animals.

Introduced in 1927, accepted as a basis for comparing and classifying chemical toxicity and became accepted by regulatory agencies (new drugs, food additives, cosmetics, pesticides, etc.) in 1970s.

Required up to 100 animals (both sexes) and even two species (rat and mouse).

Measurable endpoint: death.

Animal welfare considerations

	Number of animals per test	Number of deaths per test	
401[a]	Up to 25	Up to 12	
402[a]	5–7	1	Endpoint-evident toxicity
423	Average 7	2–3	
425	6–9	2–3	Stopping criteria to limit number of animals used

All three alternatives contain requirement to follow OECD Document on Humane Endpoints.

[a] Includes sighting study

The principles of the three alternative methods

	420 (Fixed Dose)	423 (Acute Toxic Class)	425 (Up and Down)
Methodology	Single bolus dose. Young adult rats (one sex). Oral gavage with constant volume or concentration, clinical observations, bodyweight, mortality over 14 days. Necropsy at termination.		
Sighting study	Yes	No	No
Dose levels	Fixed doses of 5, 50, 300, 2000 (5000) mg/kg 5 rats per dose level	Fixed doses of 5, 50, 300, 2000 (5000) mg/kg 3 rats per dose level	Starting at best estimate of LD50 (or 175 mg/kg) and using dose progression factor of 3.2, single animals dosed until one of three stopping criteria met
Aim	Identify lowest fixed dose causing **evident toxicity**	Identify lowest fixed dose causing **mortality**	Estimate LD50
Output	Range estimate of LD50 Signs of acute toxicity. Target organ(s)	Range estimate of LD50 Signs of acute toxicity. Target organ(s)	Point estimate of LD50 with confidence intervals. Signs of acute toxicity. Target organ (s)