Modern Biocatalysis

Historical Perspective and Future Directions

or

Boom and Bust?

RSC Conference
University College London
April 21, 2009

The Promise to Change the World

Modern Biocatalysis Could Solve Many Problems

- Replace traditional chemical catalysts with enzymes
- Biodegradable, based on renewable resources
- Alternative to petrochemical-based processes
- Operate at ambient temperature and pressure: use less energy and eliminate expensive process equipment
- "Green-ness": Reduce pollution and chemical hazards

Reality: An Up and Down History

Modern Biocatalysis has gone through historical cycles

- Excitement developed around the promise of biocatalysis
- Companies formed and established groups
- Period of R & D elapsed
- The reality failed to live up to the “hype”
- Disappointment followed
- Biocatalysis fell out of favor

- 3 Distinct Cycles

The Early 1980's

Modern Biocatalysis Cycle 1: Modern Biocatalysis was first "discovered"

- Age of genetic engineering companies; many were founded and promoted the idea of biocatalysis: Amgen, Genentech, Genetics Institute, Genex, Cetus, MBI, Celgene, Biotechnica, Chiroscience

- Large chemical companies got involved: Degussa, Dow, DuPont, Celanese, DSM, WR Grace, Shell, BP, Exxon, Tanabe, Ajinomoto, Kyowa Hakko, Novo, Degussa, Monsanto

- Products: Amino acids, Pharma Intermediates, Monomers, PHB, Food Ingredients

The 1980's: What Happened?

- Some amino acids, including L-met by enzymatic resolution and L-asp and L-phe for aspartame and D-amino acids for antibiotics were successfully commercialized (Degussa, Monsanto, DSM, Kaneka)

- A few chiral intermediates for pharma were resolved using lipases

- The larger chemical companies never found volume applications and many laid off entire groups they had built up

- Amgen, Genentech, GI, and other biotech companies abandoned efforts to commercialize enzymatic chemical processes changed focus to therapeutic proteins. Cetus switched to diagnostics and PCR; Novo (now Novozymes) refocused on industrial enzymes.

- Some chemical biotech companies failed: Genex

The 1980's: What Went Wrong?

- Very few enzymes were readily available other than a few lipases and acylase => very narrow chemical scope

- Cloning new genes was still difficult and time consuming; many processes used wild-type strains => low productivity

- Multi-year projects; Process development was too slow and costly

- Protein engineering was talked about (dreamed about) but not practiced; key tools and technologies were still lacking

- High throughput screening had not been developed

The Early 1990's

The Revival of Modern Biocatalysis: Cycle 2

- Cloning of genes became more rapid and common
- Protein crystallography expanded
- The use of protein engineering based on crystal structures to guide changes in proteins was initiated, created new optimism

- Large chemical companies built/rebuilt biocatalysis groups: Dow, DuPont, BASF, Gist-Brocades-DSM, Monsanto, Degussa

- Pharma companies established biocatalysis groups for synthesis of chiral intermediates: Roche, Glaxo-SmithKline, Lilly, BMS, Rhone-Poulenc, Novartis, Merck, Schering Plough

- New biocatalysis companies were started or gained momentum: Thermogen, Celgene, Allelix, Chirotech, [Boehringer-Mannheim]

The Early 1990's: What Happened?

- A few more processes to produce pharma intermediates were commercialized at GSK, Roche, BMS, Lilly, especially for antibiotics

- Lipases and other hydrolases continued to be the most exploited enzymes because few others were readily available (still)

- Only companies that could clone and express targeted enzymes themselves succeeded in other reactions, and successes were limited

- The large chemical companies never found cost effective applications and laid off entire groups--again

- Biotech companies abandoned efforts to commercialize enzymatic chemical processes; changed focus to therapeutic proteins--again

The Early 1990's: What Went Wrong?

- Still relatively few available enzymes other than hydrolases
- Protein engineering was too slow (too rational?) and had a low success rate
- No ability to sort through large numbers of mutants without a selection method; high throughput screening not yet established
- Still too expensive: cost typically not competitive with chemical alternatives
- Still too slow: Process development with enzymes typically took longer than chemical alternatives

The 2000's: Current Cycle

Modern Biocatalysis' Third Wave

- Important new technological breakthroughs had emerged
  - Shuffling
  - Oligonucleotide and gene synthesis
  - High-throughout screening
  - Genomics and rapid gene sequencing

- New biocatalysis companies were started: Diversa (now Verenium), Juelich Fine Chemicals, Maxygen=>Codexis, BioCatalytics, IEP, Direvo, BioVerdant, Proteus, BRAIN

The 2000's: What Is Happening?

- Biocatalysis is considered more seriously and more often

- Selected chemical and pharma companies making larger commitments and/or expanding biocatalysis groups: DuPont, BASF, DSM, Merck, GSK

- Availability of enzymes is increasing dramatically, with small companies leading

- Opportunities for both chiral and non-chiral compounds

- Large increase in established biocatalysis processes

- New focus on engineered whole cells: fuels, commodities

The 2000's: What Is Different This Time?

- Shuffling and efficient methods for creating genomic diversity allow enzyme variants to be generated rapidly and pathways to be engineered, with control over where mutations are introduced

- High throughput screening methods have been refined

- Genomics and sequencing of genomes have exploded, creating vast resources of genomic data that can be "mined"

This combination of technological breakthroughs =>
Large increase in the number of available enzymes
Broad range of reaction alternatives
Rapid, significant improvements in enzymes and pathways
Lower-cost production; Now meeting faster development time-lines
Heavy investment in biofuels and bioindustrials

Is progress slowing---or worse?

Skepticism and Misconceptions Persist

Major Hurdle: Skepticism

Second Major Hurdle: Misperceptions and Biases

Handling Enzyme Stability

Example using Directed Evolution: GDH for cofactor recycling developed at BioCatalytics

Multiple amino acid substitutions: Stability improved by 10-100 fold, allowing large decreases in enzyme required in higher temperature reactions and aqueous-organic 2-phase systems

Example using Immobilization:
Covalently bound transaminase for unnatural amino acid synthesis: Improved from 100:1 product:enzyme to more than 1000:1 product:enzyme

Large Improvements in Productivity

Low productivity has been a common complaint against biocatalysis, with good reason: dilute, high loadings

Nature provides a lot of diversity

Metagenomics combined with HTS have tapped vast natural diversity

⇒ Discover more productive biocatalysts

We are no longer limited to what nature provides

Modern methods of laboratory enzyme evolution have allowed large (100-1000-fold) improvements to be made in activity and operability at high substrate concentration

=> Create more productive biocatalysts

Regenerating Redox Cofactors

About 10-15 years ago this was a common criticism
Today, at least 50-100 compounds are produced by stereoselective enzymatic reduction coupled to a nicotinamide cofactor recycling system

Four basic methods:

Formate DH (Formate → CO_2)
Driving force: Essentially irreversible oxidation of formate to CO_2

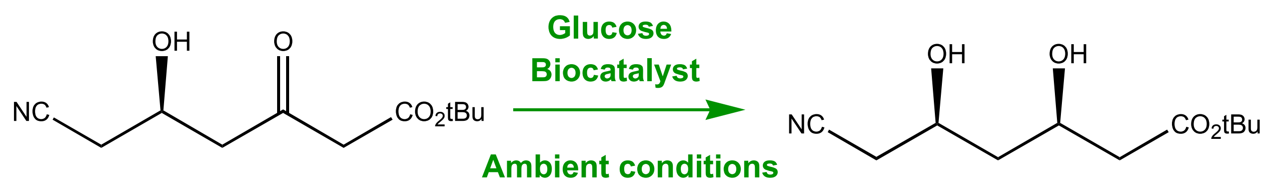
Glucose DH (Glucose → Gluconic Acid)
Driving force: Hydrolysis of gluconolactone to gluconic acid

Phosphite DH (Phosphite → Phosphate)
Driving force: Thermodynamics of phosphite oxidation

KRED-Regeneration (Isopropanol → Acetone)
Driving force: Large excess of isopropanol, acetone removal

Example: Production of TBIN

Stereoselective Reduction Step

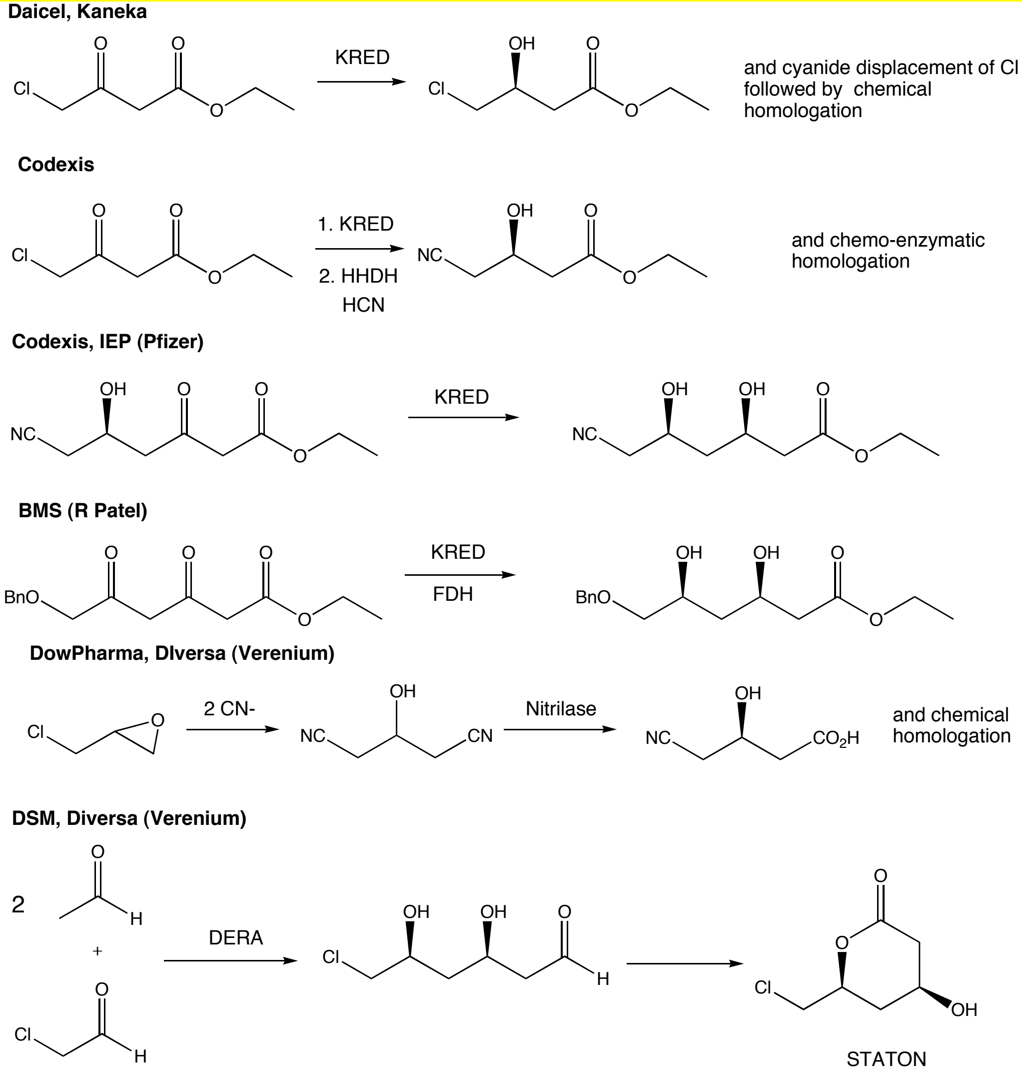


Material	Quantity
Glucose	**Approx. 1000 kg**
NADP+	**0.8 kg**
KRED	**9 kg**
Glucose DH	**1 kg**
Ketone	**1025 kg**
Diol Produced	**1000 kg**

Developed by Codexis
10s of tons per year

Data adapted from D. Rozzell, PharmaChem, October 2008, 2-3.

Biocatalytic Alternatives Have Increased



For atorvastatin side chain and intermediates, processes have been developed by multiple companies using 4 different enzyme chemistries:

- ❖ Ketoreductases with cofactor recycling
- ❖ Halohydrin dehalogenase
- ❖ Nitrilase
- ❖ Aldolase

David Rozzell, April 21, 2009

Biocatalysts: What is the Cost Contribution?

Guideline Range

Product/Enzyme: 100-1000 kg/kg
Bulk Enzyme Cost: $2500-20,000/kg

Cost contribution range: $2.50-200/kg

Bringing Biocatalysis into the Mainstream

To truly be considered as a mainstream technology, biocatalysis must be a first-line option, not an alternative that is tried after everything else has failed.

Three trends are helping:

Greener Processes

Wide Availability of Better Enzymes

Process Intensification

Nothing succeeds like success

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Biocatalysis: What About the Future?

In the Near Term

- Ketone reduction: Virtually all will be possible biocatalytically
- Transaminases: Produce a range of chiral amines
- Ene reductases: Reduce certain C=C stereoselectively
- Nitrilases: Mild, stereoselective nitrile hydrolysis
- Halohydrin Dehalogenase: Stereoselective epoxide opening
- Amine Oxidation: Stereoselective; desymmetrization
- Aldolases: Stereoselective C-C formation without activation

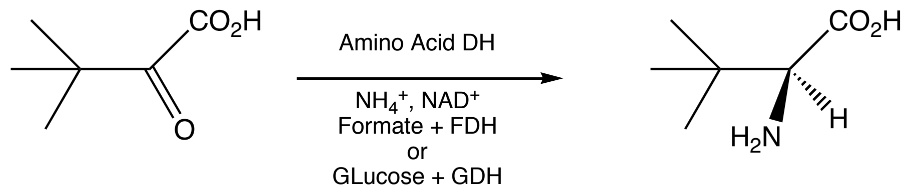
Key Reactions for Future Development?

<u>In the intermediate term we can expect to see:</u>

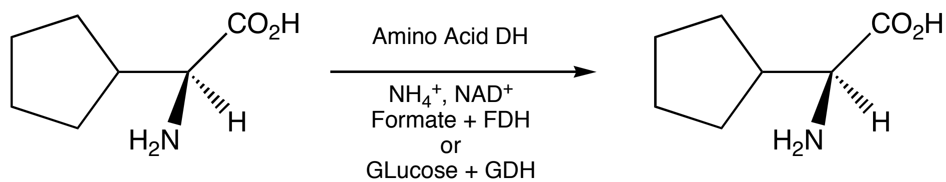
- Hydroxylation (P450s, others)
- CO_2H ----> CHO: Rosazza CAR and analogs
- Reductive Aminase: Any ketone to a chiral amine (US Patent 7,202,070; early reports by X-Zymes)
- Industrial: Production of moderately-priced monomers, modification of polymers
- Integrating biocatalysis with other disciplines

Reductive Amination

Currently

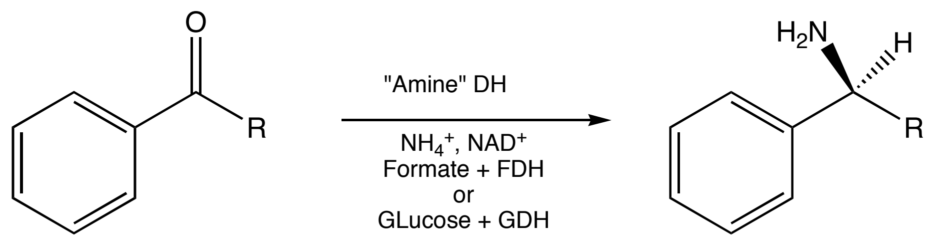


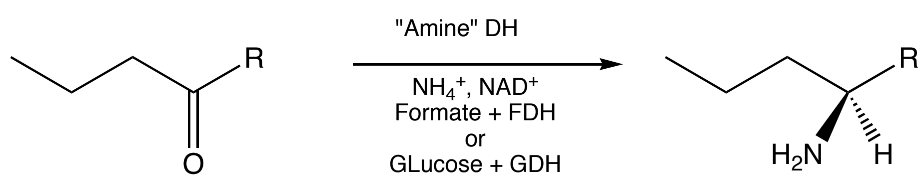
L-tert-Leucine



L-Cyclopentylglycine

What About





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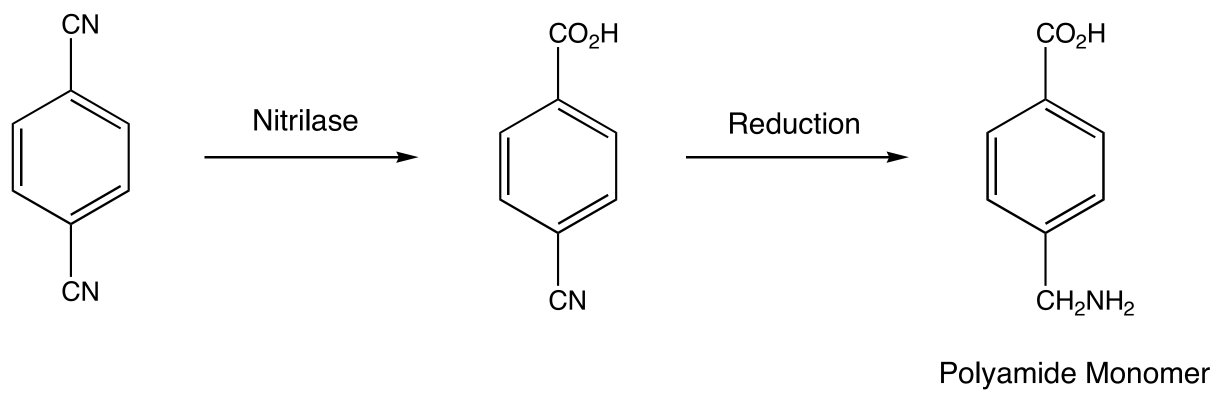
Future Direction: "Greener" Monomer Synthesis

Selective Nitrilase-Catalyzed Hydrolysis of Di-nitriles



- Near perfect selectivity for mono-hydrolysis product leads to high monomer purity
- Mild conditions
- Avoid use of harsh caustic or corrosive mineral acid

SOLIDUS

High Throughput, Predictive Toxicity Screening on a Chip

Solidus Biosciences Inc

Solidus Biosciences, Inc.

From Macro-scale to Micro-scale

Integrating biocatalysis with other disciplines

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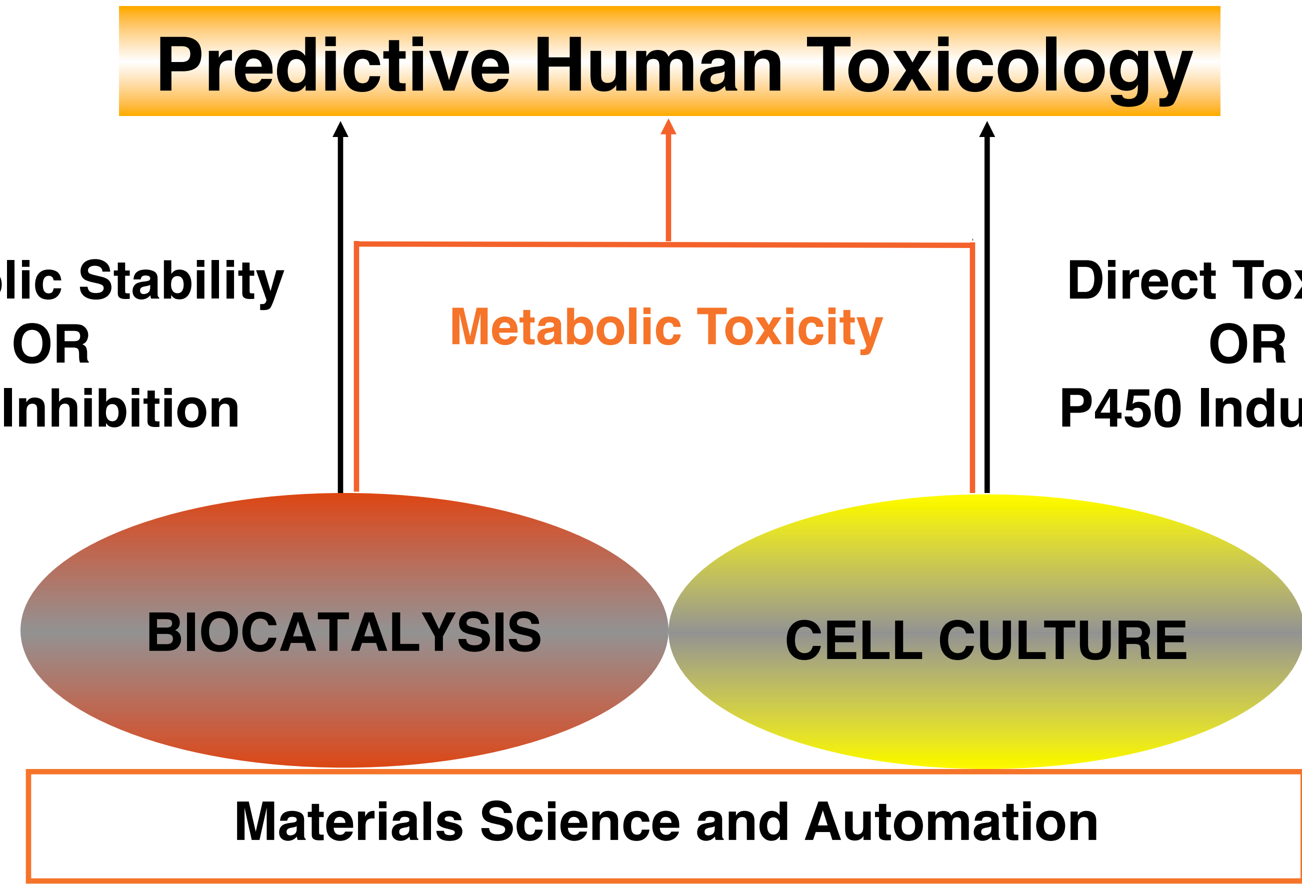
Interdisciplinary Platform



Lee et al. *Proc. Natl. Acad. Sci. USA*, 102, 983 (2005),
Lee et al. *JALA*, 11, 274 (2006)
Lee et al. *Proc. Nat. Acad. Sci. USA*, 105, 59-63 (2008)

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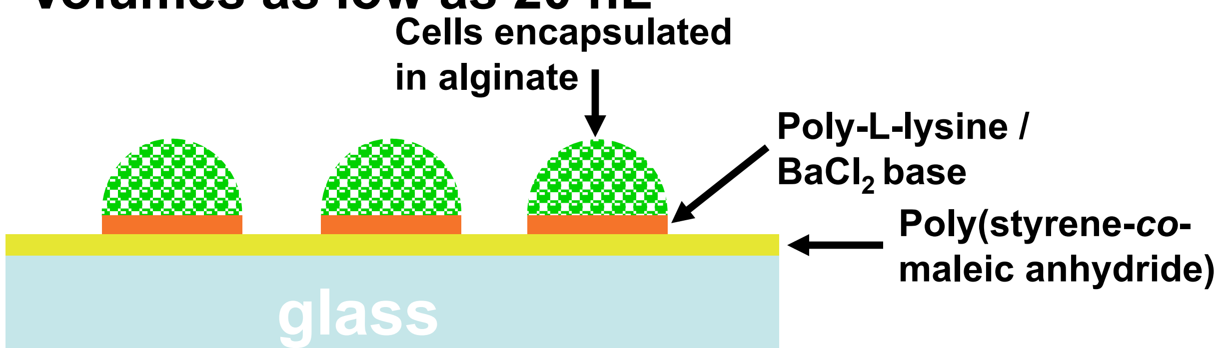
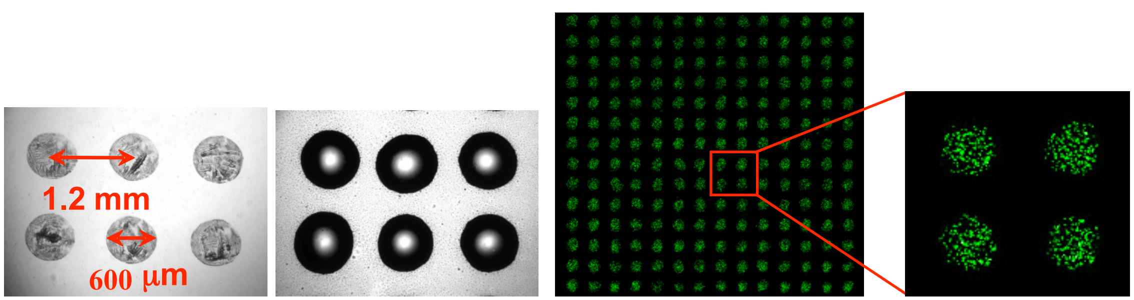
The DataChip

SOLIDUS

- Cells are spotted onto functionalized glass slides
- Spatially addressable pattern of cells encapsulated in a 3D hydrogel matrix
- Volumes as low as 20 nL





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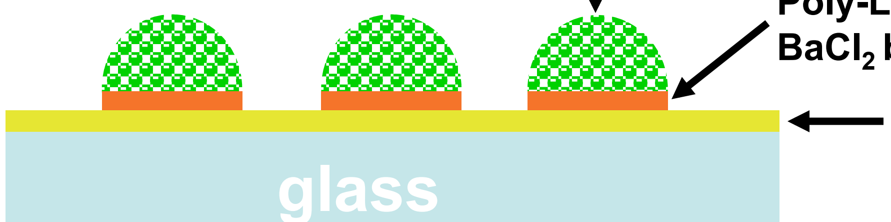
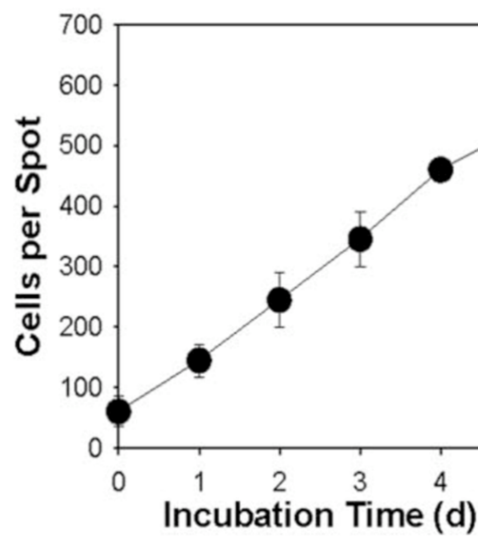
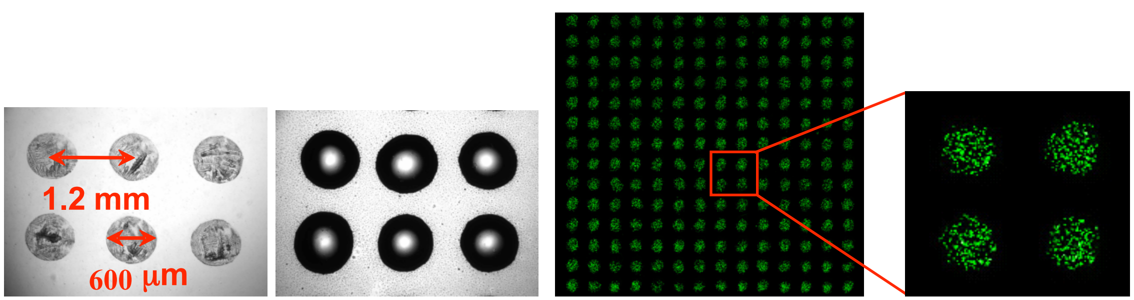




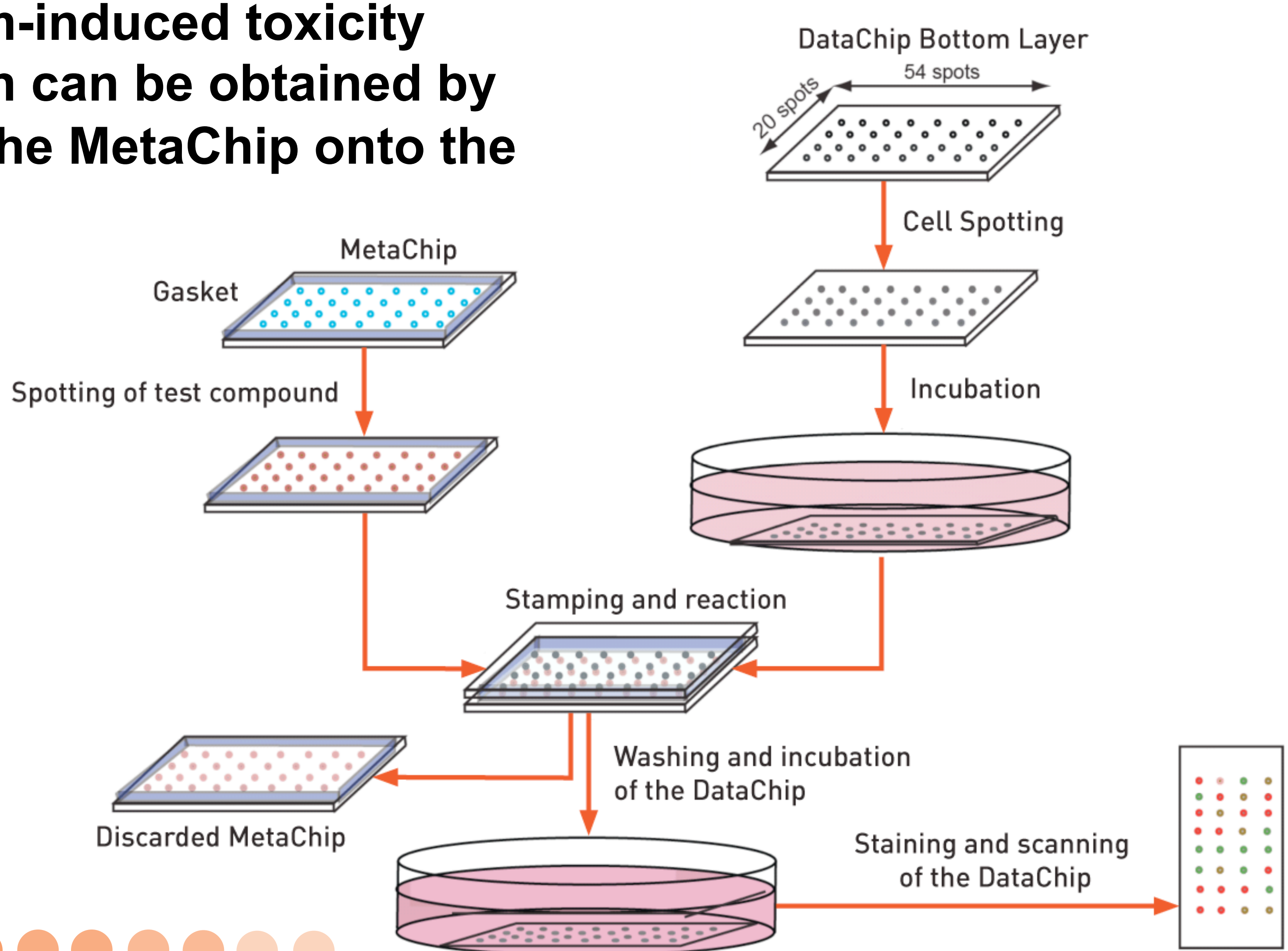


DataChip can support cell growth of multiple cell types for up to 5 days

Lee et al. *Proc. Nat. Acad. Sci. USA*, 105, 59-63 (2008)

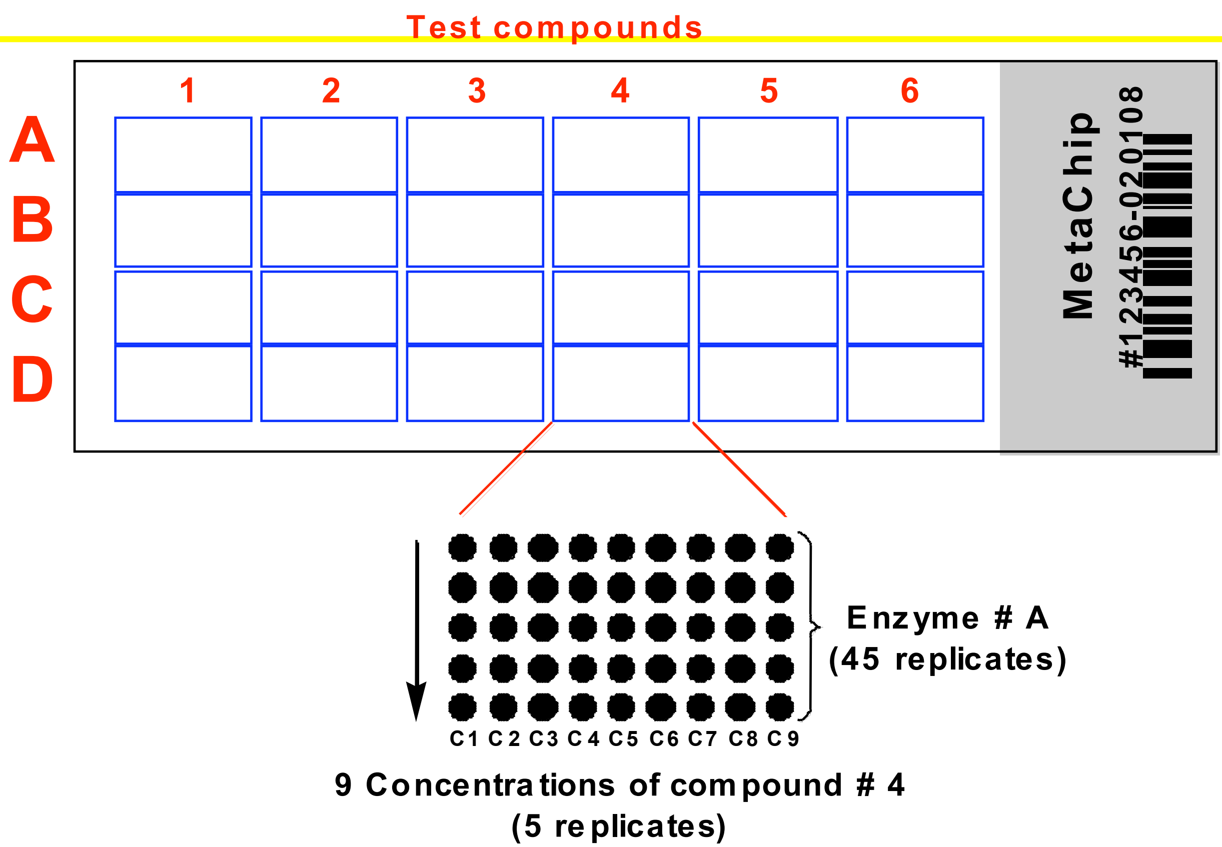
Combining the DataChip/MetaChip

Metabolism-induced toxicity information can be obtained by stamping the MetaChip onto the DataChip



MetaChip Design for ToxCast

SOLIDUS



- **6 compounds spotted per slide**
- **24 dose-response curves generated per chip**

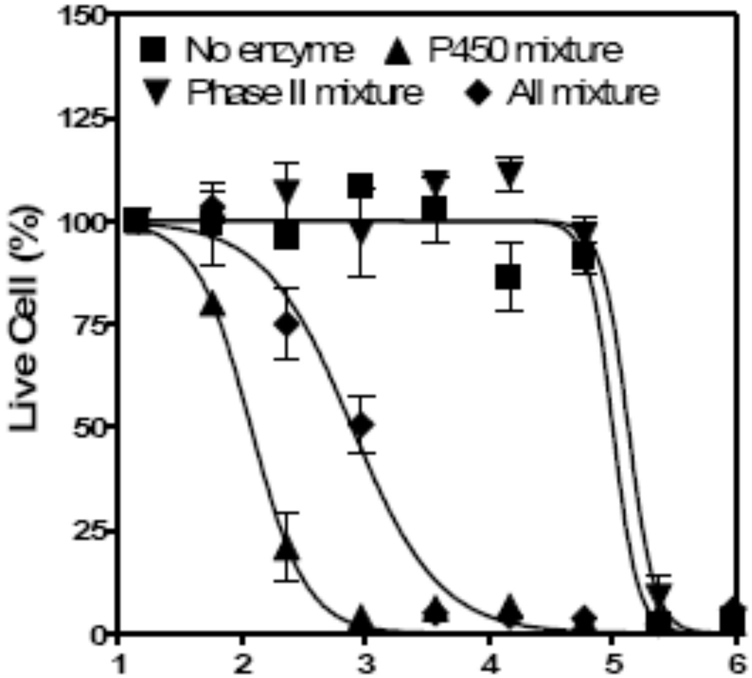
	Enzymes
A	None
B	P450 Mix
C	Phase II Mix
D	All Mix

P450 Mix: CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP2E1, CYP1A2, CYP2B6

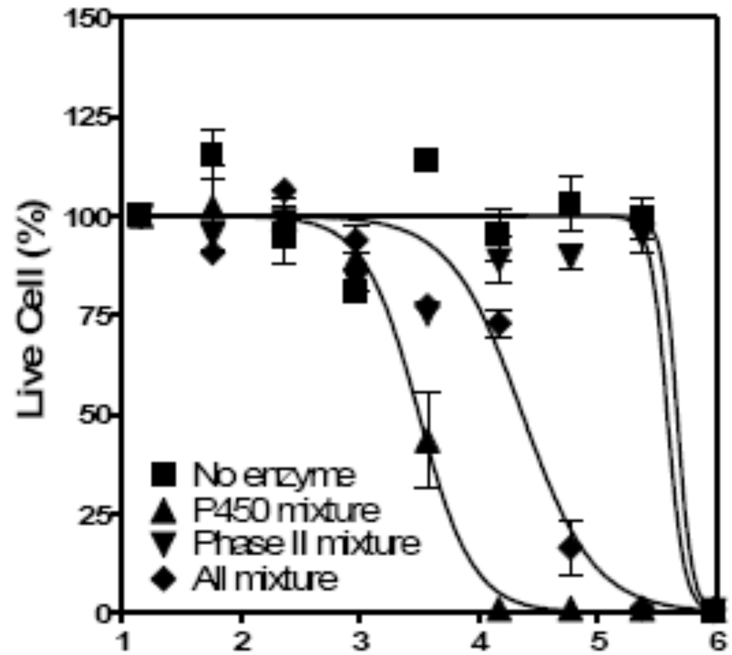
Phase II Mix: UGT1A1, UGT1A4, UGT2B4, UGT2B7, SULT1A3, SULT2A1, GST

Metabolic Toxicity Identified

SOLIDUS



Log [Diquat Dibromide (nM)]



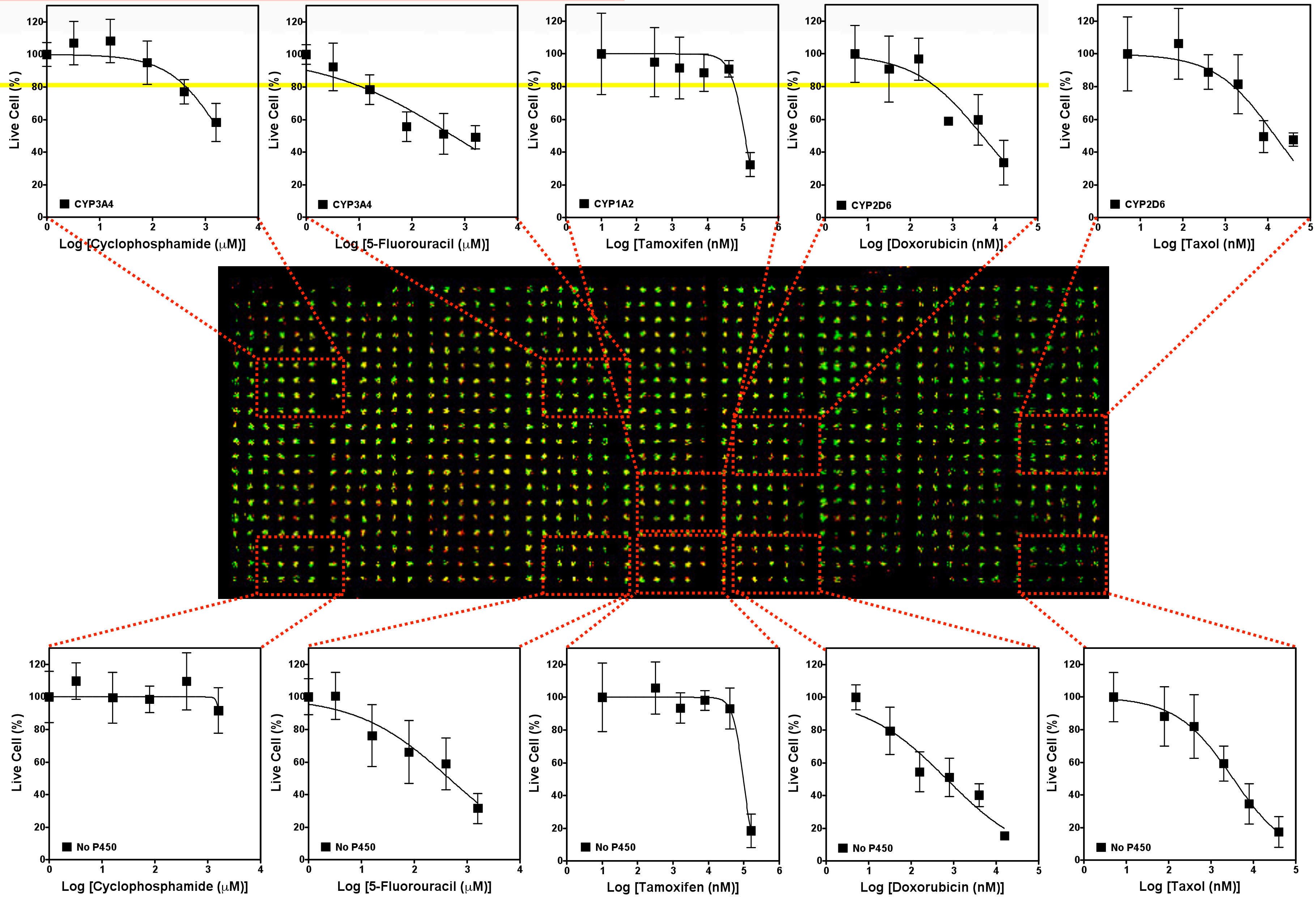
Log [Napropamide (nM)]

Assay	IC_{50} (μM)
No Enzyme	104
CYP450 Mix	0.1
Phase II Mix (UGT, GST, SULT)	136
CYP450 + Phase II Mix	0.7

Assay	IC_{50} (μM)
No Enzyme	470
CYP450 Mix	3
Phase II Mix (UGT, GST, SULT)	390
CYP450 + Phase II Mix	24

SOLIDUS

Spot density = 1,080/slide; Hep3B Cells



Nine compounds, 5 P450s or mixtures, 6 conc, 4 replicates

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Acknowledgements

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