High Throughput Screening
for Biomolecule Discovery

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Outline

✧ High Throughput Screening (HTS)
  Biochemicals-based assays
  Cell-based assays

✧ 3-D culture cell-based fluorescence assays for cytotoxicity analysis and drug screening
  Comparison between 2-D and 3-D cultures
  Effects of cell density and morphology on drug efficacy
  Microbioreactor array for high-throughput screening
Stages of drug discovery

1. Disease identification
2. Therapeutic Target
3. Lead Identification
   - High throughput screening
   - In vitro and in vivo pharmacology
4. Preclinical trials
   - Animal test
   - HTS
5. Clinical Trials
6. FDA Approval

High-throughput screening (HTS):
Lead identification and Preclinical toxicology
High Throughput Screening

The Screening Continuum

**HTS:** 10,000 to 100,000 data points/day

**Ultra HTS:** >1,000,000 data points/day

From a poster by RR Tice et al of the National Toxicology Program HTS Initiative, 2007
## High Throughput Screening

### Microtiter plates

<table>
<thead>
<tr>
<th>Plate Type</th>
<th>10,000 Compounds/day</th>
<th>100,000 Compounds/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-Well plate</td>
<td>125 Plates/day</td>
<td>1250 Plates/day</td>
</tr>
<tr>
<td>384-Well plate</td>
<td>32 Plates/day</td>
<td>313 Plates/day</td>
</tr>
<tr>
<td>1536-Well plate</td>
<td>8 Plates/day</td>
<td>78 Plates/day</td>
</tr>
</tbody>
</table>

(80 compounds/plate)

(320 compounds/plate)

(1280 compounds/plate)
High Throughput Screening

-History of Development

![Graph showing the increase in functional features per square centimeter from 1964 to 1996. Key points include:
- 96 well plate
- 384 well plate
- 864 well plate
- 1536 well plate
- 60,000 "well" chip

The x-axis represents 'Functions Performed / Plate or Substrate', and the y-axis represents 'Functional Features / sq. cm.' The graph illustrates the exponential increase in capabilities over time.]
High Throughput Screening
Commercial automation systems

✧ Biochemicals-based High Throughput Screening - microchips
✧ Cell-based High Throughput Screening – microtiter plates, microfluidic chips

High throughput screening platform, Tecan

GNF systems for HTS
Microchip Biochemicals-based High Throughput Screening

Features
- Parallelism
- Miniaturization
- Multiplexing
- Automation

Types
- DNA microarrays
- Small molecules microarrays
  - chemical genomics
- Large molecules microarrays
  - proteomics

http://arrayit.com/Products/DNA_Microarrays/DNA_Microarrays_H25K/dna_microarrays_h25k.html
DNA Microarrays

Applications
- Comparative genome analysis
- Re-sequencing of DNA
- Functional genome analysis

Advantages
- High efficiency
- Inexpensive platform for HTS
- Serving as a direct link to function

Limitations
- Local contamination
- Need of relatively pure product
- Moral issues

CGH: Comparative genomic hybridization
1. Manufacture CGH microarrays
2. BACs Oligos cDNAs
3. Hybridize genomic DNA two (2) color
4. Scan and analyze

http://arrayit.com/Products/DNA_Microarrays/DNA_Microarrays_CGH/dna_microarrays_cgh.html
Large Molecules Microarrays
Proteomics

Applications
- Expression Profiling
- Serum Based Diagnostics
- Protein-protein binding assays
- Drug-target binding
- Receptor epitope binding

Limitations
- Insufficient number of antibodies
- More complicated and more fragile molecules than DNA
- Lack of recognition elements with specific interaction
- Mostly membrane-bound
Small Molecules Microarrays
Chemical Genomics

Advantages

- The capacity to induce their biological effects rapidly and often reversibly
- The ability to study essential genes at any stage in development
- To enable the combinations of multiple knockouts

• However, it is limited by the availability of appropriate compounds.

High throughput screening by small molecule microarrays

*Current Opinion in Chemical Biology. 2002, 6: 359-366*
Fragment-based drug discovery
small molecule enzyme inhibitors

Enzyme target \rightarrow Fragment libraries

Fluorescence-based thermal shift

NMR spectroscopy (WaterLOGSY and STD NMR)

Isothermal titration calorimetry (ITC)

X-ray crystallography

Synthesis

First-line screening

Competition studies

Quantitative, K_D DH

Binding mode

Optimization

Lead inhibitors (μM – nM)

Screening

Fragment hits (mM)

Validation

Design

Computational docking

Current Opinion in Biotechnology 2007, 18:489–496
Metabolism-based assays
enzyme- or cell-based assays

Enzyme identification
Metabolic stability
Toxic metabolite identification
Enzyme inhibition
Enzyme induction

Enzyme
Enzyme and cell
Enzyme and cell
Enzyme
Cell

Pharmacokinetics
Drug toxicity
Drug–drug interaction

Why cell-based HTS?

✧ Toxicity issue (Animal model: time consuming...)
✧ Multiple targets compared to biochemicals-based
✧ More representative of the real-life model
## Cellular Assays
- **Classifications and Types**

<table>
<thead>
<tr>
<th>Assay classification</th>
<th>Specific assay type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Proliferation Assays</strong></td>
<td>• Dye uptake (e.g., Almar blue, MTT)</td>
</tr>
<tr>
<td></td>
<td>• Oxygen sensor</td>
</tr>
<tr>
<td></td>
<td>• Radioactive isotope uptake</td>
</tr>
<tr>
<td><strong>Second Messenger Assays (e.g., ion channel)</strong></td>
<td>• Ion flux assay</td>
</tr>
<tr>
<td></td>
<td>• Fluorescence based assay – fluorometric imaging plate reader</td>
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<td></td>
<td>• Automatic patch clamp</td>
</tr>
<tr>
<td><strong>Reporter Gene Assays (e.g., GPCR)</strong></td>
<td>• Enzymatic assays – luciferase, β-lactamase, β-galactosidase, …</td>
</tr>
<tr>
<td></td>
<td>• Immunoassays</td>
</tr>
<tr>
<td></td>
<td>• Direct protein measurement – green fluorescent protein</td>
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</tbody>
</table>

*Current Opinion in Biotechnology 17: 619-627 (2006)*
Cell-based High Throughput Screening

✧ Conventional cell-based HTS

6 well-plate, 96 well-plate, 384 well-plate, 1536 well-plate ...

✧ Microchip cell-based HTS

HTS in 96 well-plate

Increasing foscillin concentration

Increasing *Metridia* substrate concentration

1536 well-plate
Ready-To-Glow Secreted Luciferase System, Clontech

3D data chip platform, Solidus, Bioscience
Secreted *Metridia* Luciferase

1. Clone response element/promoter of interest into pMetLuc-Reporter vector

2. Transfect host cell line

3. At desired time points, assay luciferase activity
   - Transfer media sample to 96-well plate
   - Add substrate reaction buffer
   - Assay luciferase activity in a luminometer

Cell-based High Throughput Screening

HighRes MicroStar

HTS BSL2+ System

HighRes Biosolutions
Cell-based High Throughput Screening

Dose-Response Modeling of High-Throughput Screening Data

- Test compounds of interest
- Identify mechanisms of action
- Develop predictive models for human toxicity

Cell-based High throughput Screening

High throughput metabolism-induced toxicity analysis (MetaChip)

Cell-based
High Throughput Screening

Goals of the HTS Program

✧ To prioritize substances for further in-depth toxicological evaluation

✧ To identify mechanisms of action for further investigation (e.g., disease-associated pathways)

✧ To develop predictive models for \textit{in vivo} biological response (predictive toxicology)

National Toxicology Program (NTP), 2010
Cell-based
High Throughput Screening

Predictive models for *in vivo* biological response

- **2-D cell culture**
  - Easy operation
  - Loss of tissue morphology
  - Errors in predicting tissue-specific responses

- **3-D cell culture**
  - Better mimics of *in vivo* microenvironments
  - Better link between single cells and tissues
  - Quantification of cell proliferation is a challenge
Cell Morphology

in 3-D fibrous matrix vs. on 2-D surface

3-D

Cow luteal cells

2-D

Murine ES cells
Cell culture in 3-D microenvironments

3-D \textit{in vitro} cell models
- Better mimics of the \textit{in vivo} microenvironments
- Better link between single cells and organs

3-D microenvironments
- Encapsulation in gel
- Porous scaffolds (fibrous matrices)
3-D Culturing in Fibrous Matrix

Comparison with 2-D systems

- Provides a greater surface area, higher cell density
- Allows for a spatial distribution, analogous to that found in *in vivo* tissue
- Cells continue to grow, not limited by confluence as in monolayer (2-D) cultures, no contact inhibition
- Localized microenvironments, conducive to cellular maturation, stimulating proliferation, regulating differentiation
- Maximizes cell-cell interactions by allowing greater potential for movement of migratory cells
- Mimics 3-D tissue structure and function
Detection: cell quantification

Conventional assay techniques:

✧ Direct method: Cell counting using a hemocytometer
✧ Indirect method: detection of chemical or biological molecules

Reporter gene:

✧ Green fluorescent protein (GFP)
✧ Red fluorescent protein (dsRed)
✧ EGFP co-expressed with dsRed (Brooke J, 2002)
Microplate with microelectrodes

- Non-invasive and online
- High adaptability for automation
- Low sensitivity
- Limited to 2D monolayer cultures
- Difficult to related to a specific cell function

Toxicology in Vitro 2006; 20(6): 995-1004
Live Cell Fluorescence Assays

✧ Uncover biological issues
✧ No requirement for sample preparation
✧ Real-time of monitoring rapid cellular

➢ Flow cytometer - Manual sample preparation
➢ Automated microscopic imaging system (AMIS)
    — Limited to planar images; high cost; complexity
➢ Fluorometer - Low cost, easy to use, cell layering does not impair detection, but low sensitivity
Real-time quantification: AMIS

(c) QX5 digital microscope
laminar flow hood
μsyringe
magnetic base
micromanipulator

to computer

(m) micro-beads

Openlab Data Acquisition Software
NIH Public-domain Image Processing Software and MATLAB

Real-time quantification: Fluorometer

Challenges

- Low sensitivity: background signals > signals from live cells
- GFP released from damaged cells would lead to overestimation of GFP fluorescence inside of live cells (Girard et al, 2001, Hunt et al, 1999)
- Medium component changes due to cell activities and cell debris add additional errors

Tecan well plate reader
Cell growth kinetics with GFP

Large errors on single point assays depending on time of sampling
Prototype I
96-well plate based system

✧ 6 microbioreactors on each 96-well plate
✧ Culture for 1 or 4 days prior to drug addition
✧ Multiple dosages of drugs
✧ Fluorescence at Ex 485 nm, Em 530 nm

96-well plate

CO₂ incubator
GFP signals from 2-D and 3-D systems

- Live cell fluorescence = F1 - F2
- Increase signals more than 20 times and increase SNR
Cell Proliferation Assay

Good reproducibility

With specific cell growth close to that from 3-D culture in the spinner flask

Fluorescence (RFU)

Time (h)

μ = 0.036 h⁻¹
Cell Proliferation Assay

Effect of serum on cell growth in 3-D culture

- No FBS, no cell growth
- 1% FBS supports cell growth in 3-D culture
Effects of fibronectin coating

with coating          without coating

After one day

After 4 days

Fluorescence (RFU)

Time (h)

35% enhancement of growth rate

3000
3500
4000
4500
2500
2000
1500
1000
500
0

0 50 100 150

with coating

without coating

5000
Acute Toxicity Assay

Toxicological effect: cell death kinetics

- Control
- 62.5 ug/ml
- 125 ug/ml
- 250 ug/ml
- 1000 ug/ml

Triton X-100

Relative Fluorescence

Time (min)
Drug Efficacy Assay

5-Fluorouracil (5-FU)

Fluorescence (RFU)

Time (h)

- Control
- 0.052 ug/ml 5-FU
- 0.26 ug/ml 5-FU
- 1.3 ug/ml 5-FU
- 6.5 ug/ml 5-FU
- 32.5 ug/ml 5-FU
**ESC for Embryocytotoxicity**

**Graph a**

- **ES-GFP cells**
- **Dexamethasone**
  - ○ 0 µg/ml
  - ● 4 µg/ml
  - ▲ 6 µg/ml
  - △ 8 µg/ml
  - □ 10 µg/ml
  - ■ 15 µg/ml

**Graph b**

- Relative cell growth
- Dexamethasone (µg/ml)
  - ○ Sp. growth rate
  - ▲ 100 h
  - □ 136 h

**Legend**

- IC_{50}

Embryocytotoxicity

2-D and 3-D fluorescent proliferation assays
(drugs were added to low density cells)

![Graphs showing the effect of DM, DPH, PenG, and 5-FU on proliferation rates in 2-D and 3-D assays.](image-url)
Embryocytotoxicity

3-D tissue-mimicking response assays
(drugs were added to high density cells)
# Comparison of Embryotoxicity Test

<table>
<thead>
<tr>
<th></th>
<th>Dexamethasone (weak toxicity)</th>
<th>Diphenylhydantoin (weak toxicity)</th>
<th>Penicillin G (non-toxic)</th>
<th>5-fluorouracil (strong toxicity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZEBET IC&lt;sub&gt;50&lt;/sub&gt; (phase III testing)</td>
<td>37</td>
<td>102</td>
<td>2100</td>
<td>0.09</td>
</tr>
<tr>
<td>ECVAM IC&lt;sub&gt;50&lt;/sub&gt; (phase III testing)</td>
<td>51</td>
<td>195</td>
<td>2000</td>
<td>0.065</td>
</tr>
<tr>
<td>2-D system (I&lt;sub&gt;µ50&lt;/sub&gt;)</td>
<td>32</td>
<td>30</td>
<td>4500</td>
<td>0.045</td>
</tr>
<tr>
<td>3-D system (I&lt;sub&gt;µ50&lt;/sub&gt;)</td>
<td>11.5</td>
<td>39</td>
<td>750</td>
<td>0.035</td>
</tr>
<tr>
<td>High cell density 3-D system -Tissue mimicking</td>
<td>20-200 (~ 10 times)</td>
<td>100-1000 (~10 times)</td>
<td>10,000-30,000</td>
<td>&gt;100 (&gt;1000 times)</td>
</tr>
</tbody>
</table>

Concentration unit: µg/ml
Prototype II
40 microbioreactors on 384-well plate
Drug Efficacy Test

20 microbioreactors with different drug doses

Effects of 5-FU and gemcitabine on colon cancer cell

![Graph showing the effects of 5-FU and gemcitabine on colon cancer cell fluorescence over time.](image)
Drug Efficacy Test

The conc. of both drugs used is 10, 100, 1000 times of IG50

Both low conc. gemcitabine and 5-FU increased fluorescence, while only high conc. 5-FU showed decreased fluorescence

Each curve is from duplicate or triplicate experiments
Anticancer drug study

- **5-FU**: widely used in colon cancer treatment
- **Gemcitabine**: very weak activity on colon cancer treatment

![Graph showing IC50 values for 2-D and 3-D HT-29 colon cancer cells.

IC50 (µM) vs. Drug Concentration

- **5-FU (µM)**
- **Gemcitabine (µM)**

2-D:
- 5-FU IC50: ~1 µM
- Gemcitabine IC50: ~0.001 µM

3-D:
- 5-FU IC50: ~1000 µM
- Gemcitabine IC50: ~1000 µM

HT-29 colon cancer cells
Immunohistochemistry

p27kip1: quiescence marker; Ki-67: proliferation marker

- P27kip1: 30-40%
- P27kip1: <5%
- Ki-67: 10%
- Ki-67: 10-20%
- Ki-67: 30-40%

Fresh tonsil tissue

3-D HT-29 (2 week)

2-D (1 week)
Summary

✦ GFP cells can give non-invasive real-time growth and cell death kinetics for high throughput drug screening

✦ 3-D culture showed different responses to drugs
  – More sensitive/responsive than 2-D culture
  – High density 3-D culture (with tissue-like morphology) is much more resistant to drugs
  – more similar to those *in vivo* studies
Perfusion Microbioreactors

✧ Continuous nutrient supply and waste removal
✧ Homogeneous culture environments
✧ Mixing and concentration manipulation

Figure 1: The BioFlux 200 System for live cell assays under controlled shear flow.

Figure 2: BioFlux Plate channels as viewed from beneath the well plate. Microfluidic flow cells are integrated into the bottom of an SBS-standard well plate. Each fluidic channel runs between pairs of wells and has a central viewing window for observation.
Microfluidic Bioreactors

(a) Cell Culture Medium Inlet


Microfluidic Bioreactors

Droplet microfluidic technology for single-cell high-throughput screening

PNAS. 2009, 106: 14195-14200
Microfluidic Bioreactor Array

- Double layers to form microfluidic network
- Cell culture well filled with tissue construct
**Device Fabrication: Soft Lithography**

1. **Si Wafer**
   - Spin coating photoresist SU-8

2. **Si Wafer**
   - Expose to UV through a mask with pattern, followed by developing

3. **Si Wafer**
   - Cast poly(dimethylsiloxane) (PDMS) prepolymer

4. **Si Wafer**
   - Cure and remove from mold

<table>
<thead>
<tr>
<th>Height of top layer (μm)</th>
<th>Height of bottom layer (μm)</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>420</td>
<td>500</td>
<td>6.5</td>
</tr>
</tbody>
</table>
Packaging: Frame-Assisted Assembly

Top frame

Bottom frame

Device in assembly

Assembled device at work
Normalized Growth Curves for HT-29 Static Culture in Microbioreactor Array

Normalized Fluorescence Intensity

0 20 40 60 80 100 120
Time (hr)

Normalized Growth Curves for HT-29 Static Culture in Microbioreactor Array

- 1.88e5 cells/mL
- 3.75e5 cells/mL
- 1.5e6 cells/mL
- 3.0e6 cells/mL
Perfusion Culture

H&E Staining for Sections at Different Depths of Tissue Construct
Cytotoxicity: Colon cancer cell

Normalized Fluorescence Intensity vs. Time (hr)

- 0 ug/mL
- 1 ug/mL
- 10 ug/mL
- 100 ug/mL

5-Fluorouracil

Time (hr)
0 25 50 75 100 125 150
Conclusions

✧ Cytotoxicity test using GFP cells grown in 3-D matrix in multi-well plates and microfluidic bioreactors can be used for high throughput drug screening.

✧ 3-D culture system gave higher fluorescence signal and more reliable reading for long-term culture.

✧ The GFP fluorescence in the 3-D culture gave accurate estimation of cell proliferation rate and sensitivity to drug tested.

✧ Cells in the 3-D environment (tissue) was much more resistant (less sensitive) to drug.

✧ Continuous perfusion allowed for long-term evaluation of drug effects in microbioreactors.
Animal on a chip for cytotoxicity

Naphthalene

Lung (L2 cell)

Metabolite-induced toxicity (GSH depletion)

Flow

Other tissues

Liver (HepG2/C3A)

Fat

Metabolite generation

Other Applications

Applications of Microbioreactor Array

- Cytotoxicity of natural and synthetic materials
- Gene expression in cells and tissues
- Medium optimization in cell culture process development
- Scaffold design for tissue engineering
- Stem cell differentiation
High-throughput process development using 3-D microbioreactor array

Effect of butyrate on IgG production by CHO cell culture

35% increase in IgG production with 1 to 6mM butyrate in the medium
Spinner Flask Culture
Rotation speed: 90 rpm
Volume: 80 mL
Basal Medium: DMEM/F12
Temperature: 37°C

50% increase in IgG production
Drug Discovery To Market

$400,000,000

78% failure rate

22.5% efficacy failure
Microfluidic CD Biochips for Enzyme-Linked Immunosorbent Assays

Detection of pathogens:
- E. coli O157: H7
- Salmonella
- Compylobacter

Detection of cancers, immuno- and infectious diseases, food allergens, and environmental pollutants
Questions

Largest university with over 63,000 students

Go Bucks!
Acknowledgements

National Science Foundation – NSEC; NIH, USDA, DOE, ....
CD-ELISA

- Automation
- Parallel

US patent Application No. 11/556,132