RASL-Seq: A Gene Expression Platform to Identify Toxicity Mechanisms and Adaptive Responses

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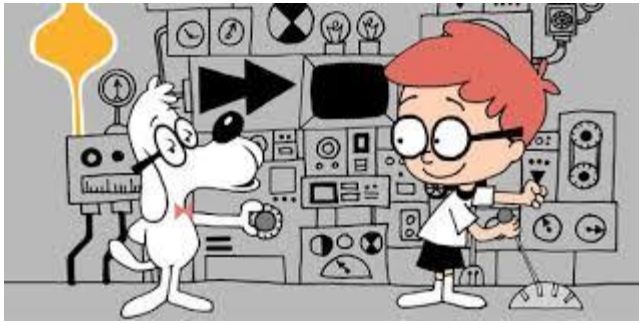
EPA'S COMMUNITIES OF PRACTICE

NCATS

NIH National Center for Advancing Translational Sciences

National Heart Lung and Blood Institute

Summary



- RASL-Seq Selected, Results
- RASL-Seq platform works well
- Industrialization proceeding
- Selecting 1,400 genes to represent the genome(s)
- Goal: HT Gene Expression Core Facility
- How to apply it? Elucidate modes and mechanisms of toxicities or AEs. ‘Secondary screening’
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Goals

- A technology that will quantify mRNA responses in hundreds-thousands of genes
- Throughput > Thousands of samples to address: many compounds x multiple cell lines x multiple concentrations x multiple time points
- >> Gene Expression Core Facility at NCATS
- Low variance, intra- & inter-experiment, will enable data interpretation and construction of a rich reference database
- Data analysis pipeline

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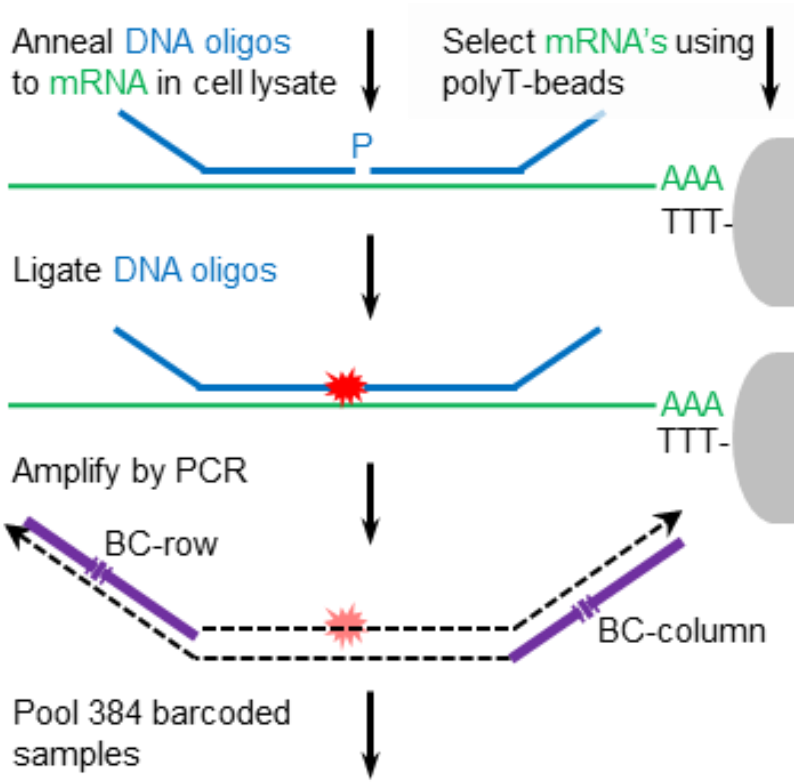
RASL-Seq Schematic



RASL-Seq Characteristics

RASL-Seq has emerged from evaluation of six technologies based on:

- **Multiplex:** ≥1,000 genes/sample, and 384 samples/sequencing reaction
- **Throughput:** (384 samples/run x ~10 runs/week)
- **Economy**: roughly $10.43/sample or $4,000/384 sample-run, including NextGen sequencing
- **Accuracy:** uses 3 redundant assays/gene
- **Reproducible:** avoids chaotic cDNA synthesis step. Intra-experiment $R^2 \geq 0.99$
- **Gene-Specific**: % mispairing events (after removing 5 promiscuous assays, of 360) is 0.26%

RASL-Seq invented by Xiang-Dong Fu Lab, Stanford U. Li H et al. PNAS 2012;109:4609-4614

Collaboration between BioSpyder (J. Yeakley & J. McComb) and NCATS to optimize RASL-Seq. Includes SBIR grant from NCATS.

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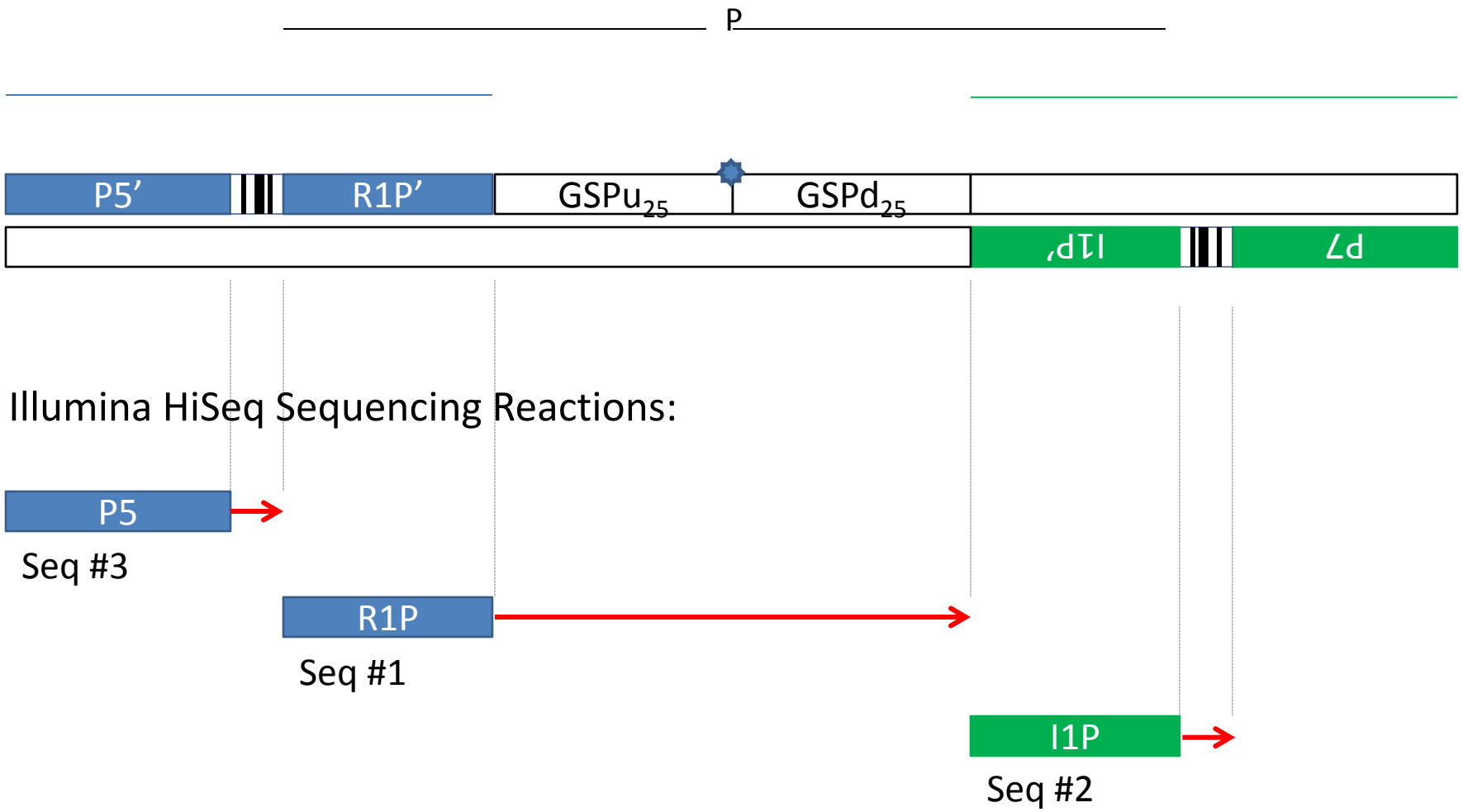
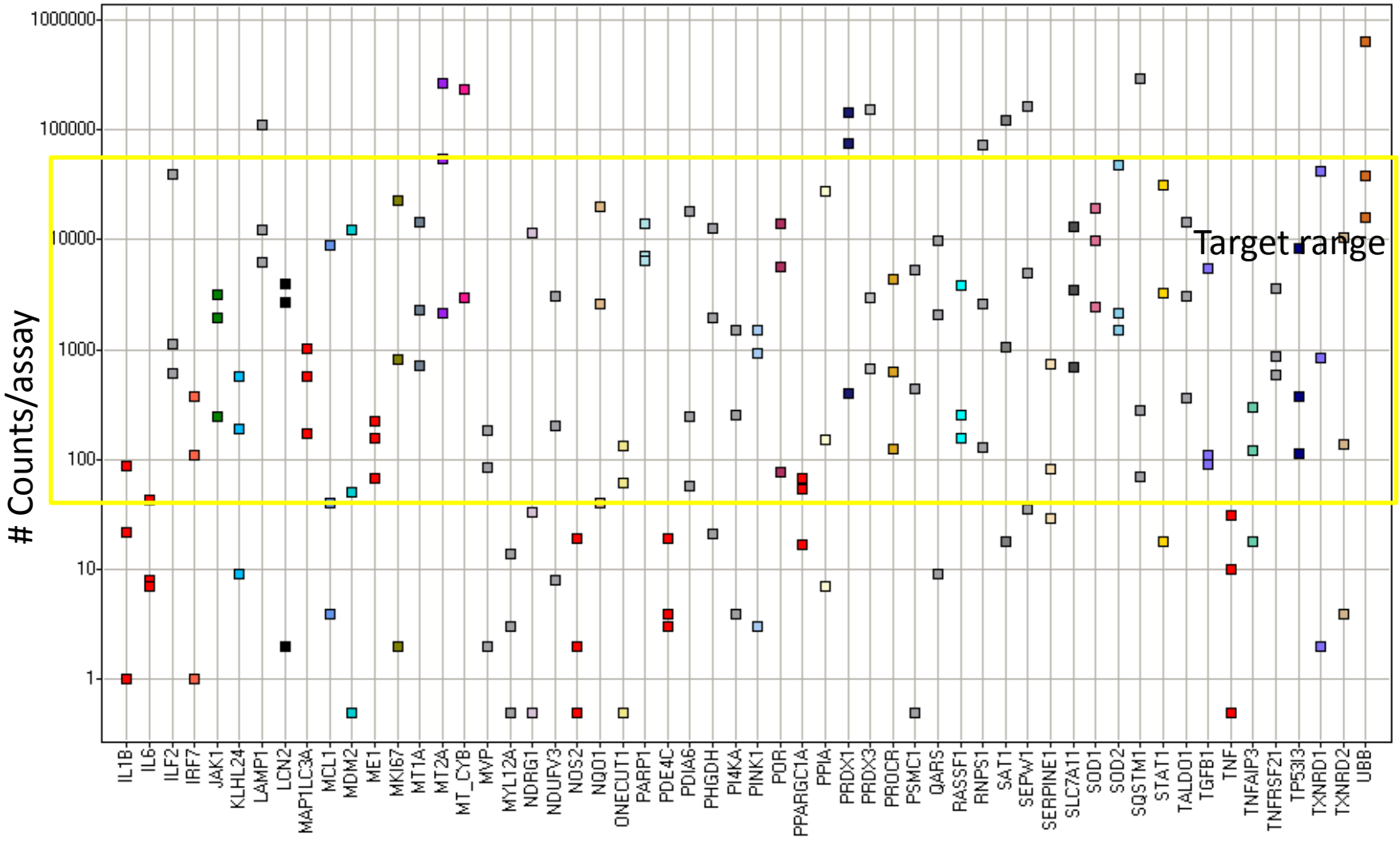
RASL Product Sequencing



3 RASL-Seq assays/gene vary widely in # counts

⇒ Remove 'greedy' assays

⇒ ignore assays that give low counts



RASL-Seq Progress March 2014

1st Gene set 120 genes (x 3 assays/gene) =360 assays. 13 'greedy' assays removed, and 5 non-specific (mispairing) assays removed.

- All steps performed by hand, using 384 samples, in microplates.
- Biomek FX robot installed 18 March 2014
- Good yields from PCR step in all five 'runs'
- 1-of-3 sequencing primers has been problematic. Two datasets now have resolved that issue for MiSeq instrument (24 or 50 samples ⇨ 11 million high-quality seq). Excellent data!
- Testing on HiSeq2000 instrument set for April 2014. Expect 150 million sequences/lane. 150 million sequences/384 samples = 390,000 sequences/sample.

2nd Gene set: 320 genes (x 3 assays/gene) 960 assays being designed

3rd Gene set: ~1,000 genes being selected by working group.

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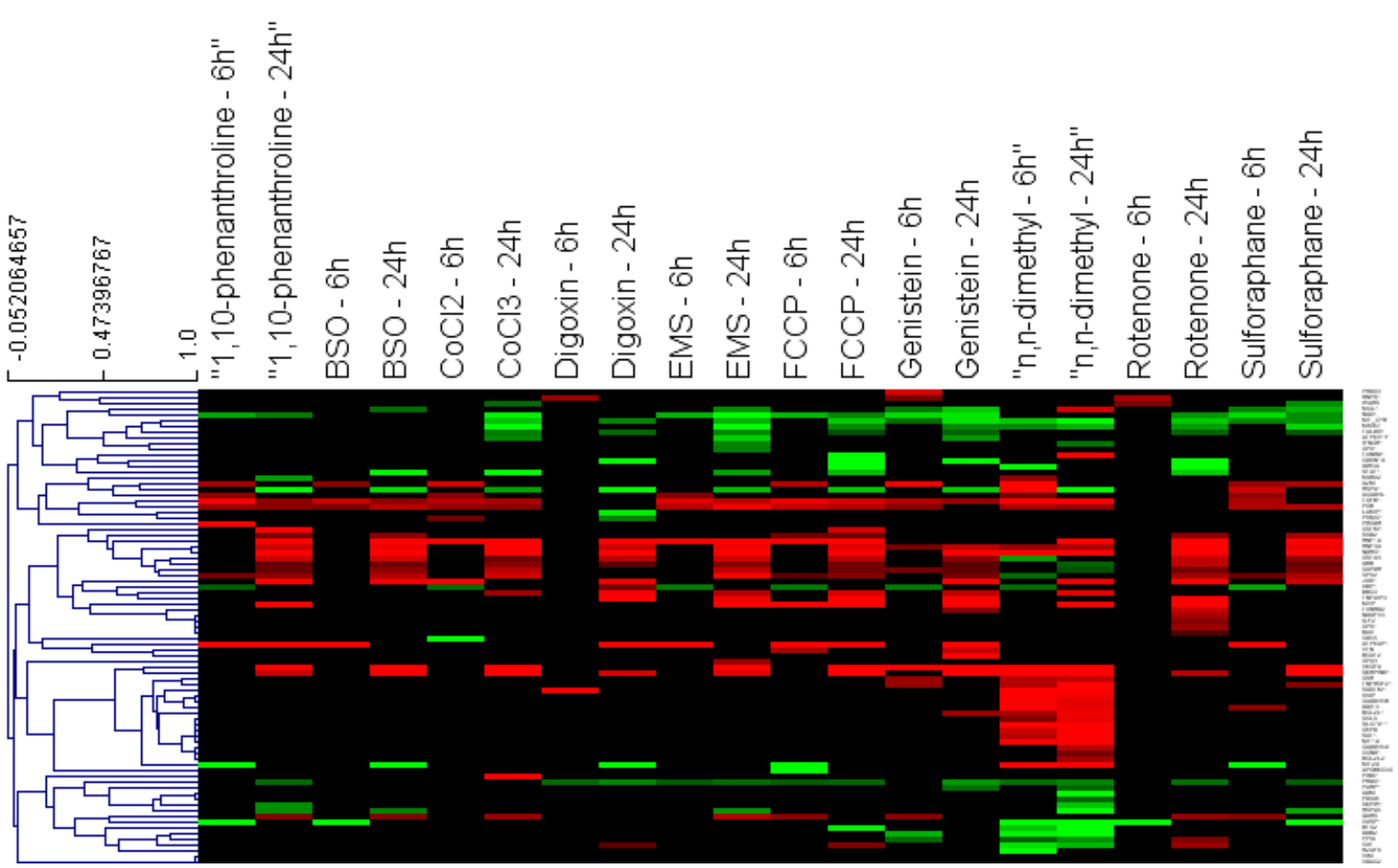
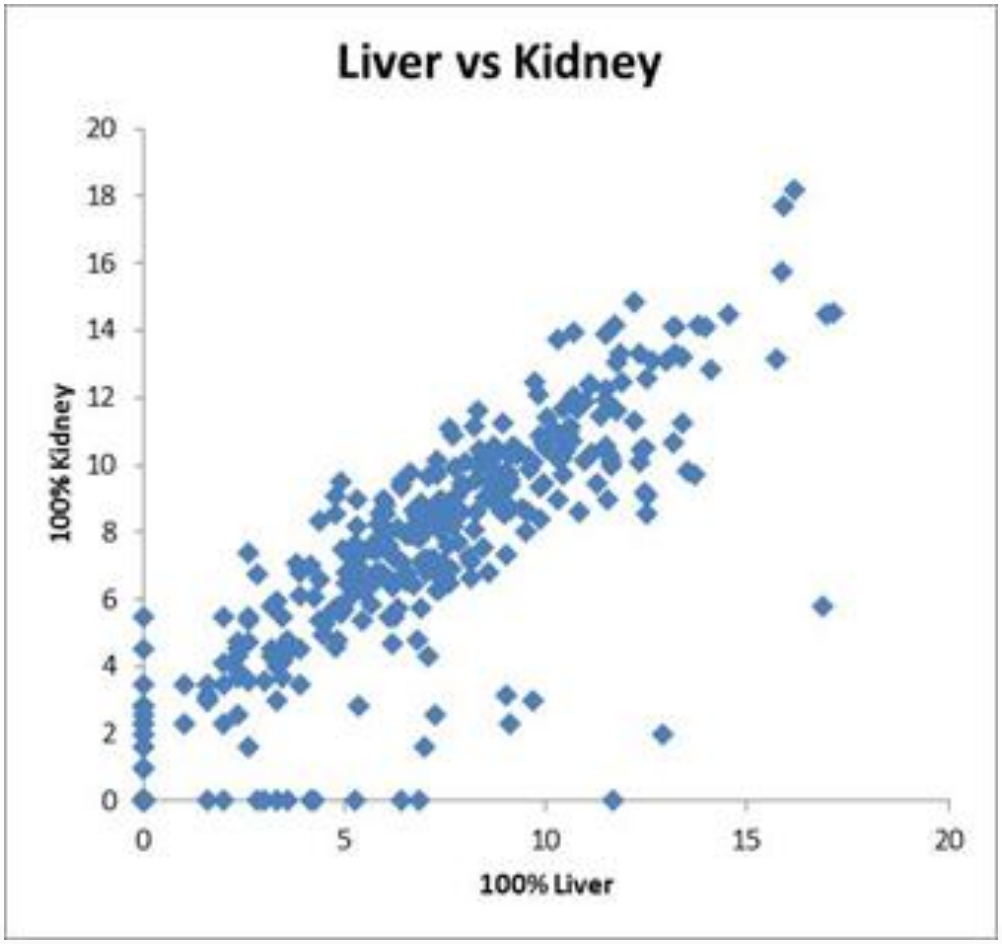
RASL-Seq Results

50-of-384 samples > 5 million reads on MiSeq

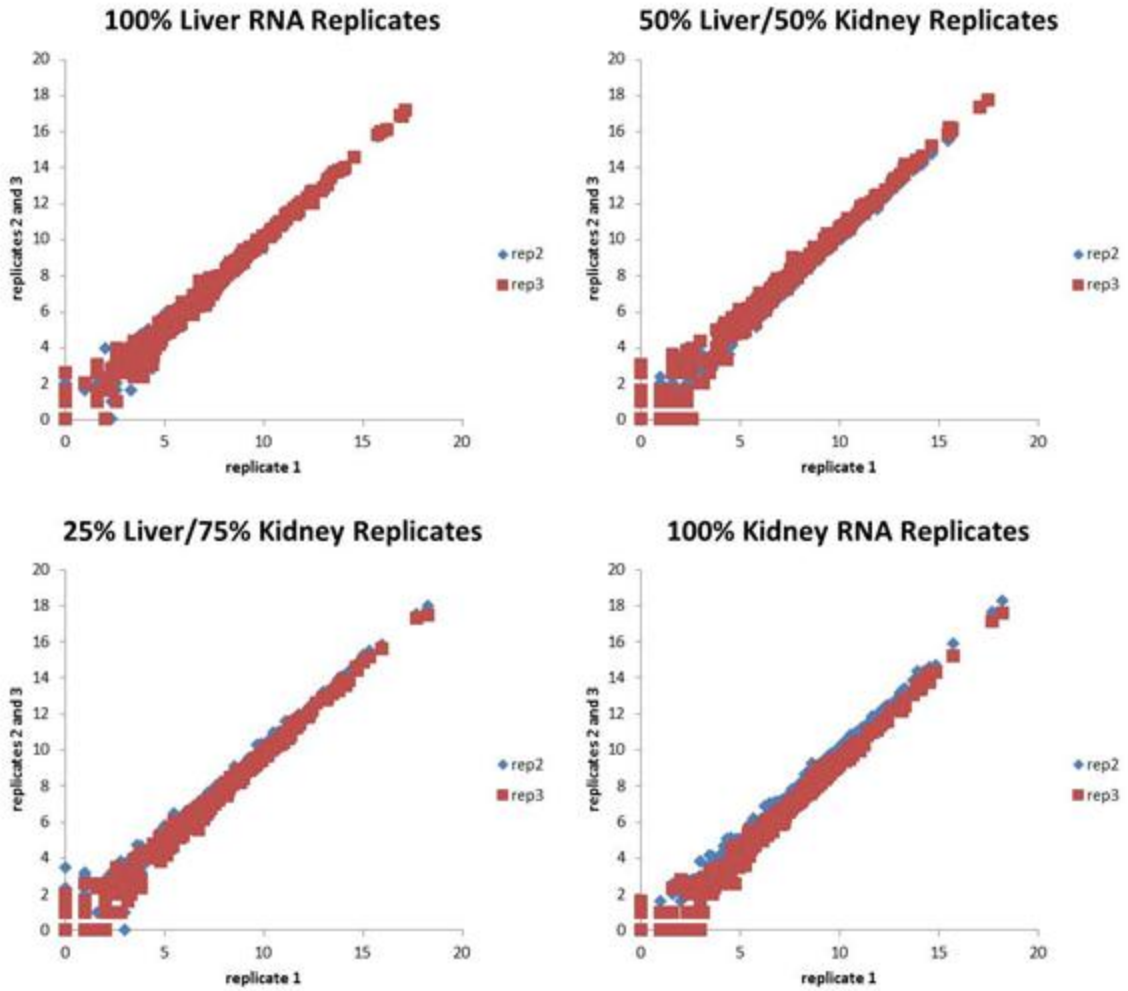


RASL-Seq Describes Differential Expression in Liver vs Kidney for First 120 Genes



Data courtesy of J. Yeakley, BioSpyder, 14 March 2014

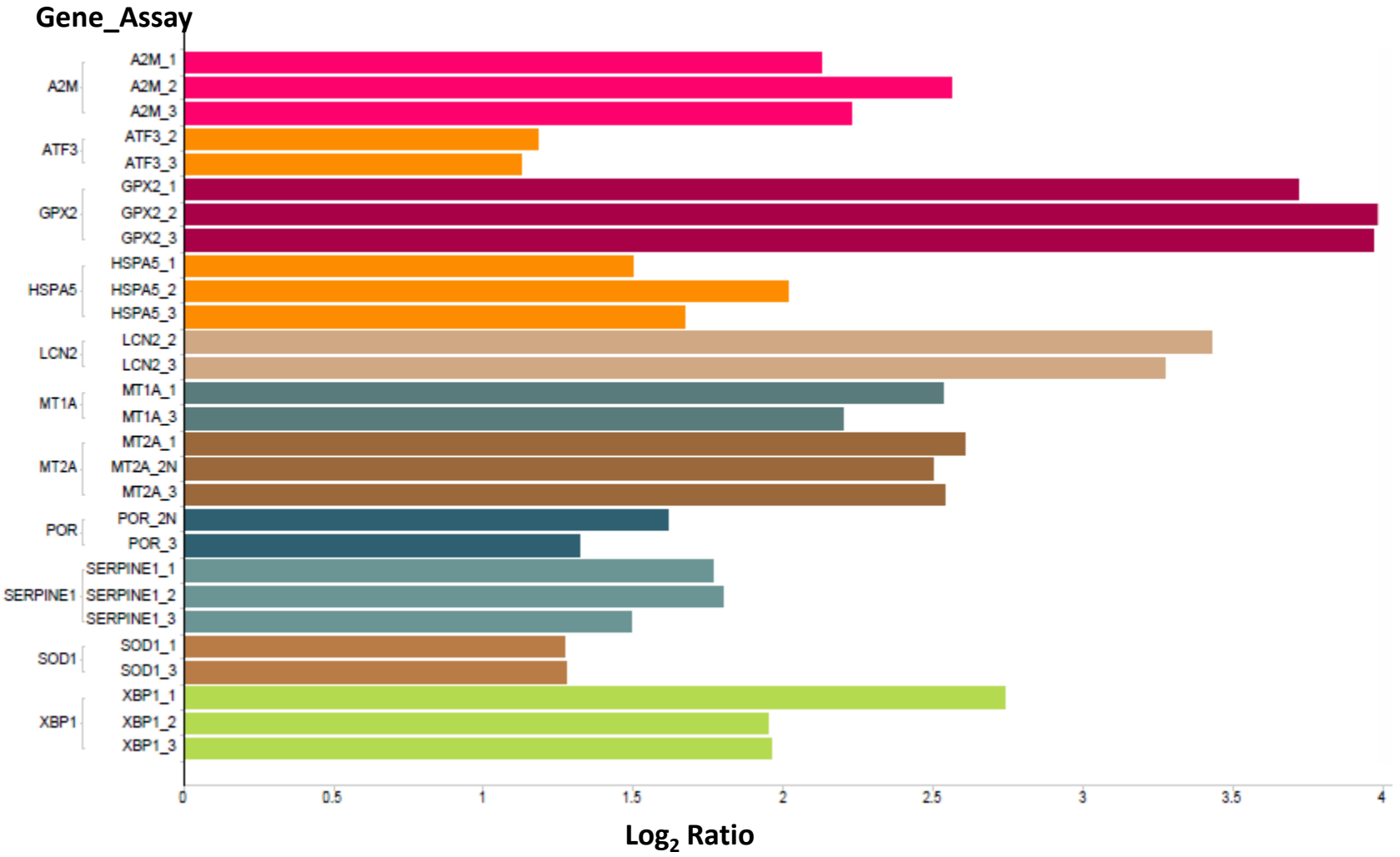
Intra-Experiment Variability is Low in RASL-Seq



The twelve replicate pairwise R^2 values ranged from 0.990 to 0.995

Data courtesy of J. Yeakley, BioSpyder
14 March 2014

Redundant RASL-Seq Assays Give Similar T/C Ratios



Tox21 Gene Set Selection

Select core set of 1000 genes to assay on all cell lines & samples.

Working Group established to select ~1,000 genes for humans…then other species. Led by Rick Paules

- to: identify ~ 1000 genes that will optimally represent the gene expression responses of the entire genome to diverse chemical and biological challenges.
- We reviewed costs. Selecting new genes and assays will cost ~$90/gene

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Typical RASL-Seq Application

Example:

- 60 compounds selected from primary mitochondrial membrane permeability screen Tox21
- Treat 3 models: (*e.g.* HepG2, LUHMES dopa-neurons, cardiomyocytes) x 2 concentrations x 2 time pts x 60 cpds = 720 treatments (2 x 384-well plates x 3 reps?).

Interpretation:

- Do cpds fall into groups that imply mechanisms?
- Do gene expression changes suggest mechanisms?
- Do cellular models react differently to same cpds?

Data Analysis & Interpretation

Data analysis pipeline:

- Filter to remove low quality sequences
- Deconvolute pooled sequences to 384 samples *via* barcodes for rows & columns
- Count matches to target genes (*e.g.* >45/50nt). Adapt NCATS extant pipeline for RNAseq data
- Perform statistical tests for significance for each assay (treated vs controls). Use median if 2-3 assays/gene?

John Braisted's scripts.
Can be automated

Data interpretation:

- Associate cpds that cause similar gene expression profiles. Does cpd profile resemble a reference perturbation profile?
  - ➢ Implies similar mode or mechanism of toxicity.
- Do early-responding genes *describe* the mode, *e.g.* DNA repair genes or E.R. stress genes?
- Clarify hypotheses by referring to RNAseq baseline data for each cell line. *E.g.* Is the hypothesized receptor/transcription factor/pathway transcribed in these cells?
- Do cellular models react differently to same cpds?

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How Would a Baseline RNAseq Dataset for Each Cell Line Inform RASL-Seq Data? Example:

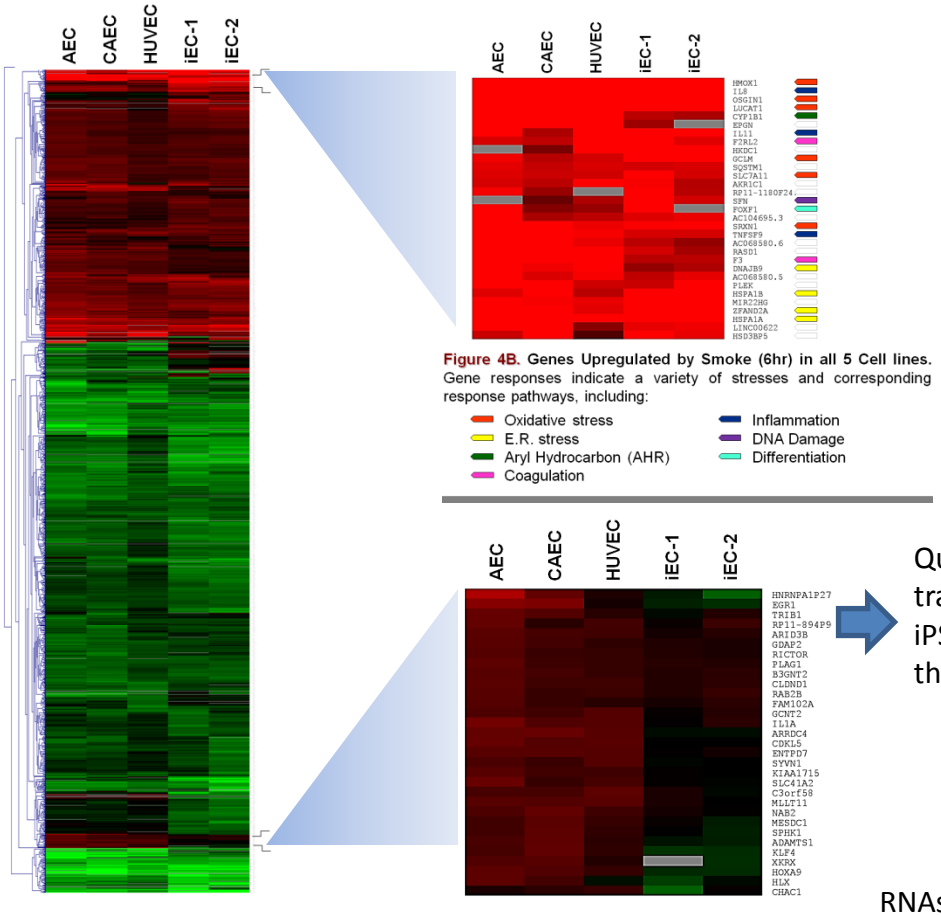


Figure 4B. Genes Upregulated by Smoke (6hr) in all 5 Cell lines. Gene responses indicate a variety of stresses and corresponding response pathways, including:

- Oxidative stress
- E.R. stress
- Aryl Hydrocarbon (AHR)
- Coagulation
- Inflammation
- DNA Damage
- Differentiation

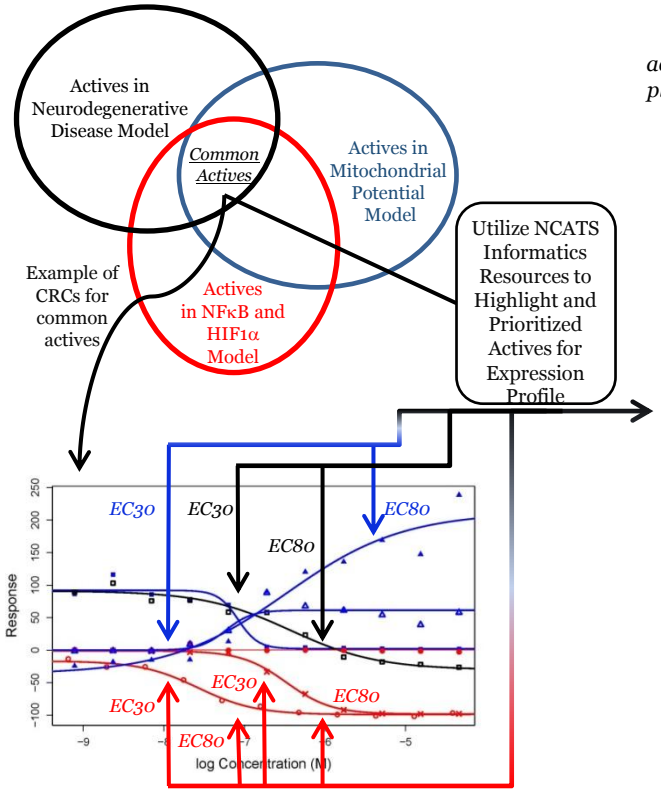
RNAseq (mRNA) Responses to Tobacco Smoke (6hr) in iPS-derived Endothelial Cells (iECs), and Primary Endothelial Cells

Question: Is a developmental transcription factor missing from the iPS-derived cells that could explain these nonresponsive genes?

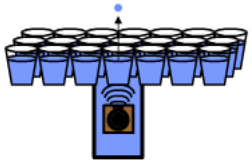
Examine reference RNAseq data

RNAseq data suggest GATA6 is expressed only in the primary cells. Testable Hypothesis

Disease Cell Models	*General Phenotype Cell Models*	*Pathway Informing Cell Models*
1) Cancer • Multiple Myeloma • Viability & Apoptosis • Rhabdomyosarcoma and Neuroblastoma • Viability & Apoptosis	**1)** Autophagy • ~~HEK 293A line~~	**1)** NFκB • Me-180 line
2) Diabetes • INS-1E pancreatic β-cells • ~~Insulin secretions~~ In	**2)** Mitochondrial Potential • Parkin line	**2)** ~~STAT3~~ • ~~HEPG2 line~~
3) Neurodegenerative Diseases • Hela line w/GFP-parkin • ~~Parkin Translocation~~	**3)** DNA Damage/Repair • P53 HCT-116 line	**3)** HIF1α • ME-180 line
4) Innate Immunity • A549 line w/GFP-RSV • RSV spread • Hela line w/GFP-Vaccinia • Vaccinia spread	**4)** TNFα secretion • THP-1 line **5)** ROS Induction/Mitigation • HEPG2 line **6)** Type 1 interferon response • *h*Fibroblast line **7)** Lipid Droplet accumulation • 3TL1 line	**4)** ~~NRF2~~ • HEPG2 line **5)** AP1 • ME-180 line **6)** Estrogen Receptor • HEK293 line **7)** Androgen Receptor • HEK293 line **8)** CREB • HEK293 line

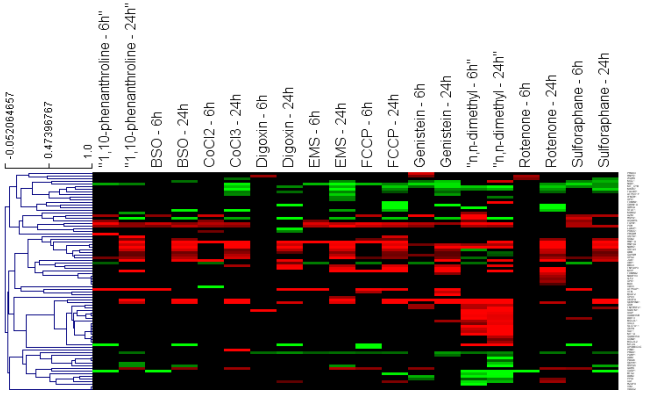


Macros written for EDC acoustic dispenser allow facile plating of each small molecule perturbagen at custom concentrations



Established protocols allow rapid RASL-Seq gene capture on 1000 genes per sample in 384-well plate format

Comparative analysis of expression signatures from selected, active and mirrored inactive perturbagens



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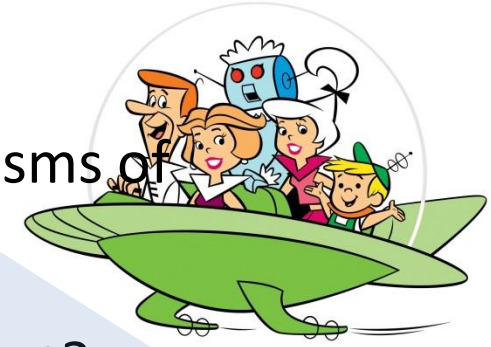

Collaborators

"Vieles ist bekannt, aber leider in verschiedenen Kopfen", W. Kollath
("much is known, but unfortunately in different heads")

NCATS

John Braisted
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David Gerhold
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NHLBI Seq Core

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