Multiplex Imaging for Single Cell Mechanistic Analysis of Steroid Receptor Functions

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Modeling nuclear receptor regulation at the promoter level

A generic and highly simplified snapshot

- Population-based biochem studies provide a wealth of mechanistic data from pooled cells.
- Cell-to-cell (or allele-by-allele) differences in pools are difficult to assess (missed!).
- Many studies use non-contextual assays (cell free and exogenous systems)
- Is it possible to visualize, and quantify, towards a systems level appreciation…cell-bv-cell?
- Is there utility for endocrine disrupting chemical testing?
Galveston Bay/Houston Ship Channel: A case study for analysis of environmental pollutants released during storms

Ivan Ruysen, PI

Mancini Lab, Project 4: Single Cell, Multi-Parametric High Throughput Platform to Classify Endocrine Disruptor Potential of Mixtures
Visualizing steroid-induced nuclear receptor trafficking in living cells (circa 1998)

David Stenoien, PhD

GFP- Androgen Receptor

~40 min after testosterone

Nuclear translocation

GFP- Estrogen Receptor

~40 min after estrogen

Subnuclear organization
Can additional functional measurements for transcription be included for improved data mining and classification of results?

- DNA targeting
- promoter NR occupancy
- protein-protein interactions
- chromatin modification
- mRNA synthesis
- *et cetera*....

Why single cell analysis?
NR expression in tumors (and cell culture): the challenge of heterogeneity

Endogenous ER AR GR

Quantifying population responses cell-by-cell
Visualizing prolactin promoter-regulated transcription by ER

Versatile cell model system: (ERα, ERβ, PR-ER chimera, AR-ER chimera*)

100 copies in 1 cluster

E2 inducible and Tam repressible

Super-PRL enhancer

~100 COPIES PER CELL

PRL Distal Enhancer

Proximal PRL Promoter

Reporter Gene

100 copies in 1 cluster

Fully-automated high content analysis for ER functions

(ERα, ERβ, PRER chimera, ARER chimera, TRER chimera*)

Bolt et al, 2013, NAR
Bolt et al, 2015, Oncogene
Trevino/Bolt, 2015, Mol Endo
Szafran et al, PLoS One 2017

A Single Assay for quantitative multiplex analysis:

- translocation
- protein levels
- promoter occupancy
- chromatin
- interactions
- mRNA (FISH)
- cell cycle (2N/4N)
- proliferation (cell count)
- toxicity index (stress)

>>1,000 commercial abs tested: visual ChIP and single cell proteomics
Evaluation of Endocrine Disrupting Potential via the Estrogen Receptor Pathway

Multiple EDC sources are present in the environment that may affect human physiology.

High throughput assays are used to determine EDC potential – ToxCast initiative.

EPA :18 (and now 4) in vitro ER assays substitute for uterotrophic assay (Judson et al., 2015, 2017)

ER Mechanistic Pathway:
1. Receptor Binding
2. Dimerization
3. DNA Binding
4. Cofactor Recruitment
5. Chromatin Remodeling
6. RNA Transcription
7. Protein Synthesis
8. Cell Phenotype
Can our platform classify biological activities of endocrine disrupting chemicals?

Stossi et al, 2014, *Chemistry & Biology*
HCA-based functional screening of BPXs on ERα and ERβ

Combined data mining summary:
- PRL arrays
- BiFC protein interaction assays
- MCF7 growth assays
- Reporter gene assays
- Endogenous ER target gene mRNA FISH

- Most BPXs bind to either ERα and/or ERβ
- BPXs have generally higher affinity for ERβ
- BPXs are mostly antagonists on ERβ while being partial agonists on ERα

Some BPXs are relatively inactive and may be candidates as BPA substitutes

Commercial contract screening has identified 2 new candidates (inactive) (Szafran et al, 2017, PLoS One)

Fabio Stossi et al, 2014, Chem&Biology
The Goal:
To characterize and manage both existing and environmental emergency-created hazardous waste sites through the development of the tools that can be used by first responders, the impacted communities, and the government bodies involved in site management and cleanup.

RESEARCH PROJECTS

Project 1: Dynamic exposure pathways under the conditions of environmental emergencies.
Goal: Study the mobilization of contaminants through laboratory and computational models.

Project 2: Novel broad-acting sorption materials for reducing bioavailability of contaminants.
Goal: Development of novel enterosorbent materials for communities at risk of exposure to hazardous substances during disasters.

Project 3: In vitro and in vivo studies of hazard, kinetics and inter-individual variability of responses to mixtures.
Goal: Develop a tiered translational experimental testing strategy for evaluating inter-tissue and inter-individual variability in responses to mixtures.

Project 4: In vitro multiplex single-cell assays to detect endocrine disruption potential of mixtures.
Goal: Develop and commercialize in vitro assays that facilitate evaluation of endocrine disruption hazards through novel high throughput imaging approaches.

SUPPORT CORES

Administrative Core
The central hub for all Superfund Center activities. Provides leadership and guidance, assuring the excellence of research, support, outreach, community engagement, translation, and training activities.

Community Engagement Core
Builds relationships with community partners to ensure community involvement in every stage of the research process to the communication of findings.

Research Translation Core
Expands the reach and impact of research by communicating key findings with stakeholders and communities with the dissemination of key data and predictions on the impacts of contamination.

Training Core
The central hub for science and practice learning by creating opportunities for the professional development of graduate students and postdoctoral fellows.

Data Science Core
Translates data produced by the research projects into useful knowledge for the community via data collection, quality control, analysis, and modeling.

Decision Science Core
Helps investigators to convert environmental and biological data into predictions of health effects and economic costs useful for risk management.

Exposure Science Core
Uses state of the art instruments to identify known chemicals of interest, as well as unknown chemicals in environmental samples to understand exposures.
Reference Environmental Toxicants Sets

- **EPA Estrogen Receptor Reference Set (Environmental Protection Agency)**: 45 agonists, antagonists and inactives.

- **ATSDR Reference Set (Agency for Toxic Substances and Disease Registry)**: 42 compounds selected by the Superfund team based on toxicity and exposure risk.

- **Reference Mixtures Set**: 16 including several mixtures based on ATSDR list (exposure, AC50, POD etc.) + commercially available standards.
ERα PRL Array - DNA Binding

EPA REFERENCES

Strong
Moderate
Weak
Very Weak
Antag/SERM

ATSDR REFERENCES

DDT, P,P'
DI-N-BUTYL PHTHALATE
DDD, P,P'
METHOXYPHCHL
DDT, O,P'

Stossi et al, in prep
Endogenous target gene expression by mRNA FISH
- quantitative, multiplexing, higher resolution and HT amenable

- Additive mechanistic data for analyses and fingerprinting
- Compatible with any target gene; amenable to combination with FISH-compatible mAbs
Epigenetic SMI screen identifies candidate molecules that enhance E2-response by increasing the fraction of firing alleles.

~60 small molecule inhibitors of epigenetic writers, readers, erasers...

Overnight treatment followed by 1hr of E2

Stossi and Mancini, submitted
High Throughput mRNA FISH

Novel low mag + high resolution
Thousands of cells/image
RNA molecule detection, multiplexing, microfluidics…

Zoomed 20x air lens
endogenous GREB1 mRNA transcripts
10nM E2, 8hr
intron probe set
DNA

Synthetic Aperture Optics

www.OpticalBiosystems.com
Summary

- Single cell-based approaches are readily-available for **fast** basic science:
  - efficient, multiplex mechanistic and phenotypic assays of **INDIVIDUAL CELLS**
  - addresses challenges posed by heterogeneity

- Multiplexed HCA/HCS-based studies and image informatics can classify EDCs

- Highly multiplexed studies demonstrate E2 induction of GREB1 transcription is linked to increased numbers of cells **and** alleles (**other genes, too**)

- Screening with inhibitors demonstrate novel epigenetic regulation of number of alleles to fire (methyltransferase coregulators CARM1/PRMT6)

- Single cell analysis requires the best antibodies. HT mAb library screening platform = custom-fit mAbs, including those for specialty assays (IHC, FISH, Immunogold) and “functional/neutralizing” mAbs
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