Optimized Experimental and Analytical Tools for Reproducible Drug-Response Studies

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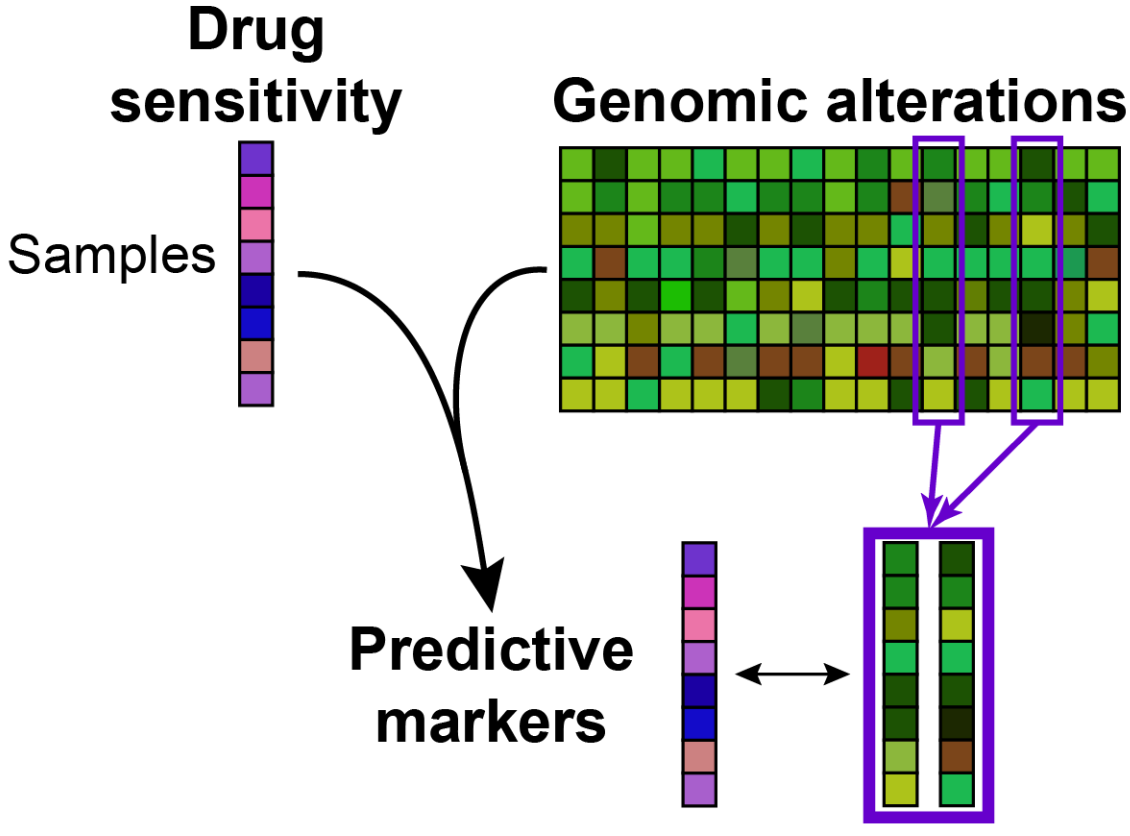
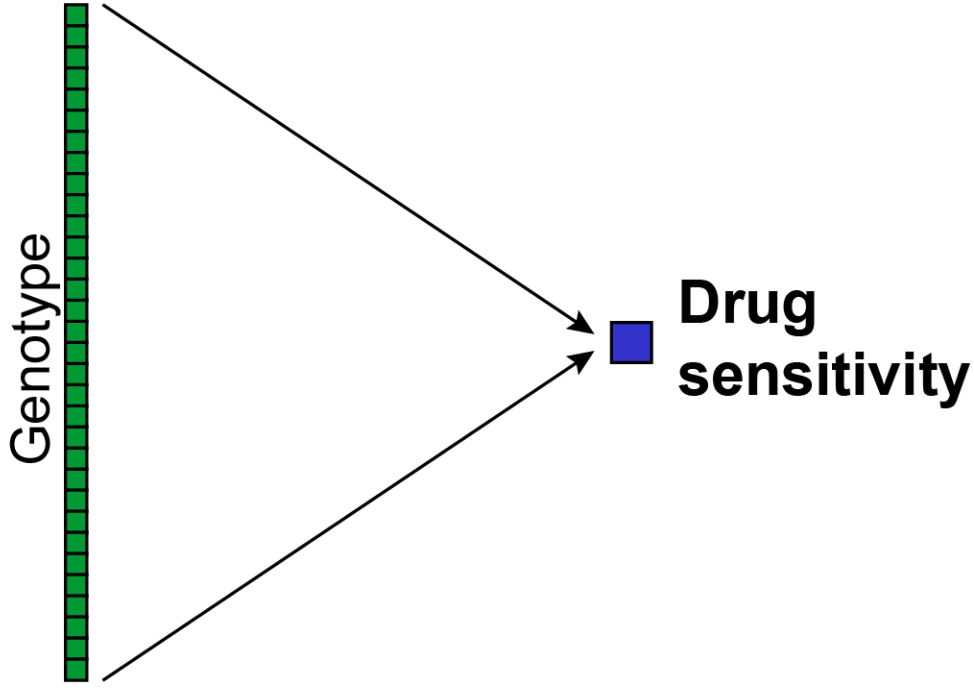
Current approaches focus on mapping drug sensitivity to genotype using screening data



Current approaches assume that genotype and drug sensitivity are directly connected



Current approaches require reproducible drug sensitivity studies

Inconsistency in large pharmacogenomic studies

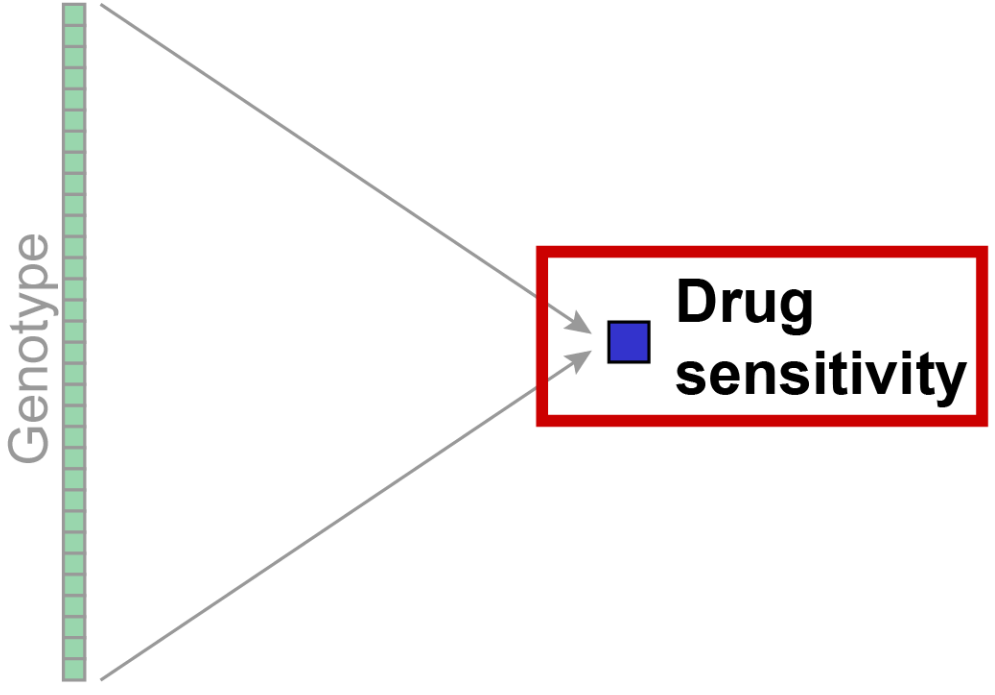
Benjamin Haibe-Kains, Nehme El-Hachem, Nicolai Juul Birkbak, Andrew C. Jin, Andrew H. Beck, Hugo J. W. L. Aerts & John Quackenbush

Affiliations | **Contributions** | **Corresponding author**

Nature **504**, 389–393 (19 December 2013) | doi:10.1038/nature12831

1. CCLE & GDC, Nature, Dec 2015
2. Haverty et al., Nature, May 2016
3. Bouhaddou et al. Nature, Dec 2016
4. Mpindi et al., Nature, Dec 2016
5. Safikhani et al., Nature, Dec 2016
6. Geeleher et al., Nature, Dec 2016

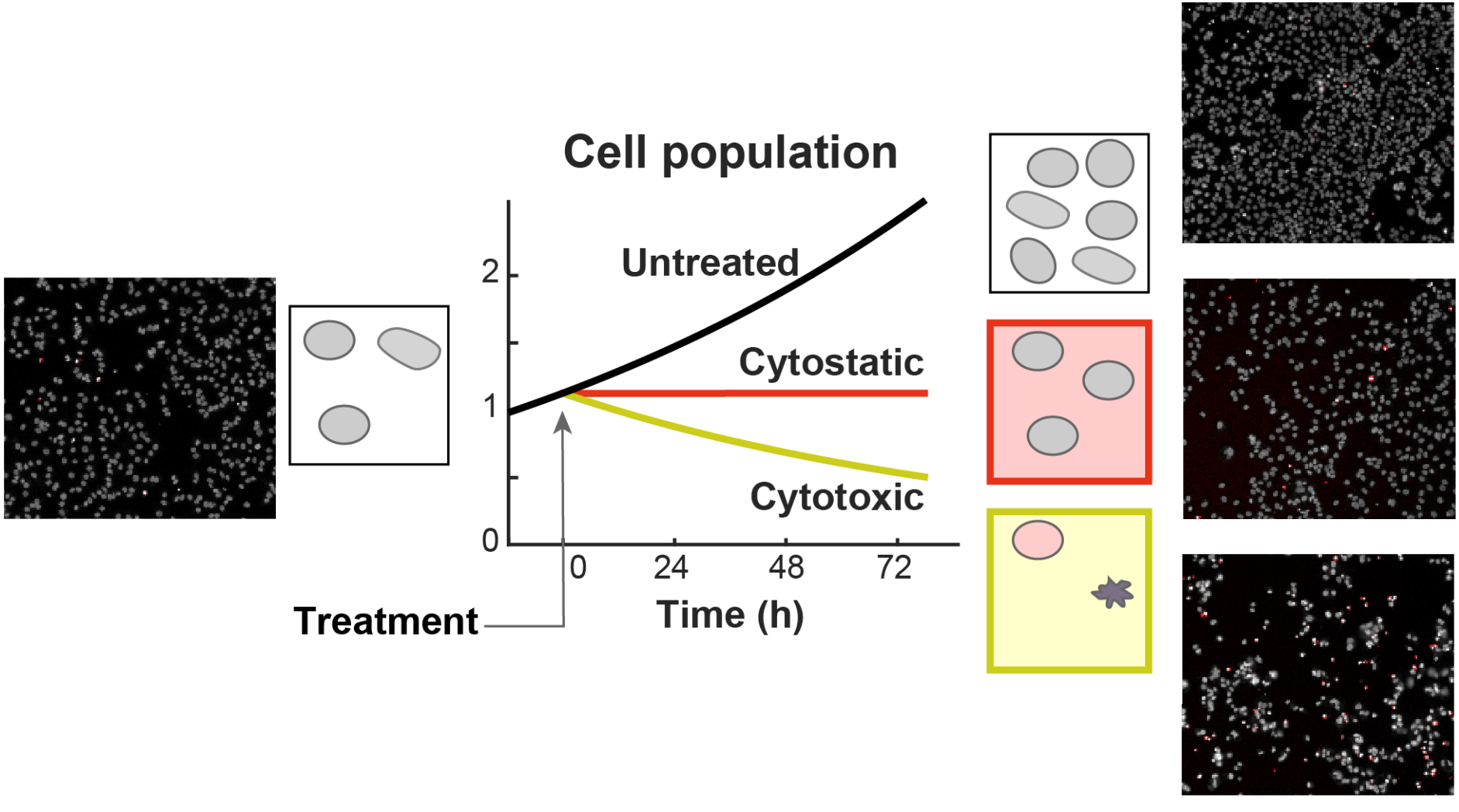
Advancing precision medicine requires improved quantification of drug sensitivity



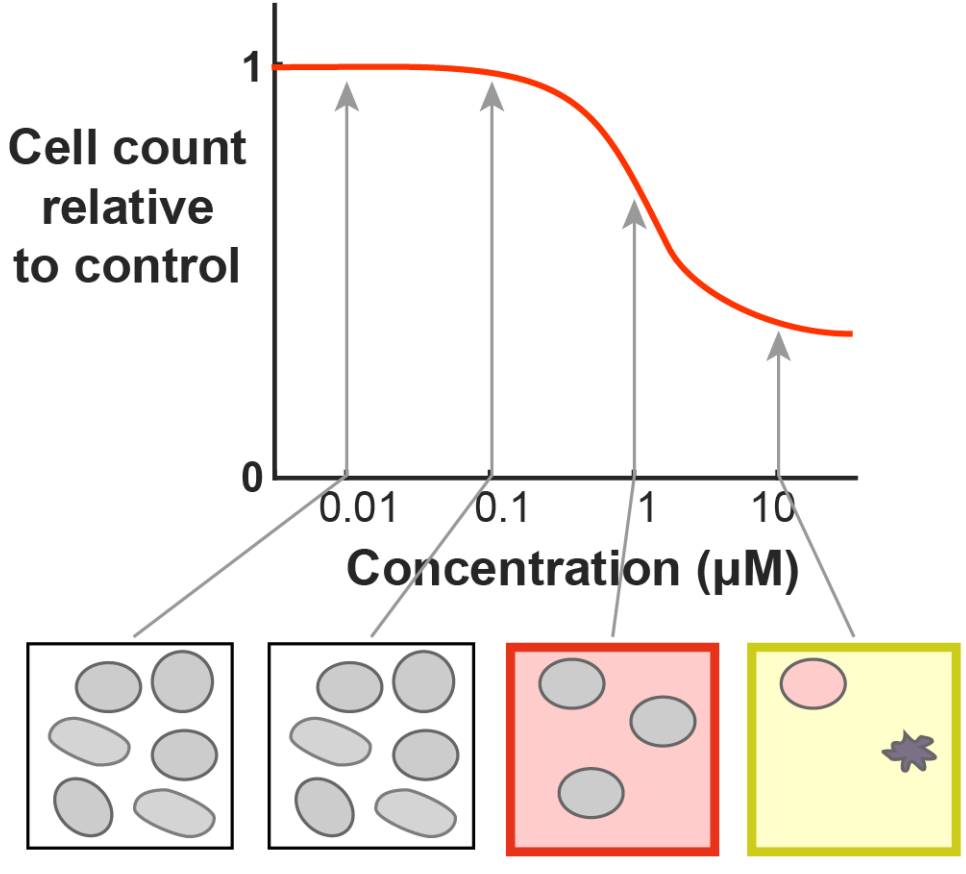
Which metrics best capture the response phenotype?

1. **Theory of drug response**
   - **Normalization**
   - **Importance of adapted metrics**
2. Experimental setup
3. Designing and analyzing experiments
4. Biological examples

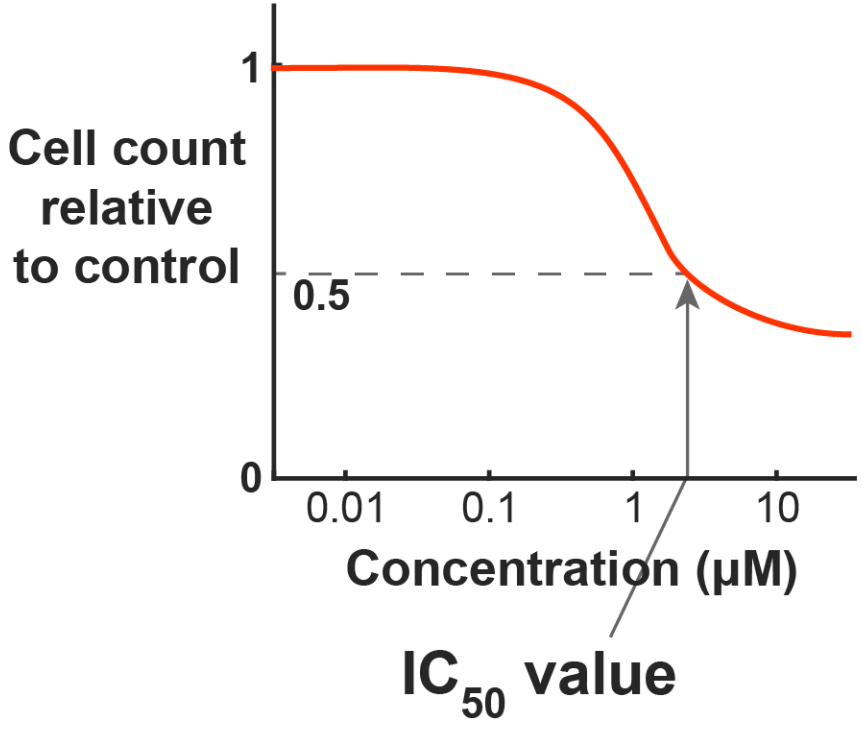
Measuring drug response is essential in pharmacology



Drug response is assayed at multiple doses

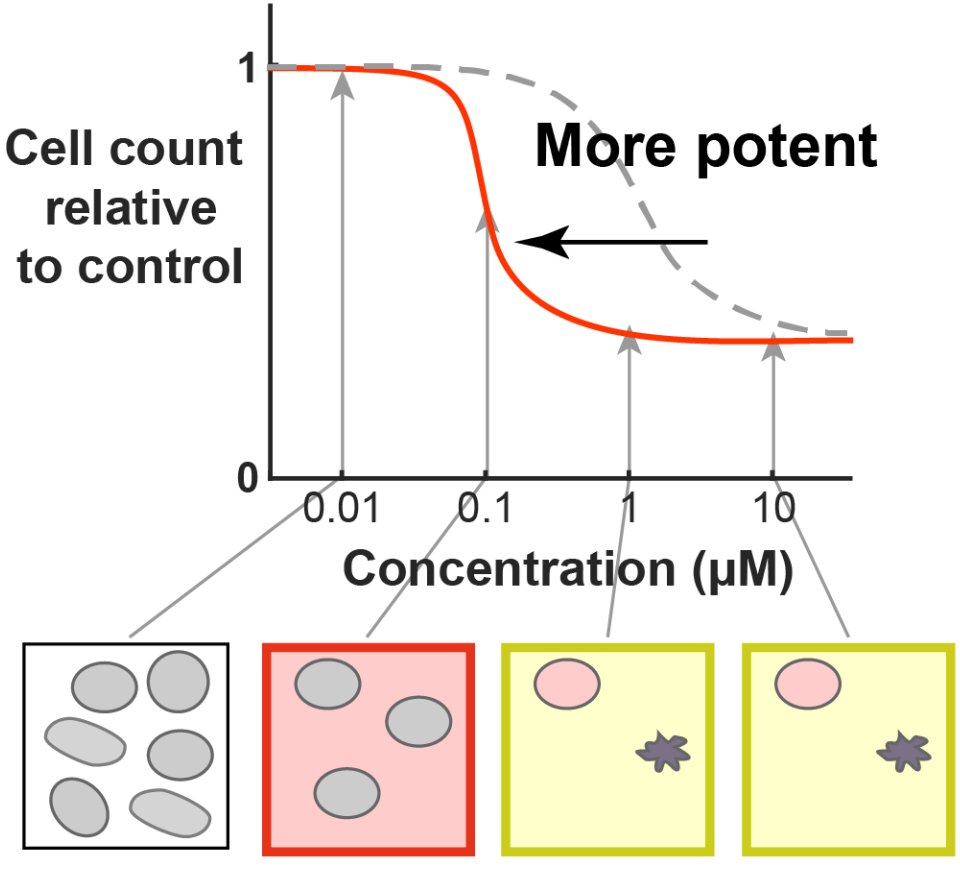


Drug response is assayed at multiple doses



IC_{50} value is the concentration at which the relative cell count is 0.5.

Dose response curves vary across cell lines

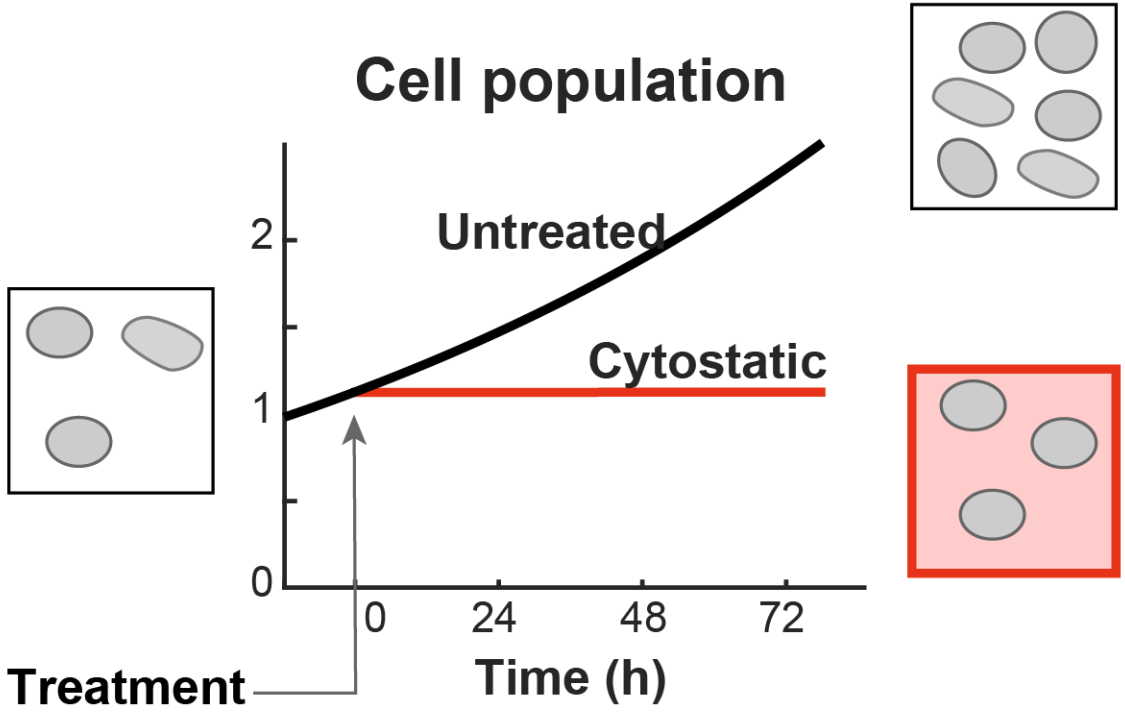


Normalization by the untreated control

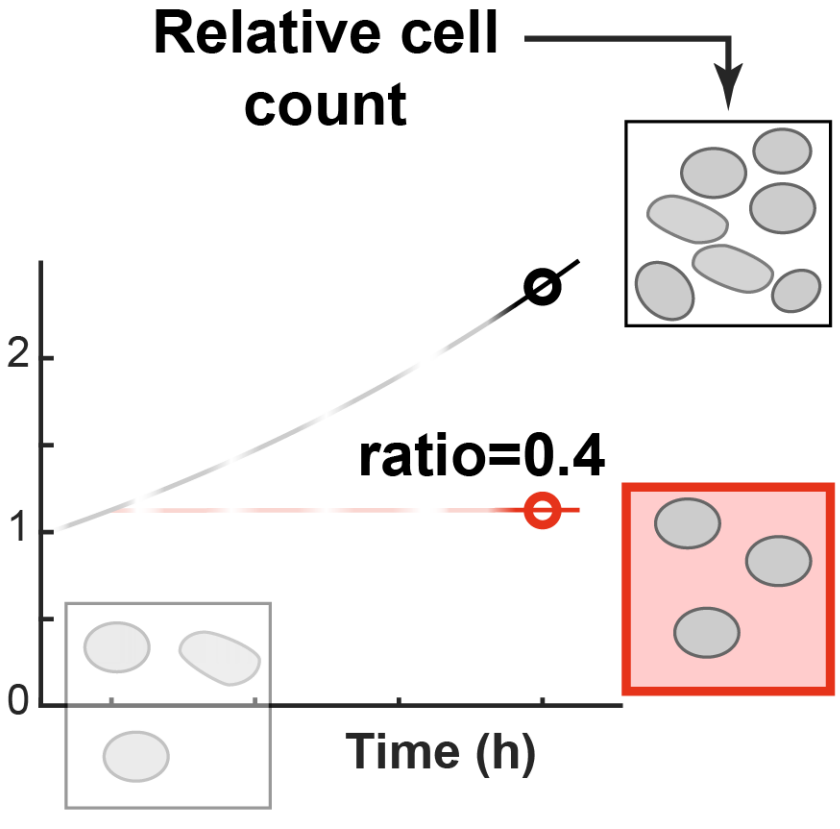
Relative cell count is valid when the untreated control remains the same:

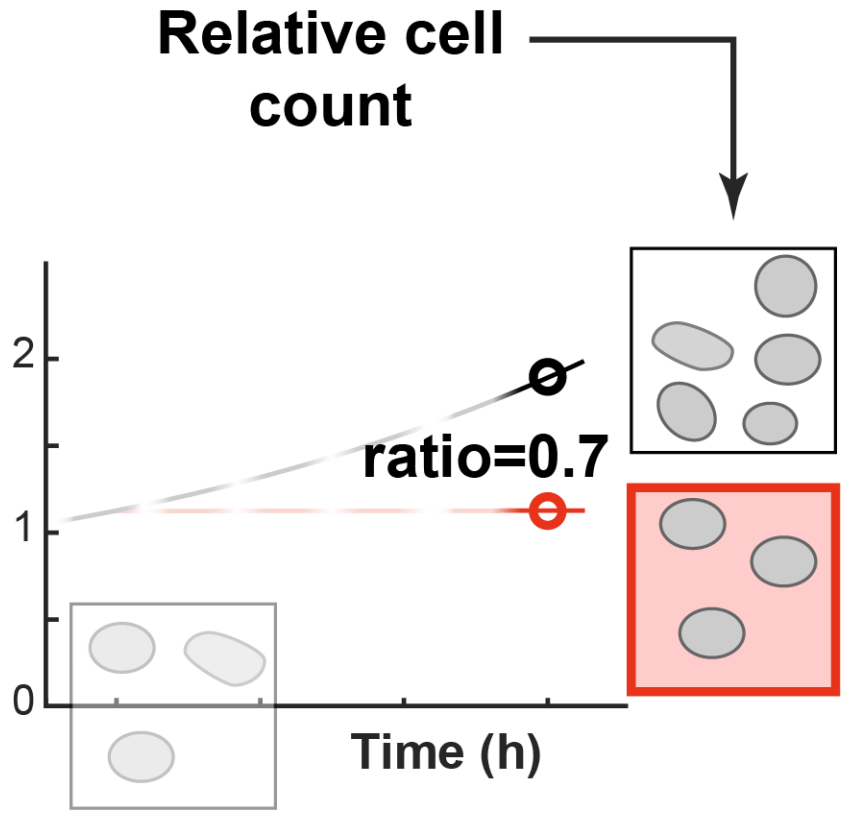
- Phenotype is not related to cell growth
- Untreated cells do not grow
- Short assays during which growth is negligible

Assays that have a growing population

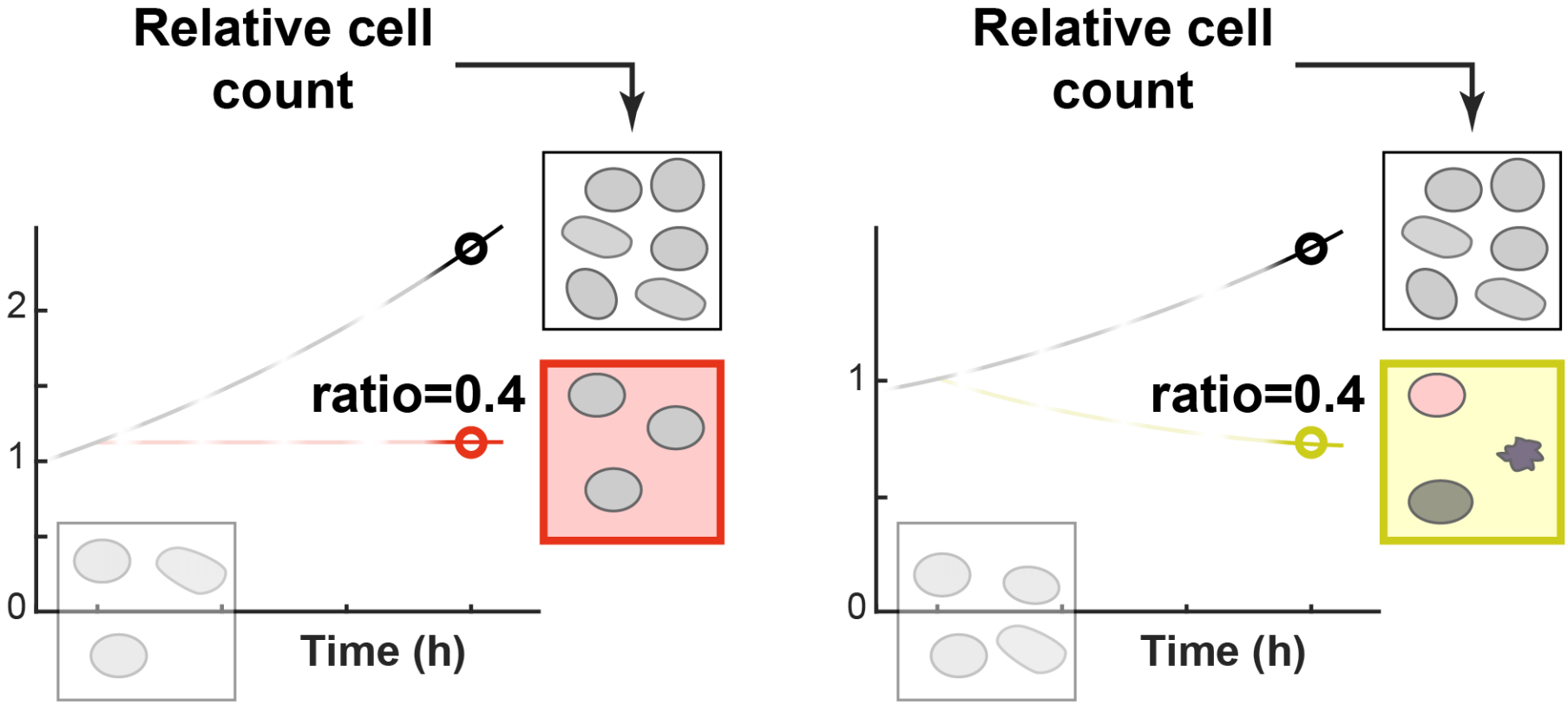


Relative cell count is biased by division rate

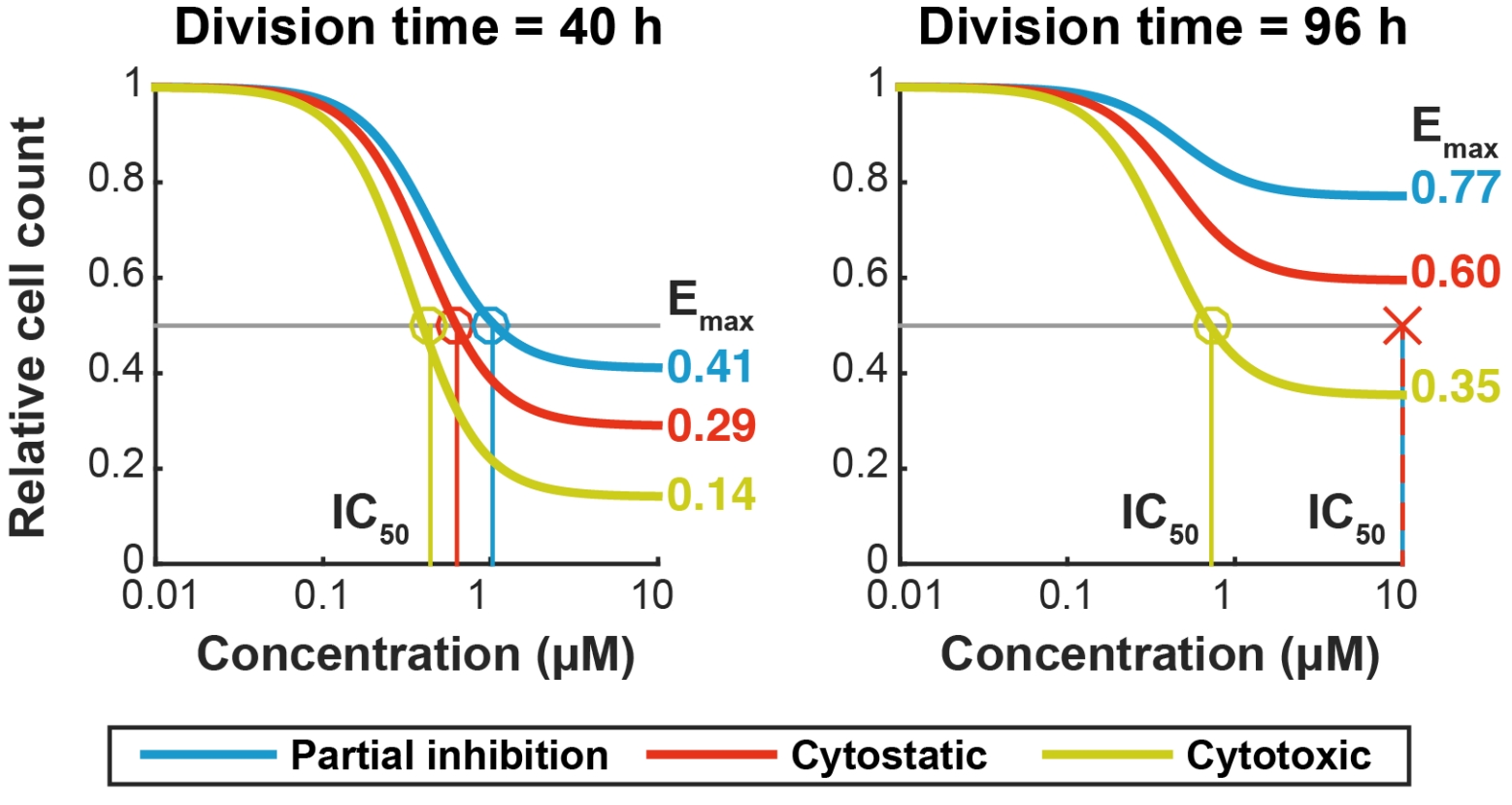




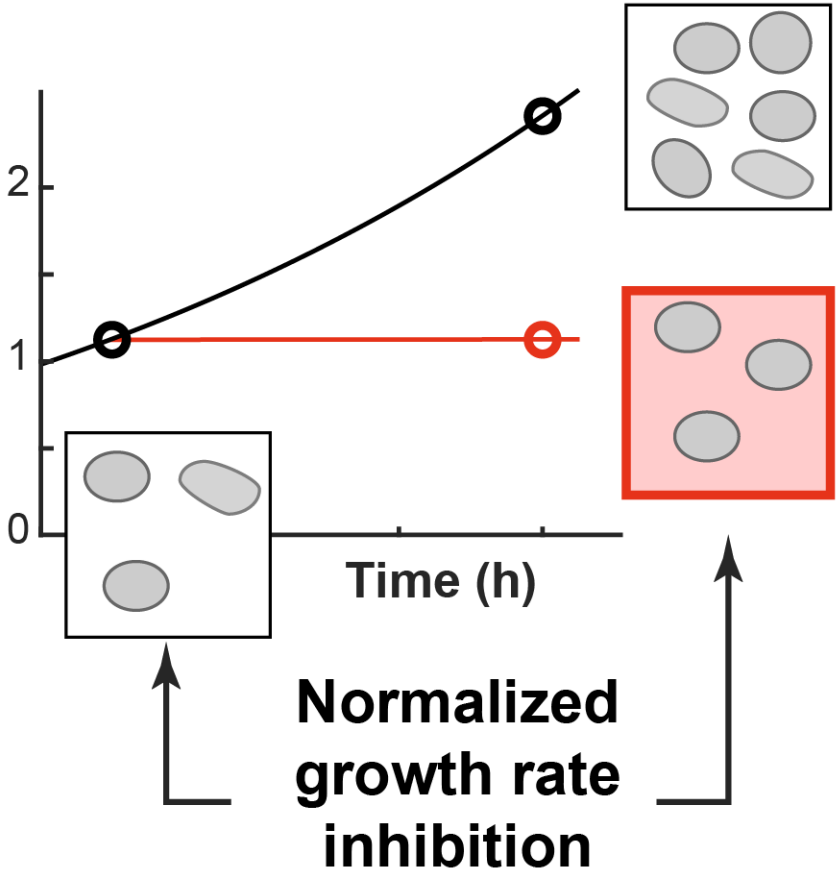
Relative cell count does not distinguish underlying phenotypes

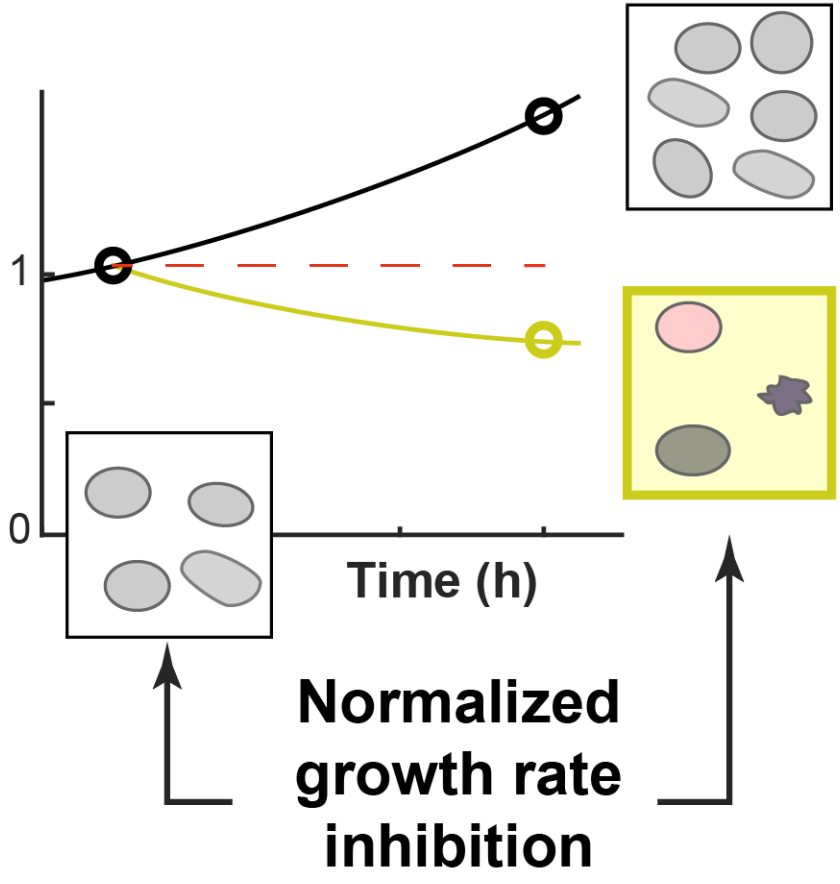


Slow growing cell lines look systematically more resistant when using relative cell count

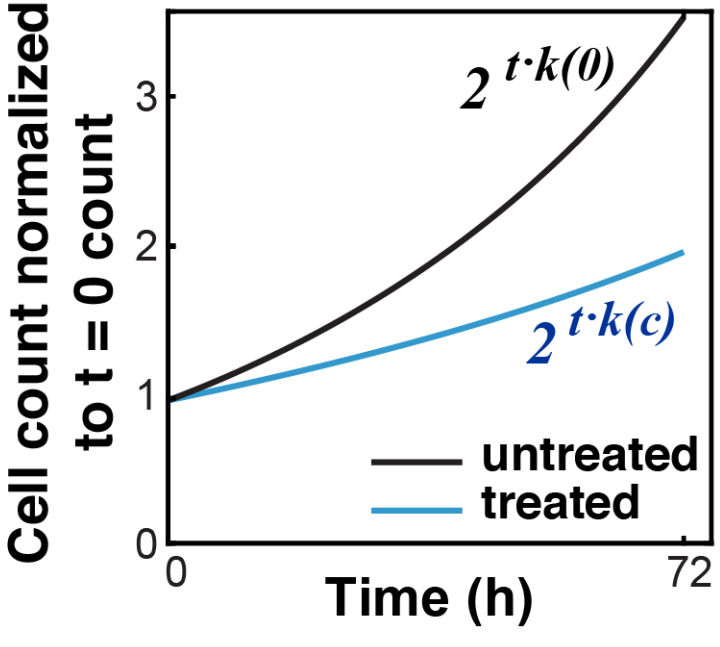


New unbiased metrics that define these underlying phenotypes are needed





Normalized growth rate inhibition (GR) value

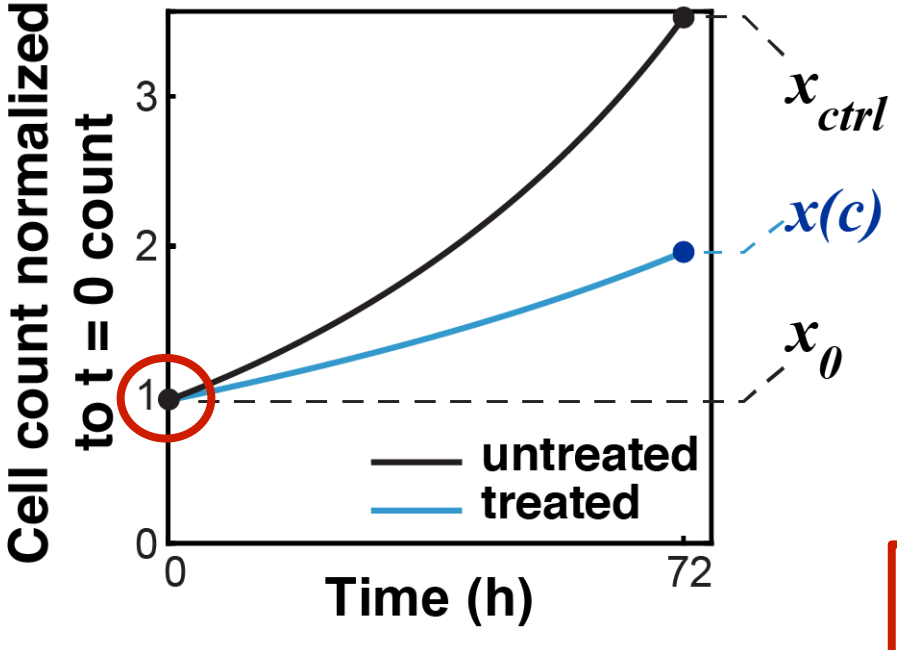


$$GR(c) = 2^{k(c)/k(0)} - 1$$

k(c) is the treated growth rate

k(0) is the control growth rate

GR values rely on three measures of cell count



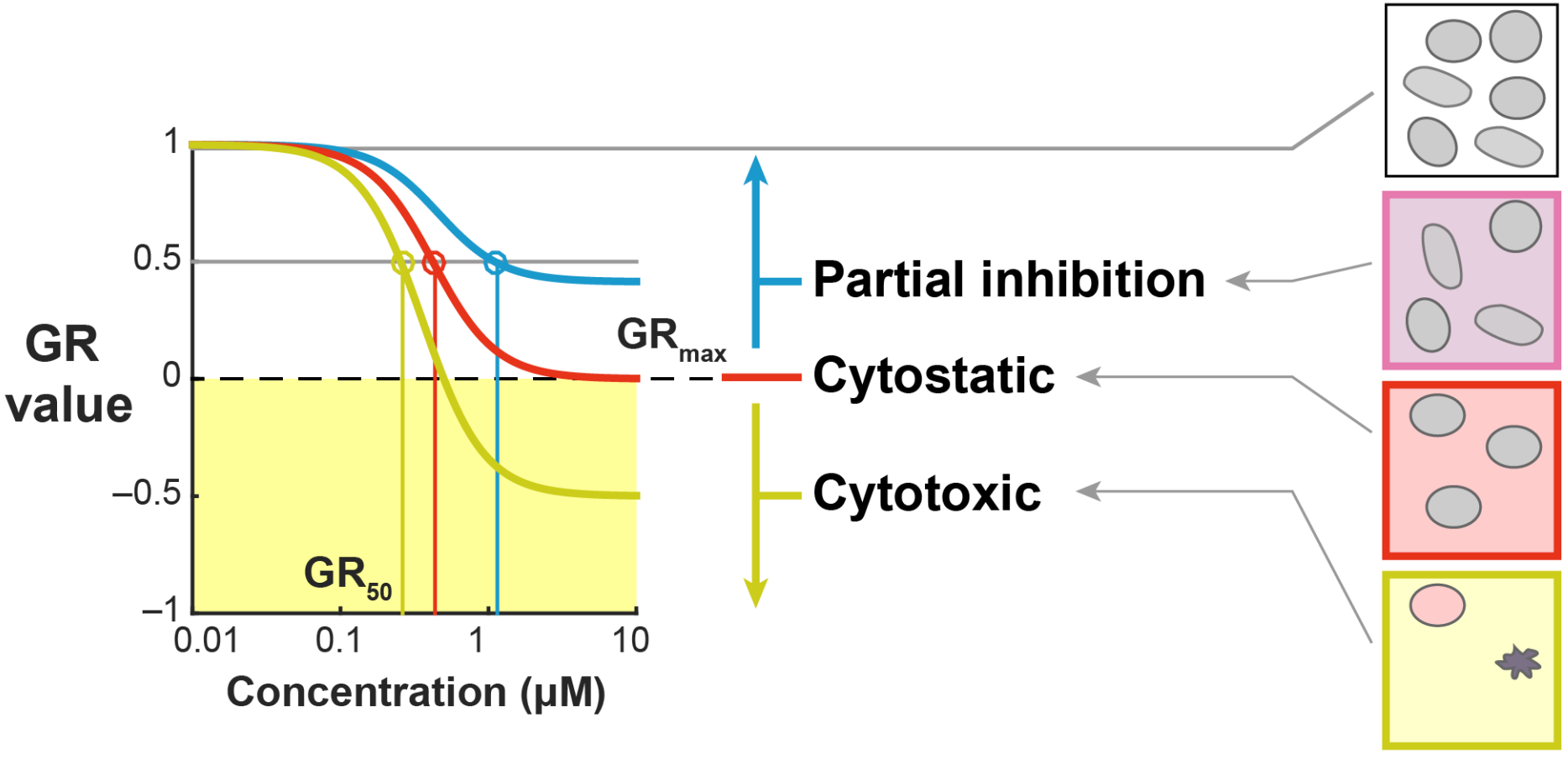
$$GR(c) = 2^{\frac{\log_2(x(c)/x_0)}{\log_2(x_{ctrl}/x_0)}} - 1$$

$x(c)$ is the treated cell count

x_{ctrl} is the control cell count

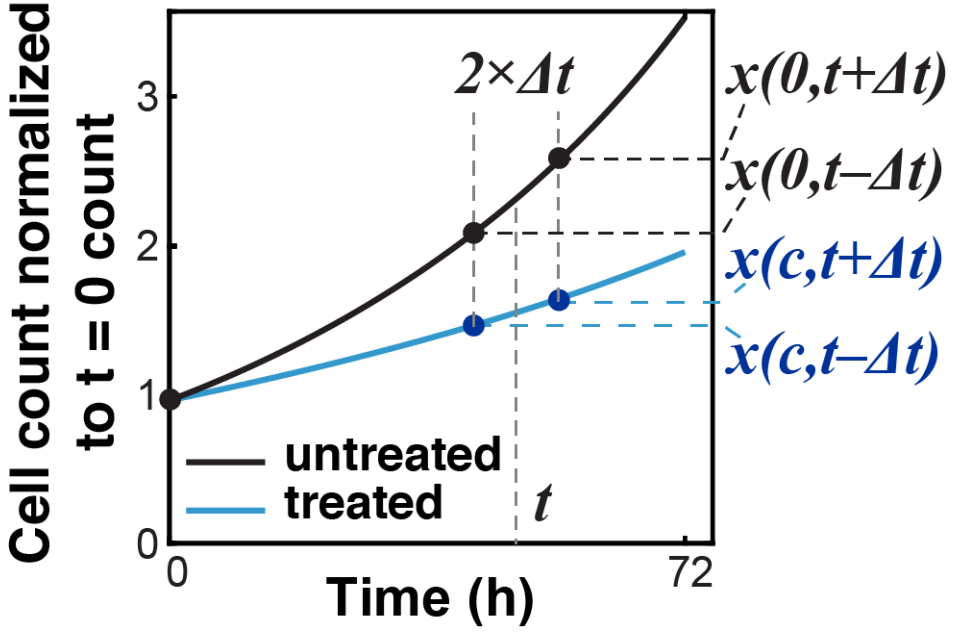
x_0 is the cell count at the time of treatment

GR values are independent of the division rate and directly relate to the phenotype



Time-dependent GR values and metrics

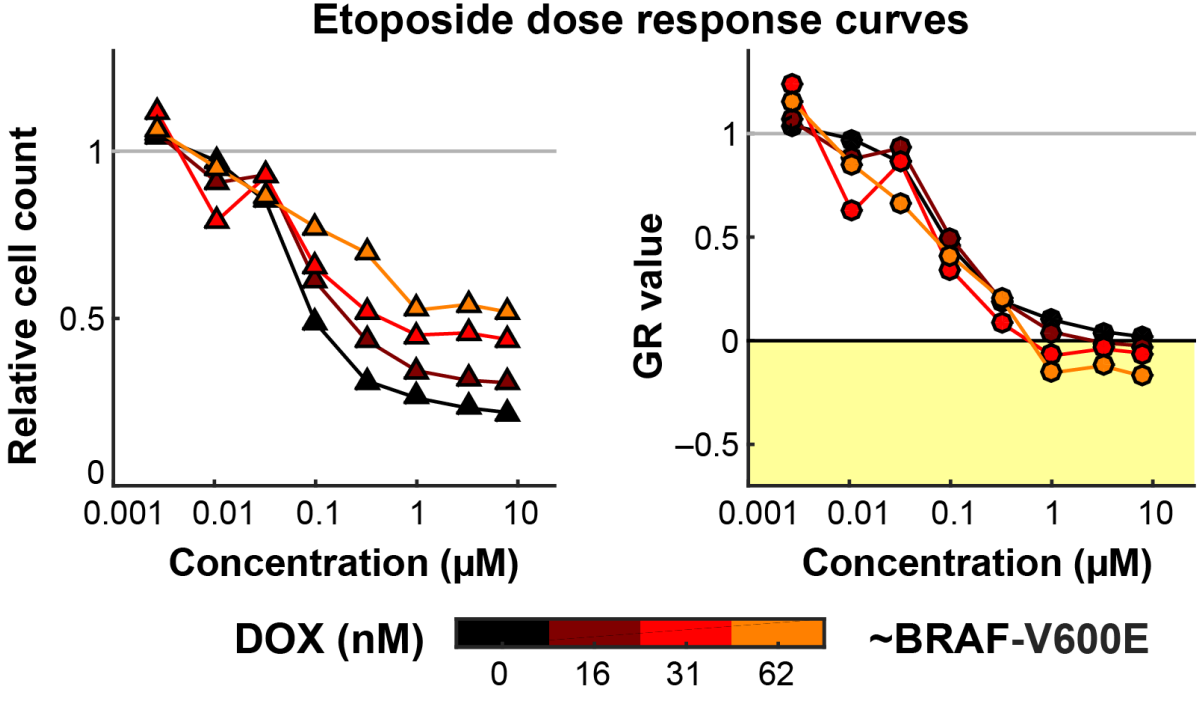
$$GR(c,t) = 2^{\frac{\log_2(x(c,t+\Delta t/2)/x(c,t-\Delta t/2))}{\log_2(x(0,t+\Delta t/2)/x(0,t-\Delta t/2))}} - 1$$

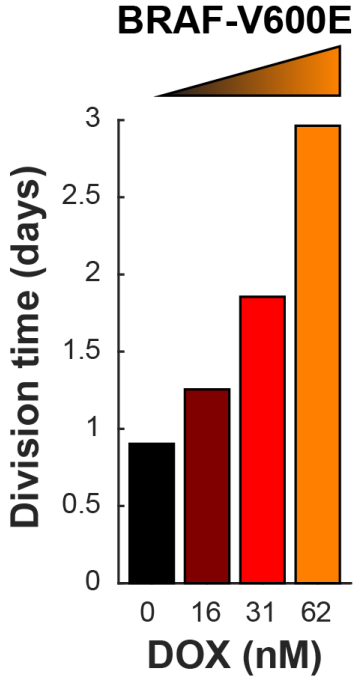


It allows evaluating $GR_{50}(t)$ and $GR_{max}(t)$ and quantifying adaptive response or late drug action.

Genetic alterations affect division time independently of drug sensitivity

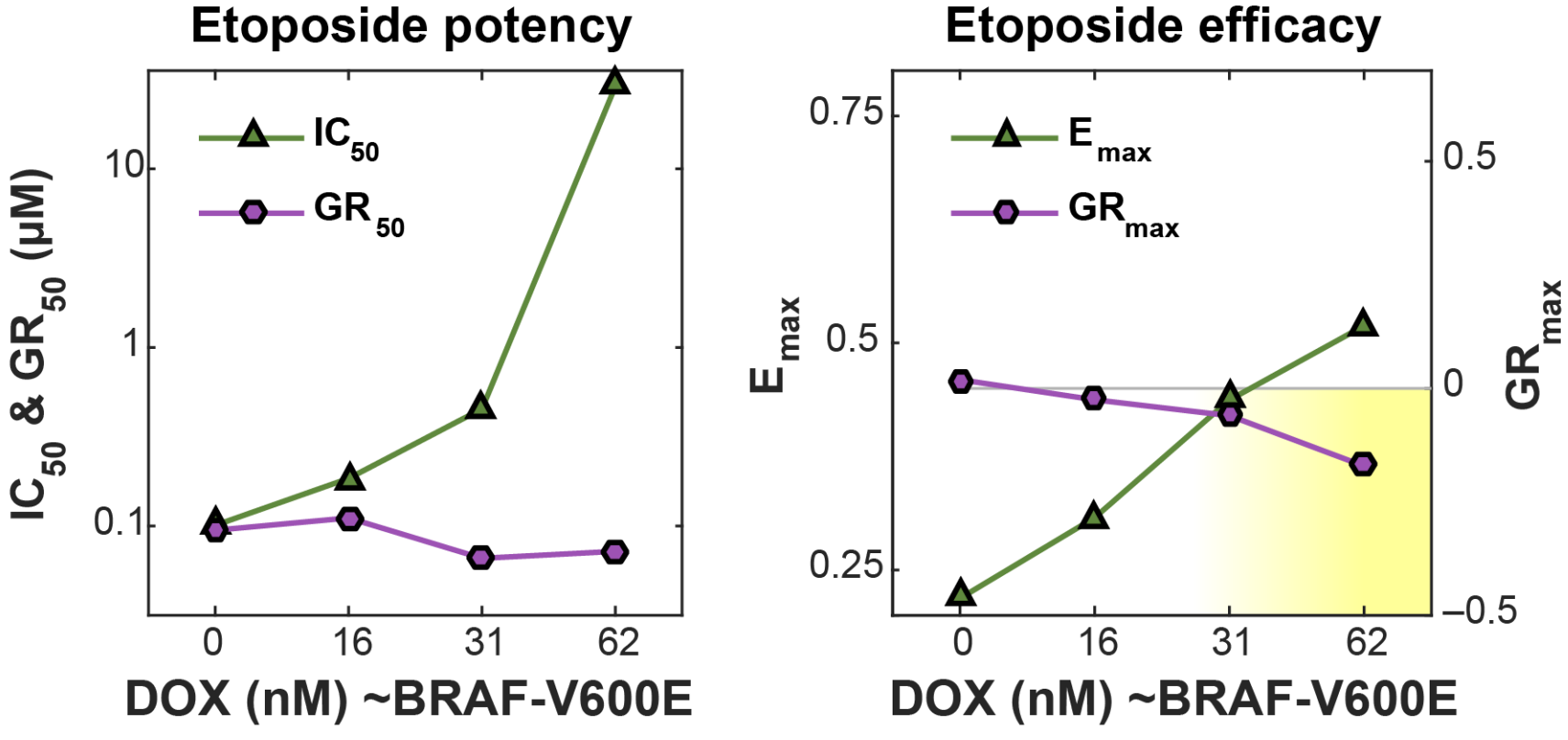
Etoposide sensitivity in HME RPE-1 cells expressing $BRAF^{V600E}$.



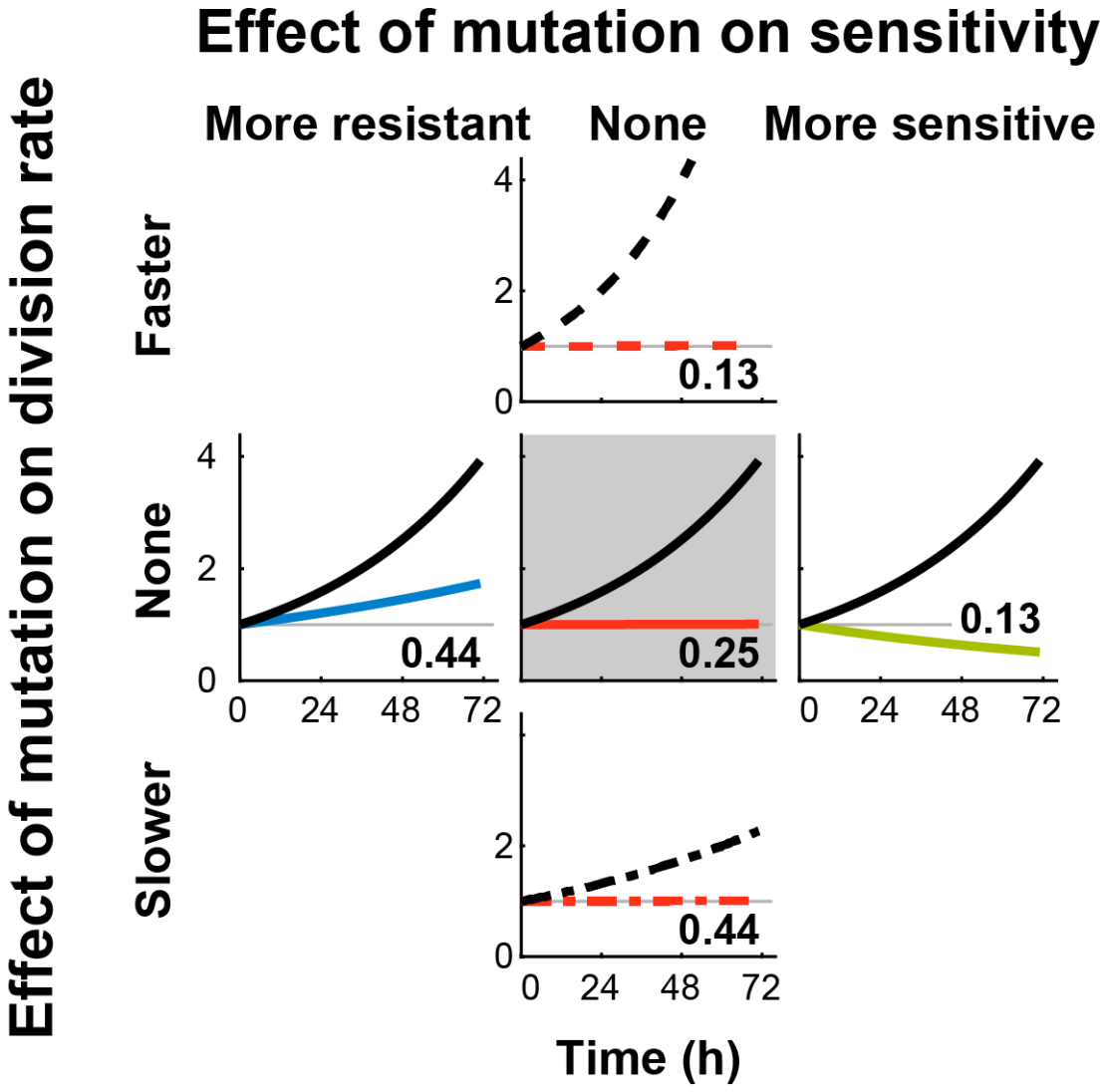


Thanks to Jia-Yun Chen for the cell line

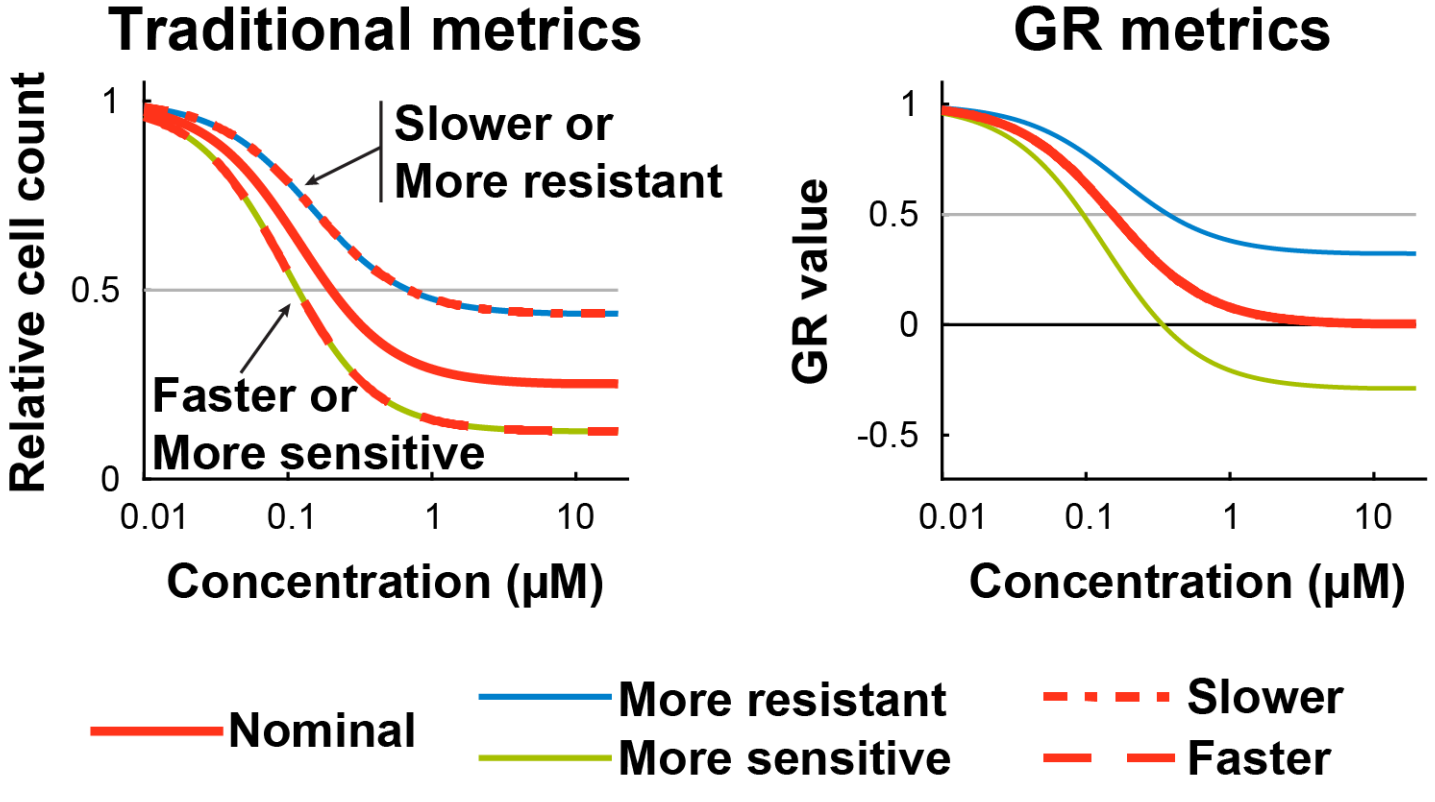
Genetic alterations affect division time which biases traditional sensitivity metrics



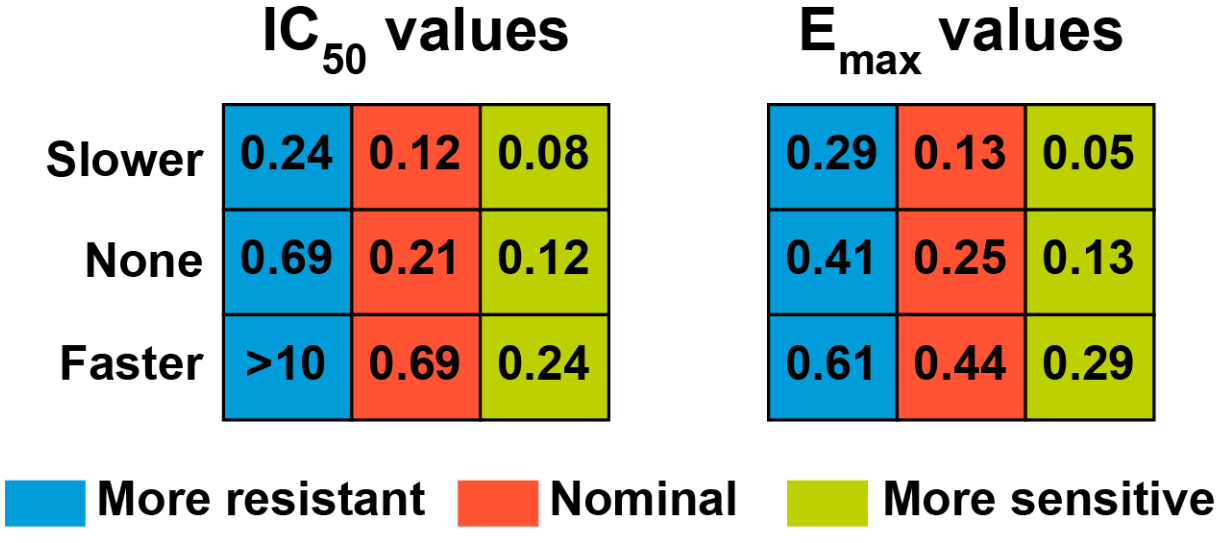
Model of interaction between mutation, division time and drug sensitivity



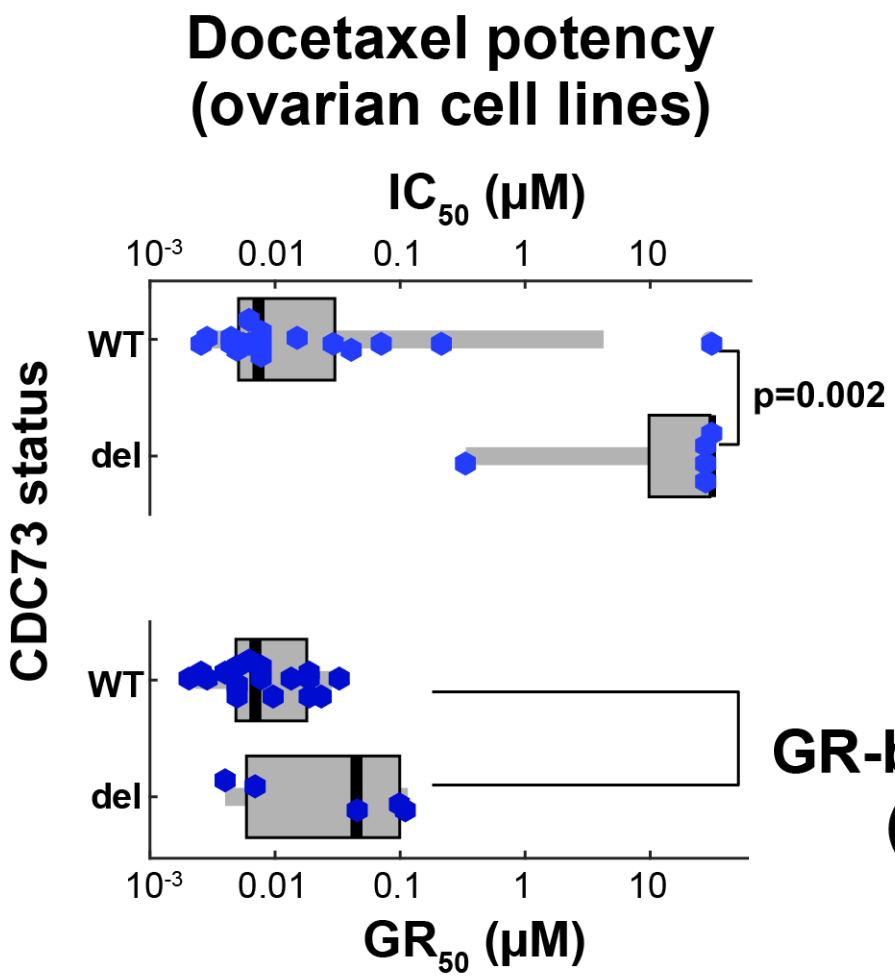
Division rate and sensitivity are confounded in traditional metrics of drug response



The bias of traditional metrics impacts pharmacogenomic studies



False-positive associations between IC_{50} and genotype are common

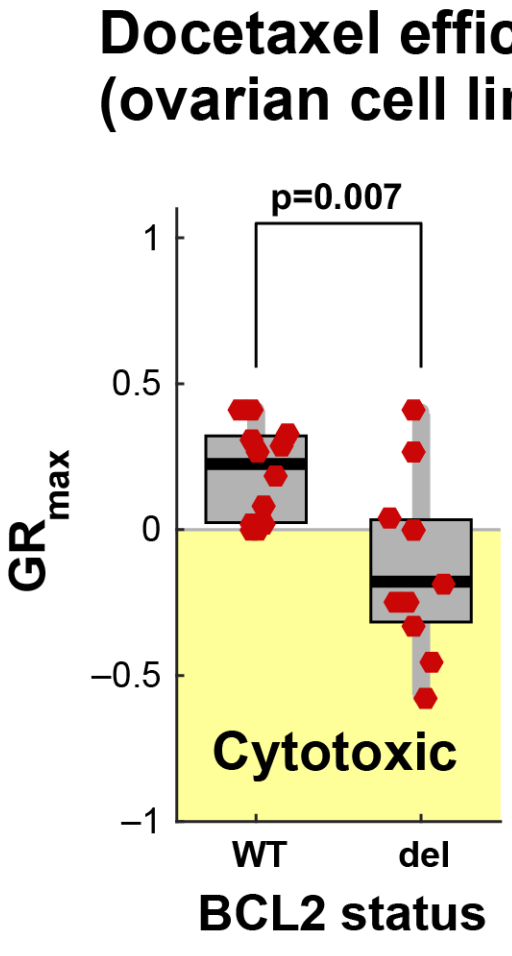


CDC73-loss slows growth, which artificially increases IC_{50} values.

	Traditional (IC_{50}) FDR<0.15	Traditional (IC_{50}) non-sign.
GR-based (GR_{50}) FDR<0.15	49	50
GR-based (GR_{50}) non-sign.	73	58276

Data from Haverty et al., Nature 2016, 533, 333-7

Efficacy (GR_{max}) correlates with genotype



Efficacy (GR_{max}) \ Potency(GR_{50})	FDR<0.15	non-sign.
FDR<0.15	18	81
non-sign.	81	58268

1. Theory of drug response

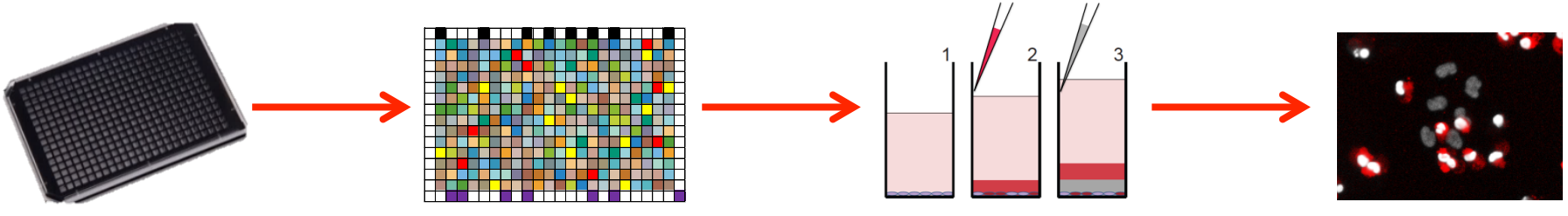
2. **Experimental setup**
   - **Growth conditions and drug treatment**
   - **Data acquisition and quantitation**
   - **Strengths and limitations**

3. Designing and analyzing experiments

4. Biological examples

Basic experimental workflow

- Grow (happy) cells
- Seed cells at appropriate densities in multi-well plates
- Deliver drugs to multi-well plates
- Stain and fix cells
- Image cells
- Extract quantitative data from images

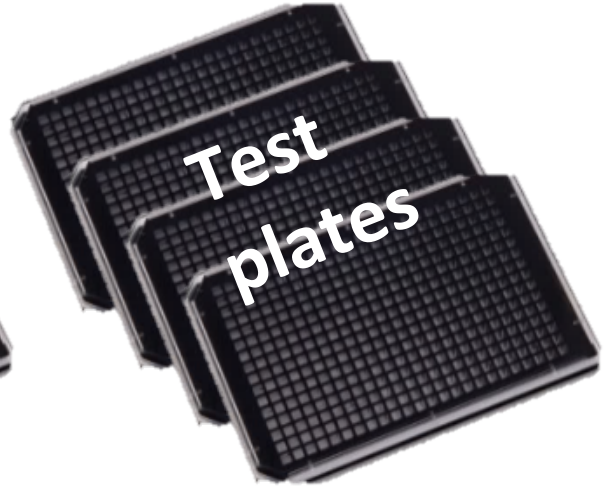


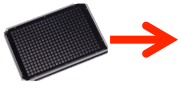
To consider *before* you start

- How many cell lines do I want to test?
  - Are they amenable to imaging?
    - Are they adherent? Do they grow in a monolayer?
  - How densely should they be seeded?
- How many drugs do I want to collect dose response data for?
  - Are they DMSO soluble?
  - What's an appropriate dose range?

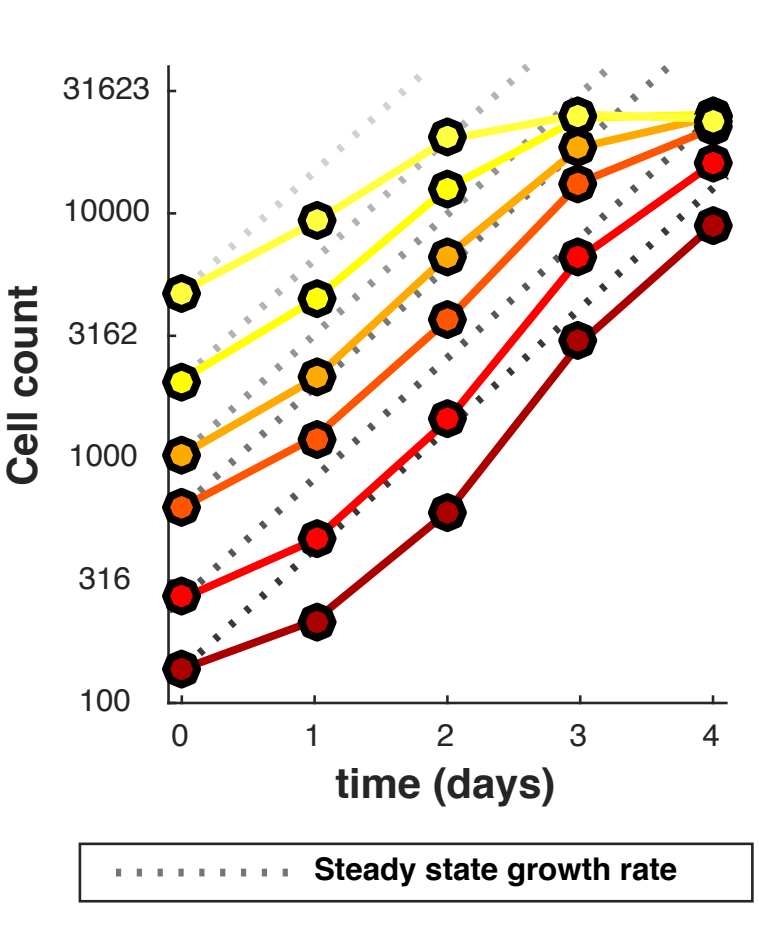
Cell seeding

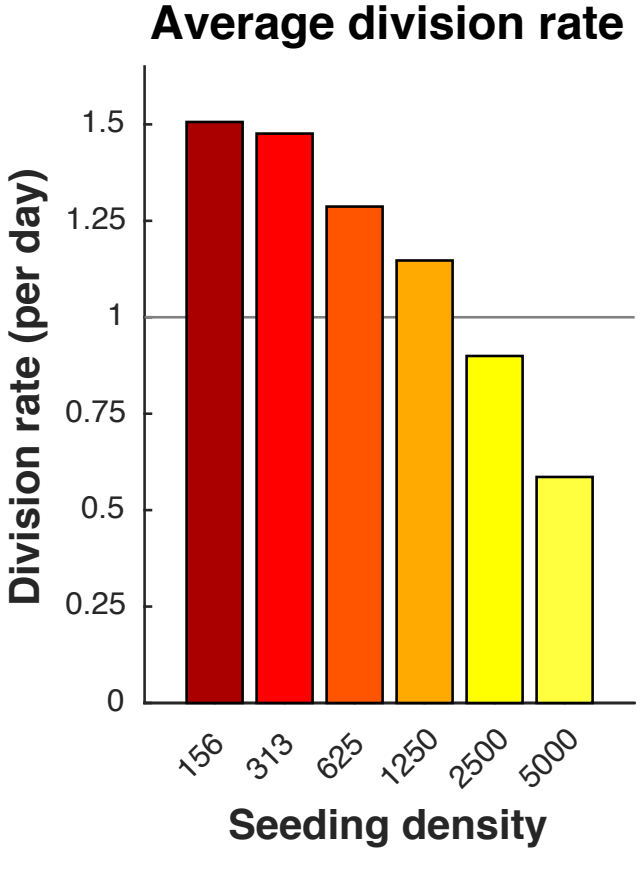
- Seed plates at an appropriate density
- Use automation if possible
- Add barcodes to plates



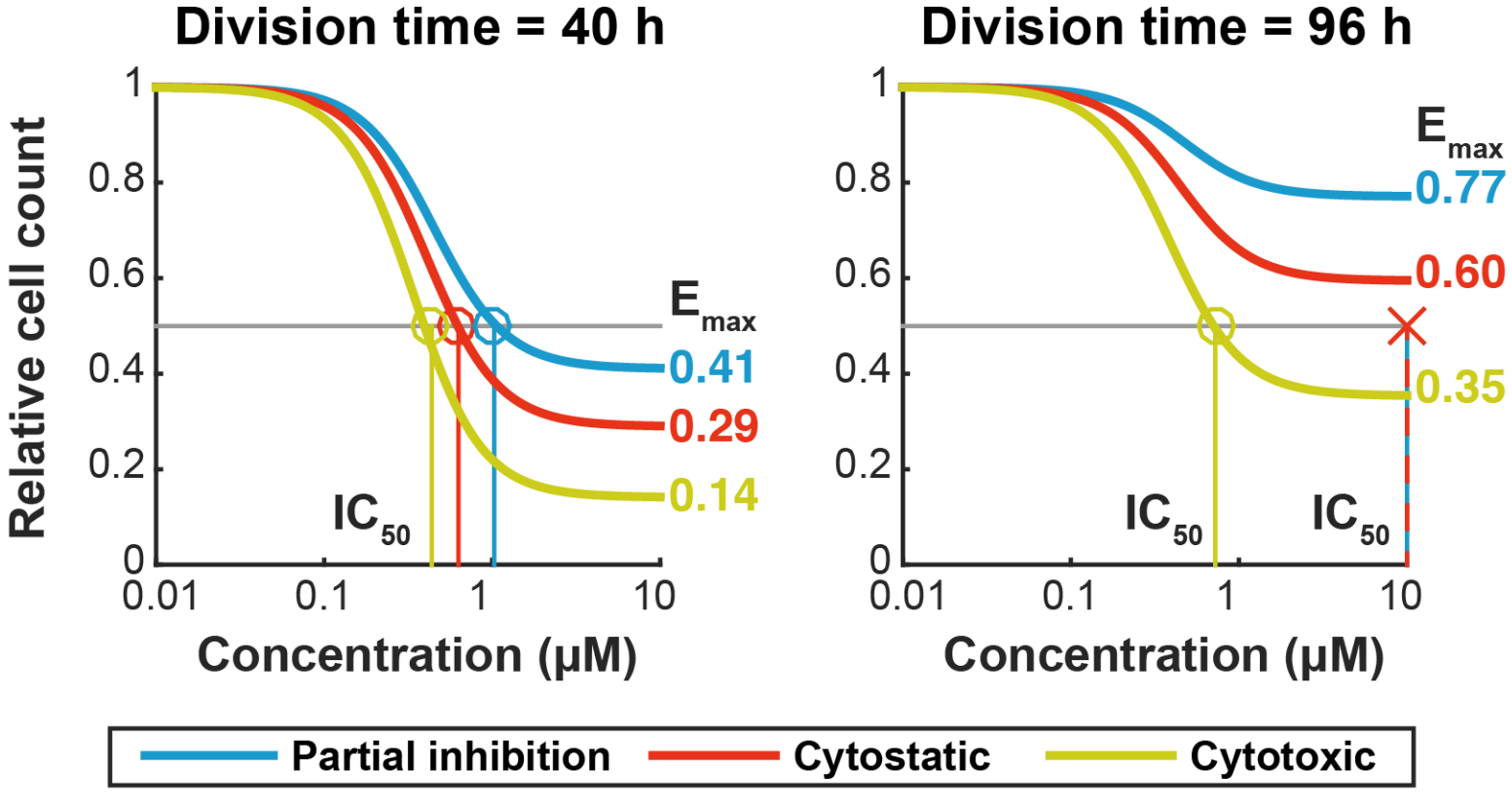


Cell seeding density influences growth rate...





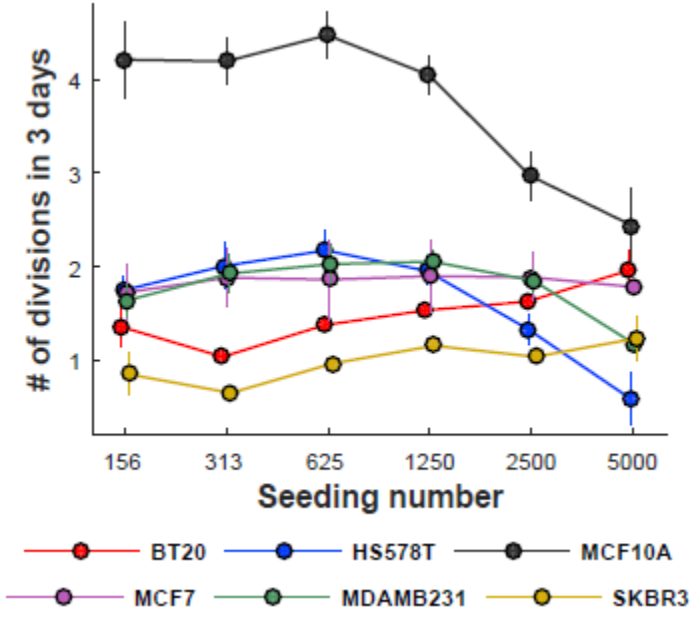
...which influences the dose response

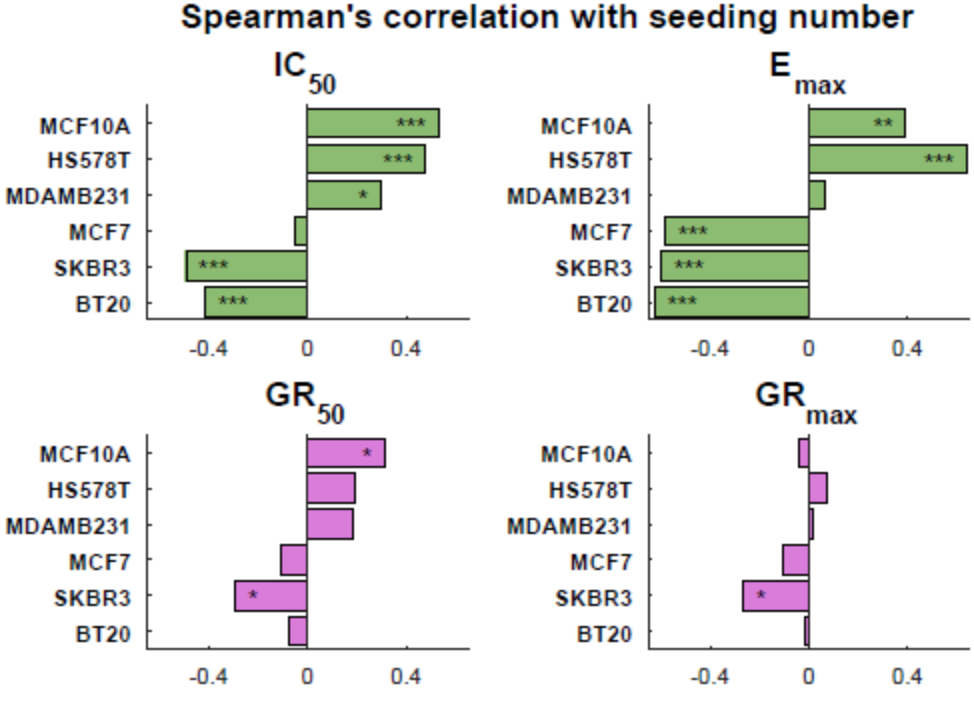


Division rate differs across densities

Seeding density affects the number of divisions.

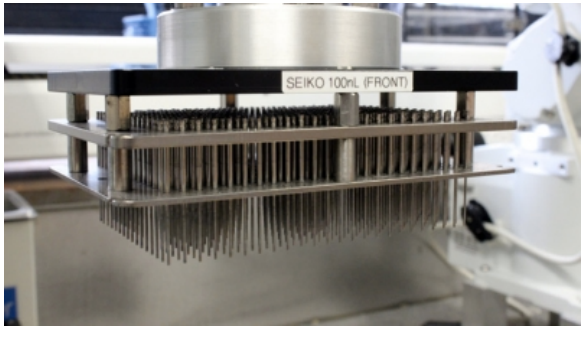
→ IC_{50} and E_{max} are correlated with density.





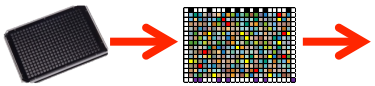
Drug delivery via pin transfer

- For simultaneous delivery of many drugs
- For large scale experiments (many cell lines, conditions)
- Facilitates reproducibility

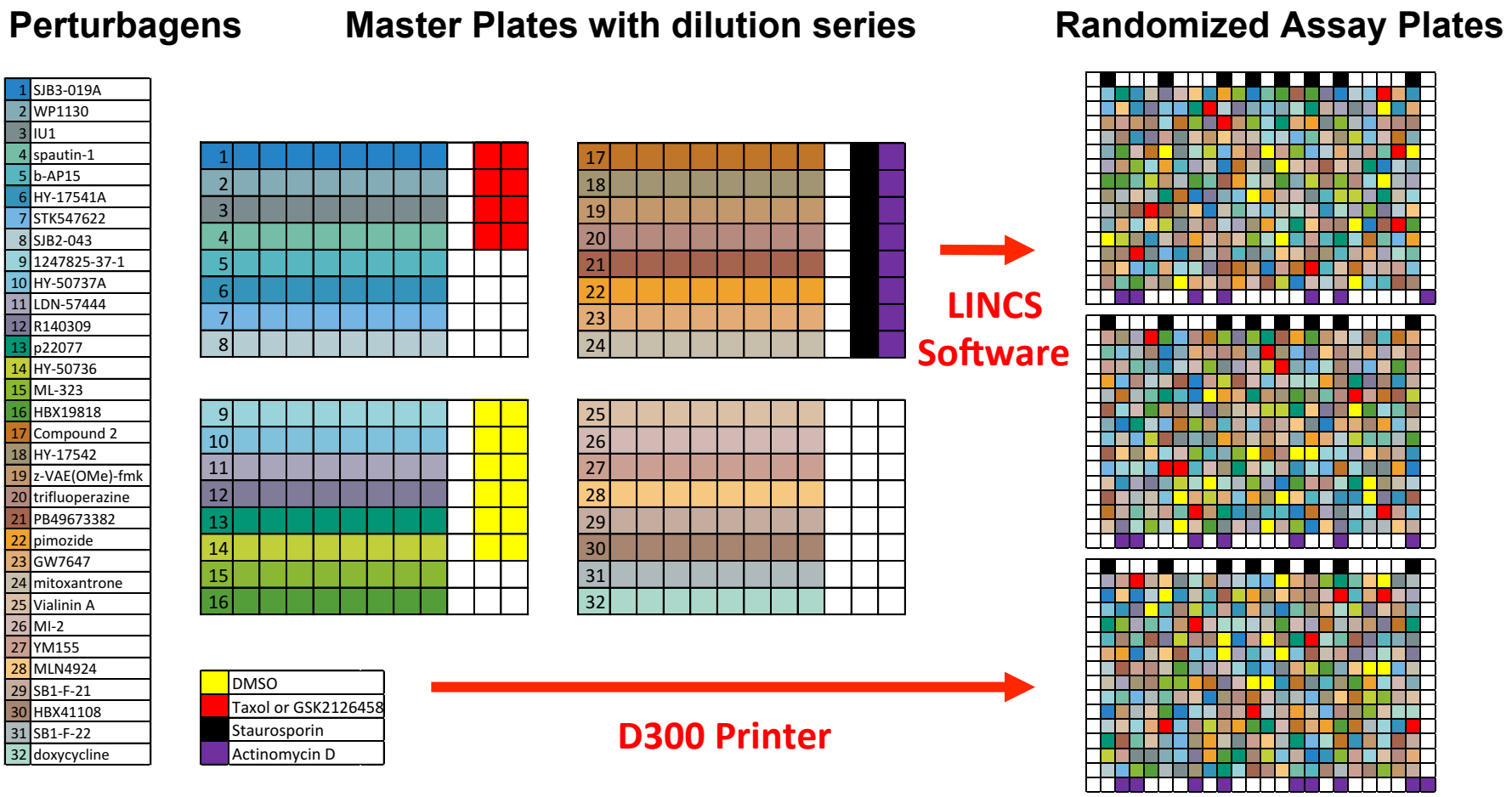


Drug delivery via digital drug dispenser

- For accurate delivery of a few drugs
- Pilot experiments- to identify appropriate doses
- Follow-up experiments
- Drugs that cannot be prepared in DMSO



Treatment randomization



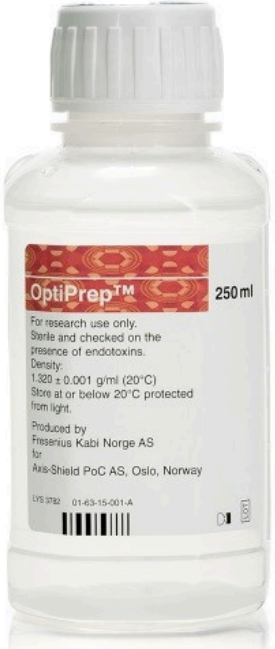
Laura Doherty

Other considerations

- Artefacts
  - Edge effects
    - Exclude outer wells
    - Use humidified secondary containers
    - Some cell lines are more sensitive than others
    - Depends on the duration of the experiment
  - Systematic bias from automation
- Randomization helps!

Dye-drop assay reagents

- Minimally-disruptive, reagent-sparing cell staining and fixation protocol



Dye-drop assay protocol

- Stain: Hoechst + LDR in 10% optiprep in PBS
- Fix: 4% formaldehyde in 20% optiprep in PBS

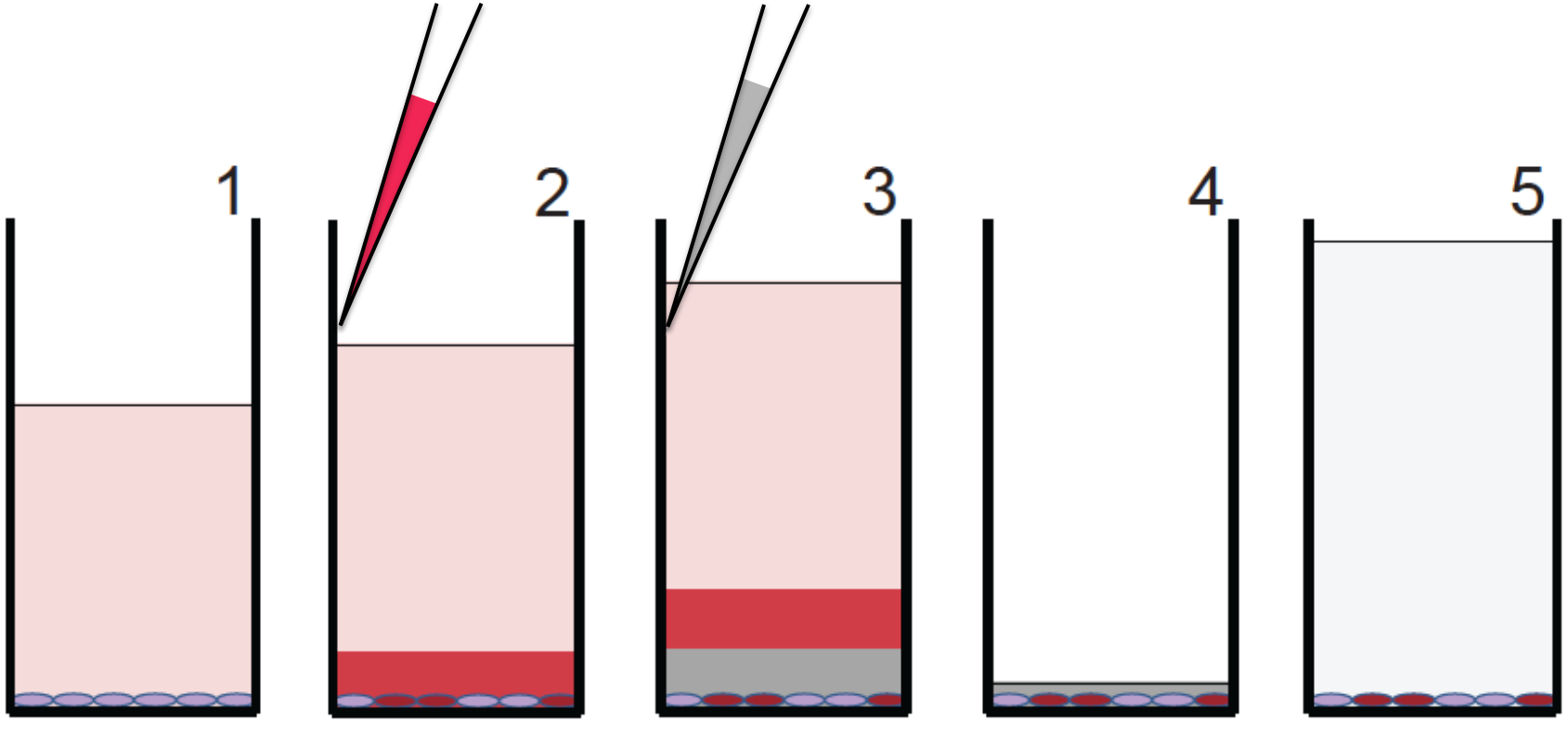


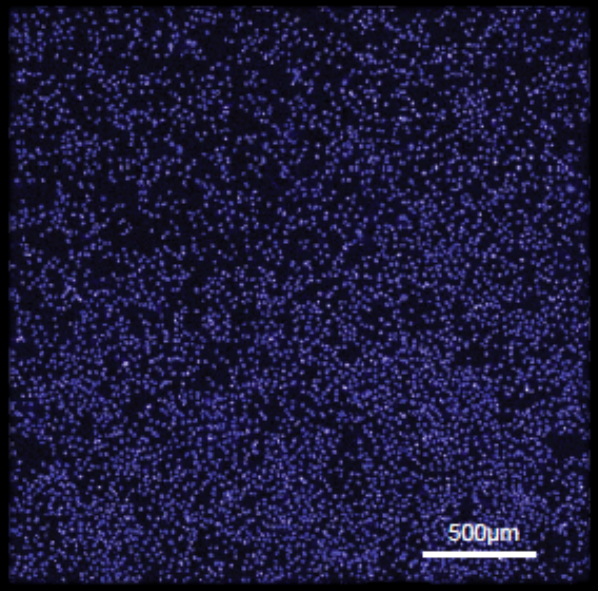
Plate washer

- Uniform and controlled aspiration and liquid dispensing

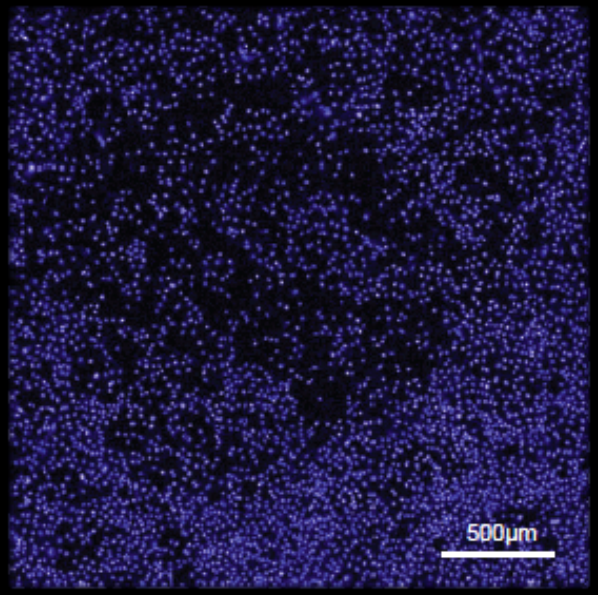
- Is repeat washing really that bad?

Repeat washing can result in cell loss…

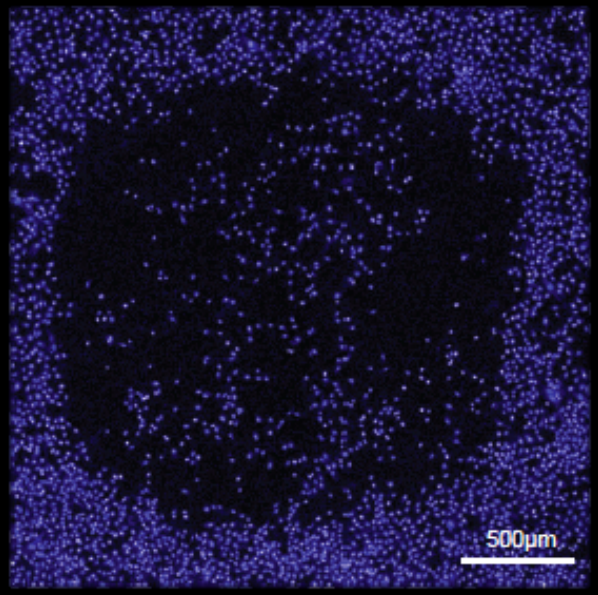
No wash



PBS wash x 1



PBS wash x 2



…that can bias your results

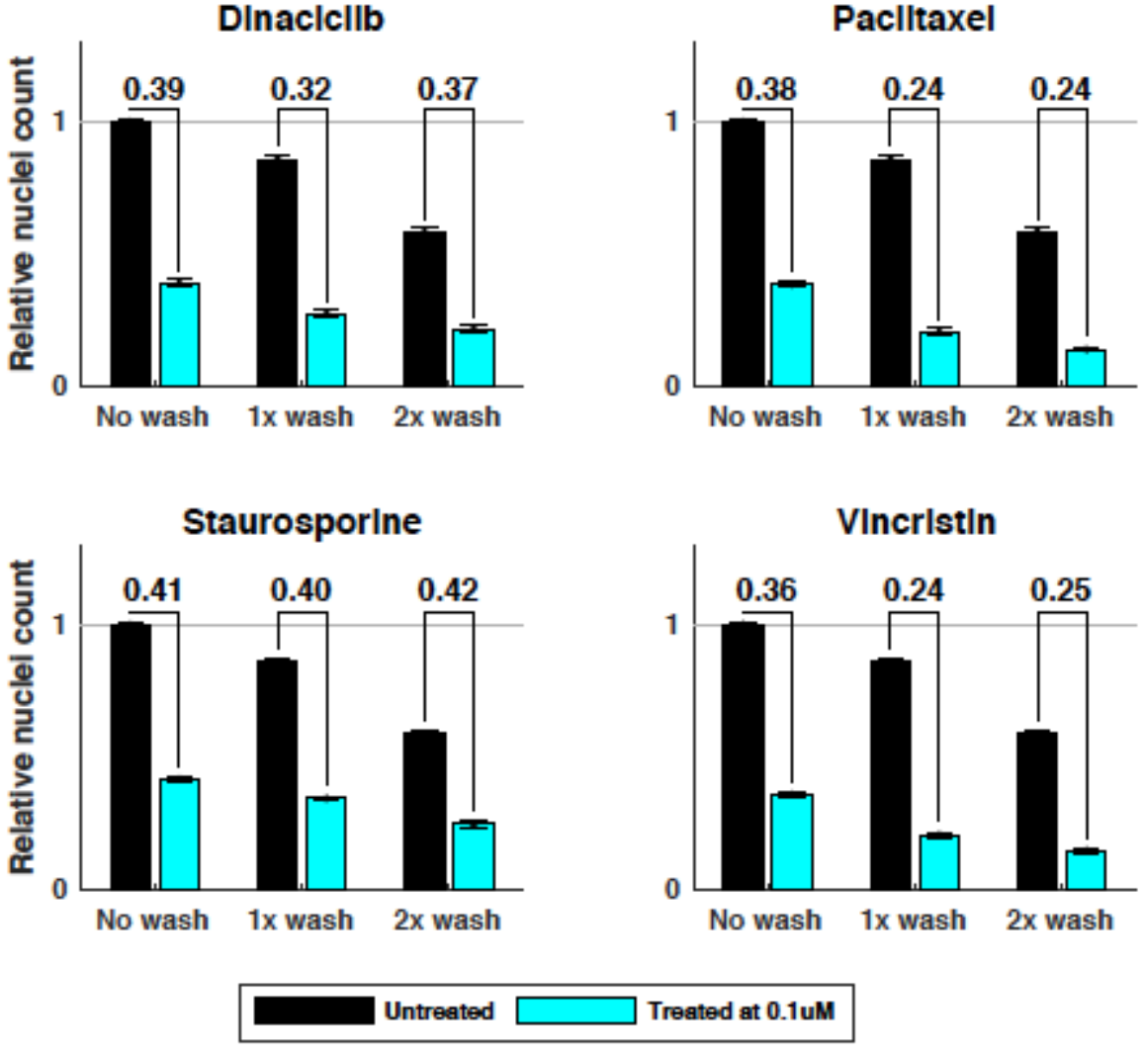
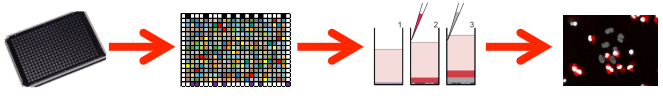


Image acquisition

- Operetta microscope with plate hotel, barcode reader & robot
  - Automated data collection for 40+ plates



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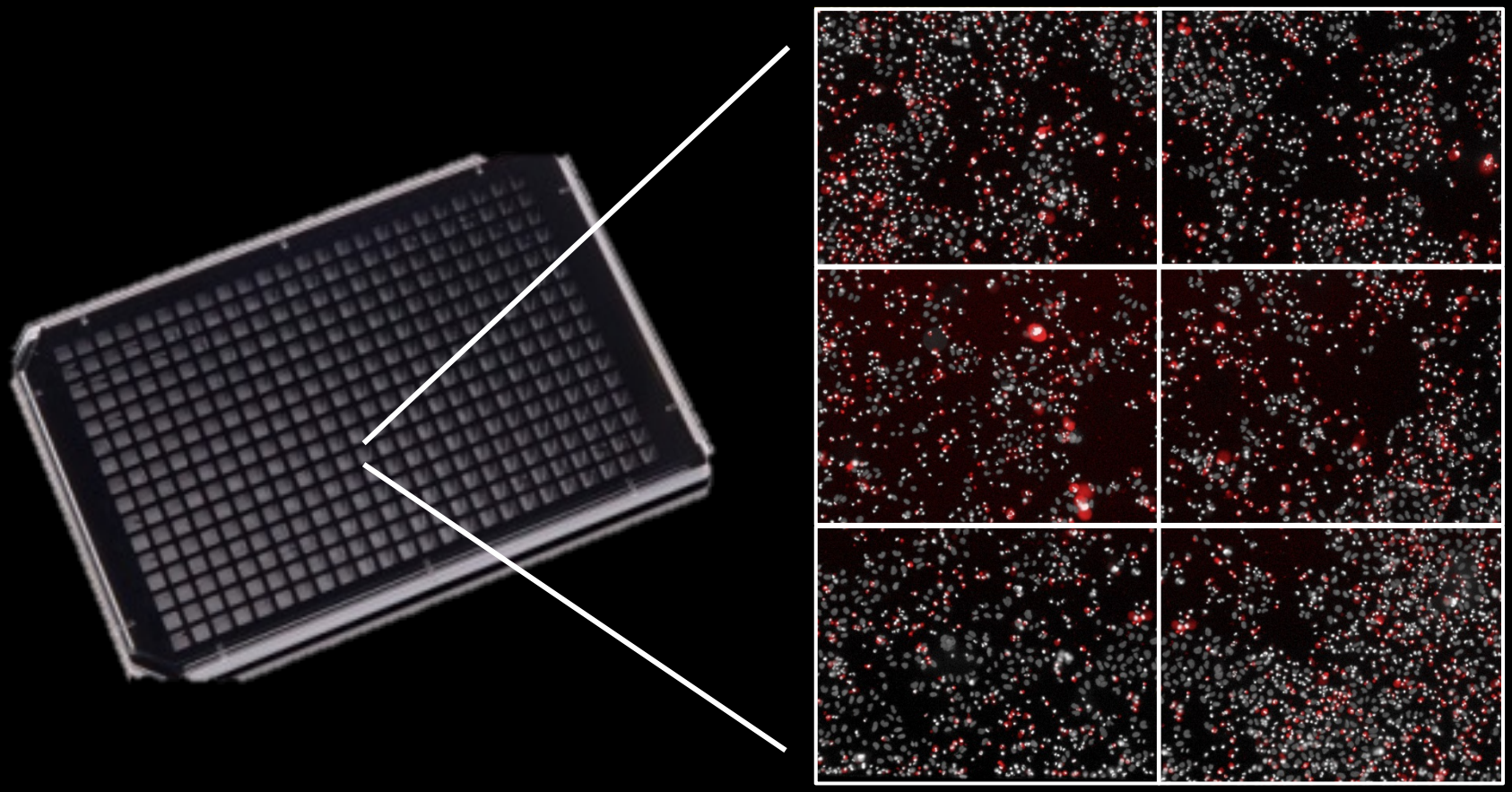
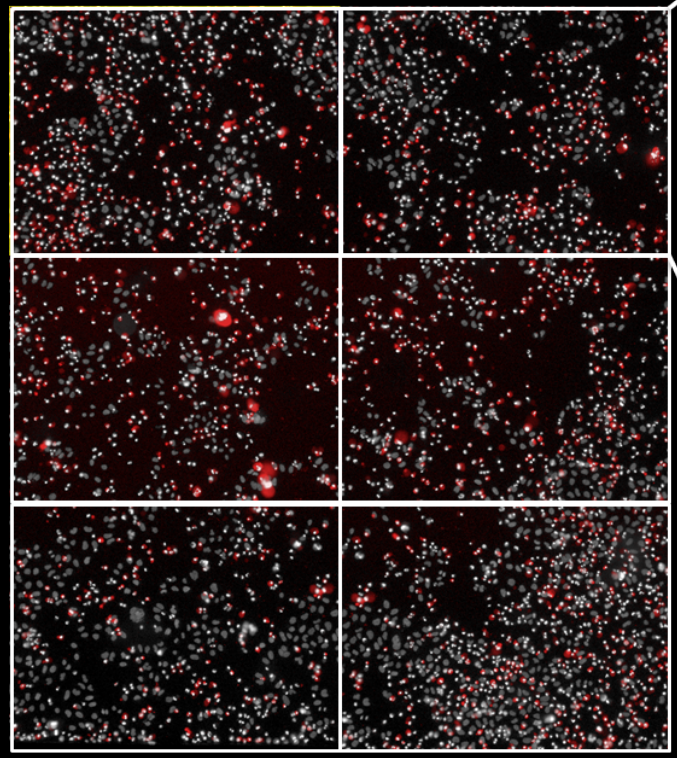


Imaging 6 fields of view @ 10x
captures *almost* the entire well

Image acquisition



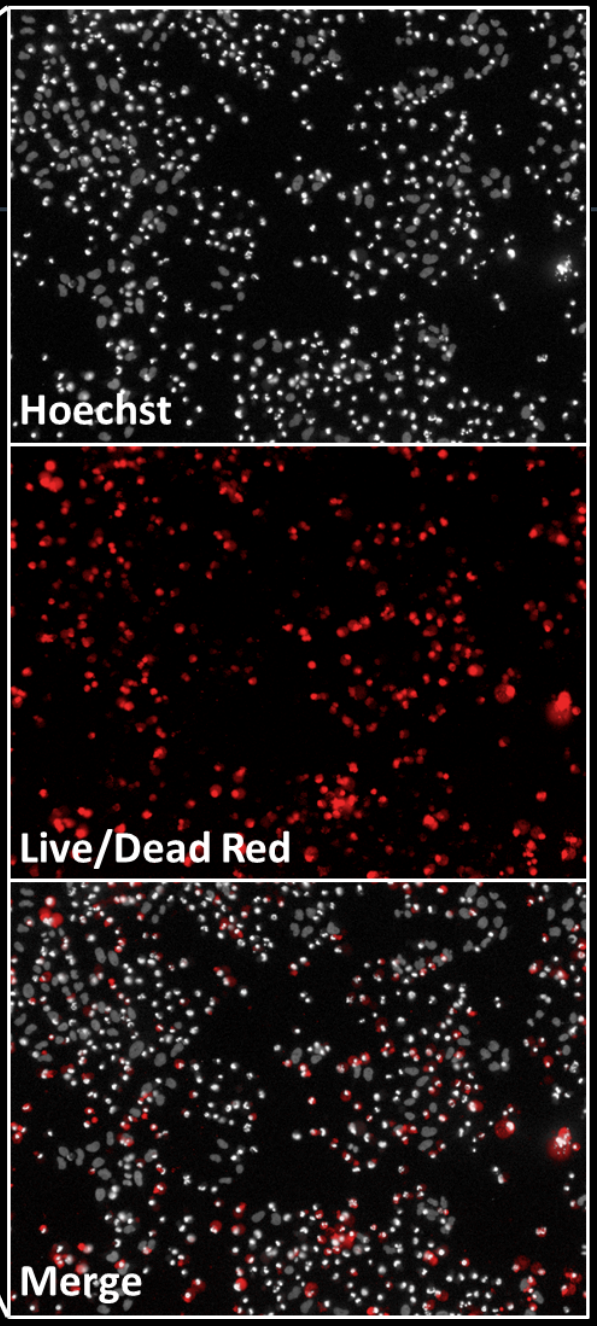
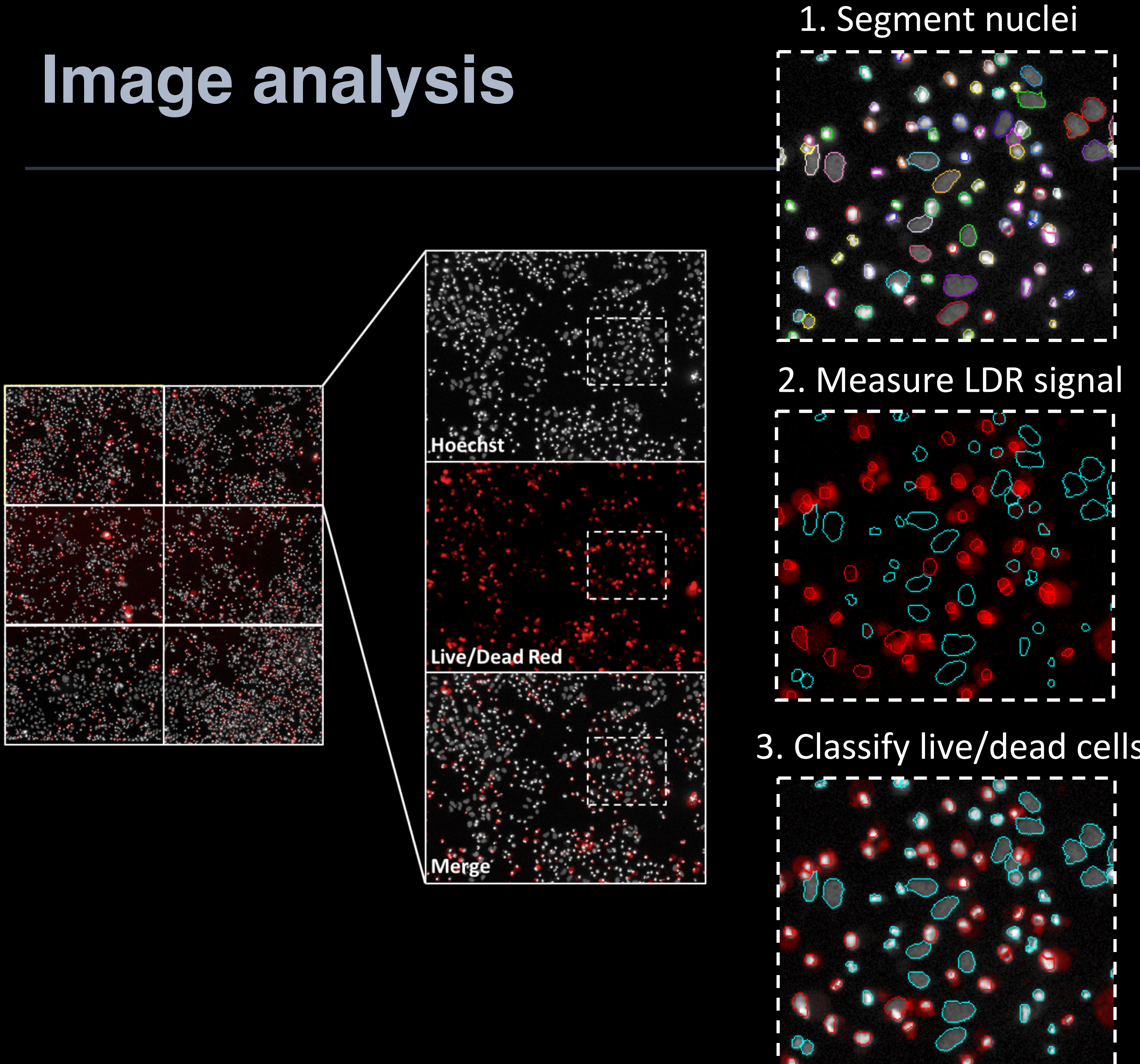


Image analysis



Well	Row	Column	Cell Line	Time point	Treatment	Dose (uM)	Cell count	Dead cell count	Cell count t=0
C2	3	2	MCF10A	72	Staurosporine	1	5091	1833	1954
C3	3	3	MCF10A	72	Staurosporine	1	5929	2137	1954
C4	3	4	MCF10A	72	Staurosporine	1	5663	2021	1954
C5	3	5	MCF10A	72	DMSO	0	8000	297	1954
C6	3	6	MCF10A	72	Staurosporine	0.316	6613	1142	1954
C7	3	7	MCF10A	72	DMSO	0	7732	329	1954
C8	3	8	MCF10A	72	Staurosporine	1	5463	2473	1954
D2	4	2	MCF10A	72	DMSO	0	8746	88	1954
D3	4	3	MCF10A	72	Staurosporine	0.316	6168	1496	1954
D4	4	4	MCF10A	72	Staurosporine	0.1	7941	636	1954
D5	4	5	MCF10A	72	DMSO	0	8529	360	1954
D6	4	6	MCF10A	72	Staurosporine	0.316	6994	1157	1954
D7	4	7	MCF10A	72	DMSO	0	8872	160	1954
D8	4	8	MCF10A	72	DMSO	0	9166	73	1954
C2	3	2	MCF10A	72	Staurosporine	1	5091	1833	1954
C3	3	3	MCF10A	72	Staurosporine	1	5929	2137	1954
C4	3	4	MCF10A	72	Staurosporine	1	5663	2021	1954
C5	3	5	MCF10A	72	DMSO	0	8000	297	1954
C6	3	6	MCF10A	72	Staurosporine	0.316	6613	1142	1954
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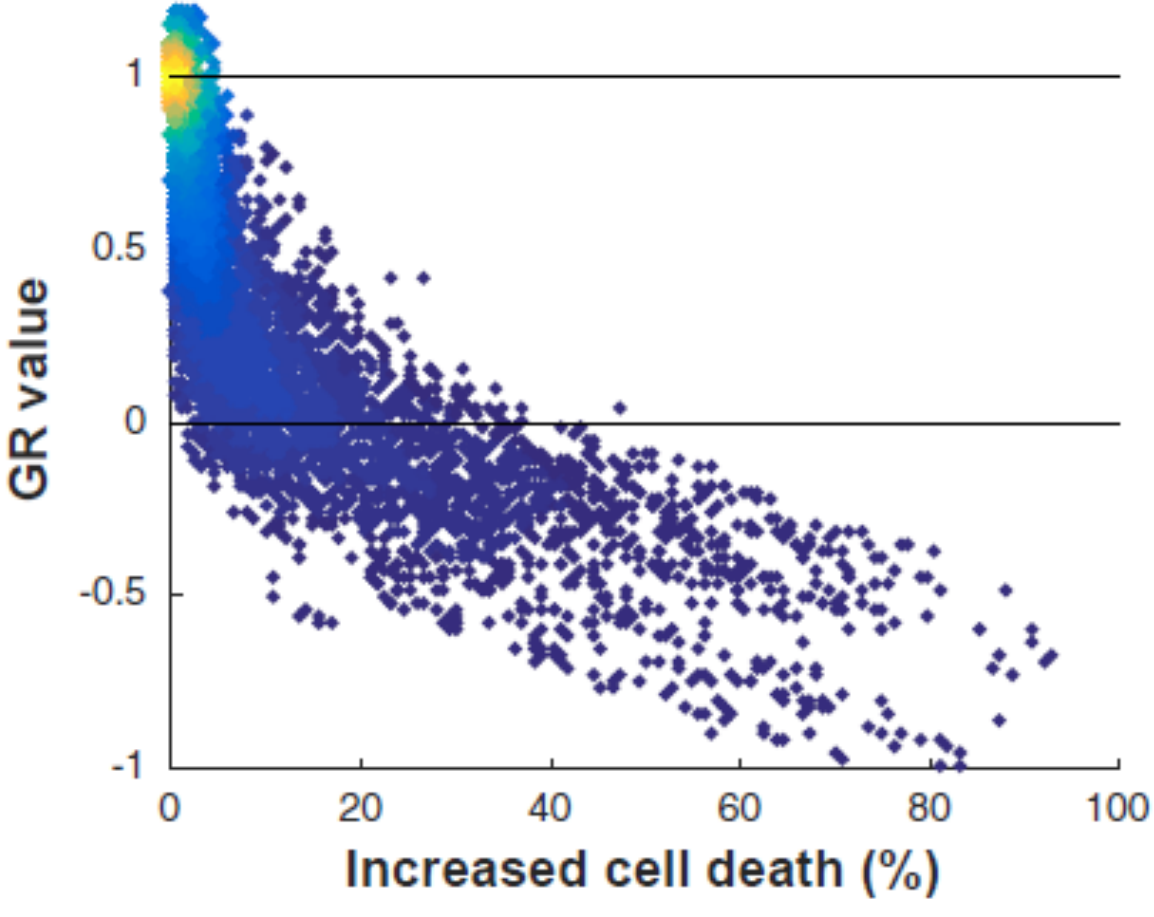
D7	4	7	MCF10A	72	DMSO	0	8872	160	1954
D8	4	8	MCF10A	72	DMSO	0	9166	73	1954

Can I just count cells?

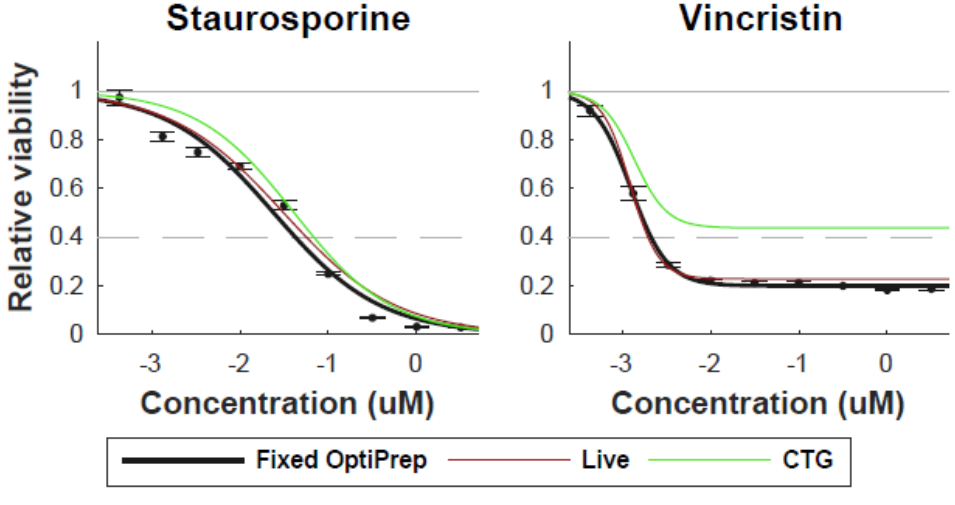


Strengths and limitations

- Imaging based
  - Best suited for adherent cells that grow in monolayer culture
- Image analysis can be time consuming
- Can go back and visually inspect imaging data
- Potential for multiplexing, immunofluorescence
- Fate of live cells unknown
- Reagent sparing
- Distinction between cytotoxic and cytostatic effects

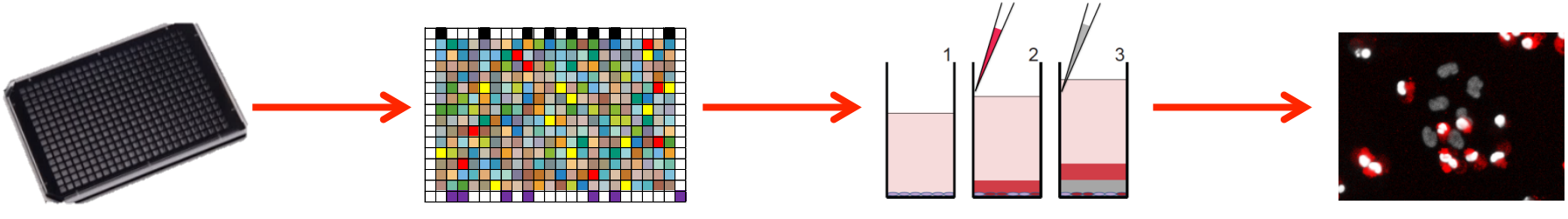
Other assays

- CellTiter-Glo etc.
  - Simple, no wash protocol
  - Luminescence read-out, simple analysis, rapid results
  - Treatment-induced changes in metabolic activity of cells can skew results
- Measurement of confluency
  - Inaccurate
  - Treatment-induced changes in morphology can skew results



Take away messages

- Include a t=0 plate
- Optimize conditions
  - Seeding density per cell line
  - Dose range per drug
  - Duration of assay
- Automate as much as possible



1. Theory of drug response

2. Experimental setup

3. **Experimental design and analysis**
   - **Scripting the design**
   - **Processing and analyzing data**

4. Biological examples

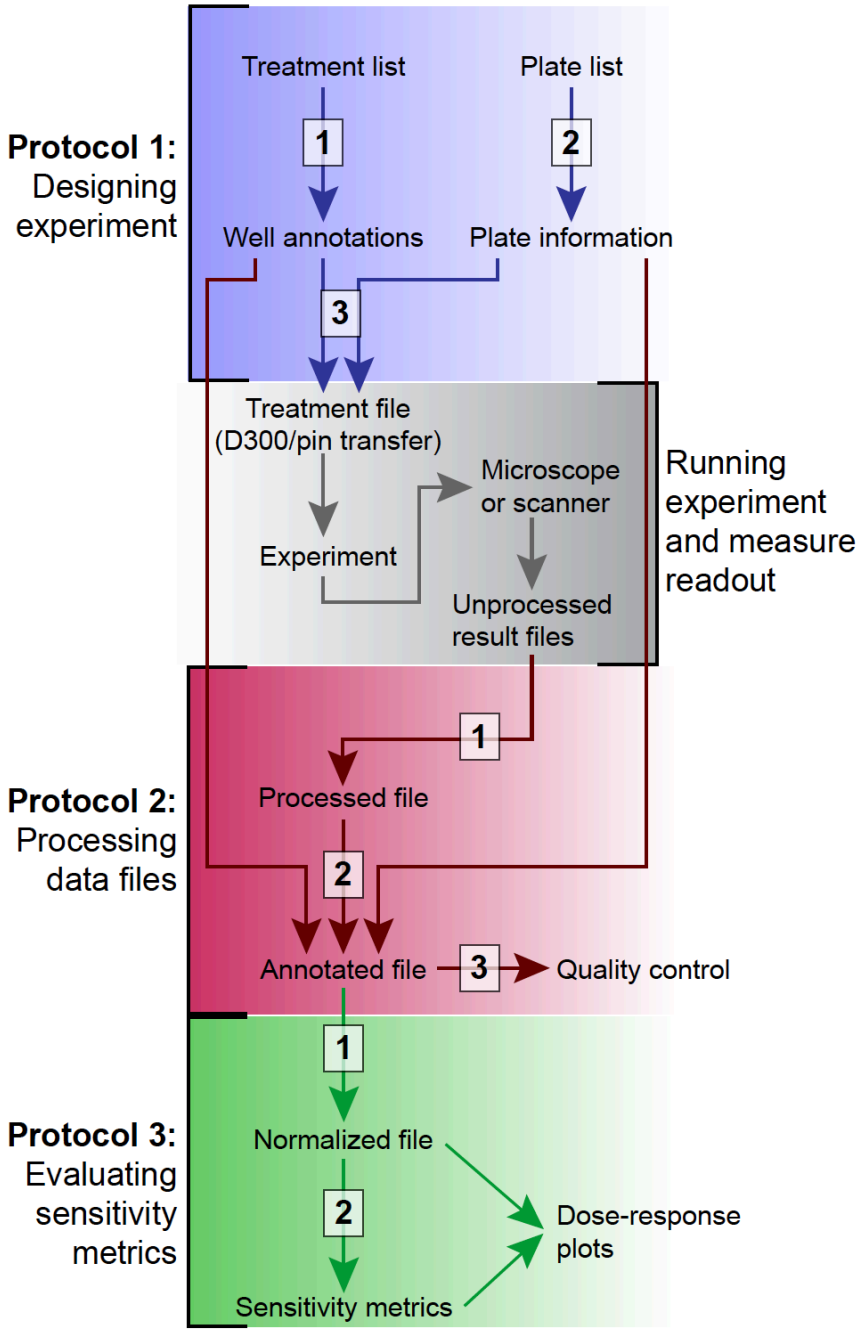
Pipeline

1. Designing experiment

   Running experiment

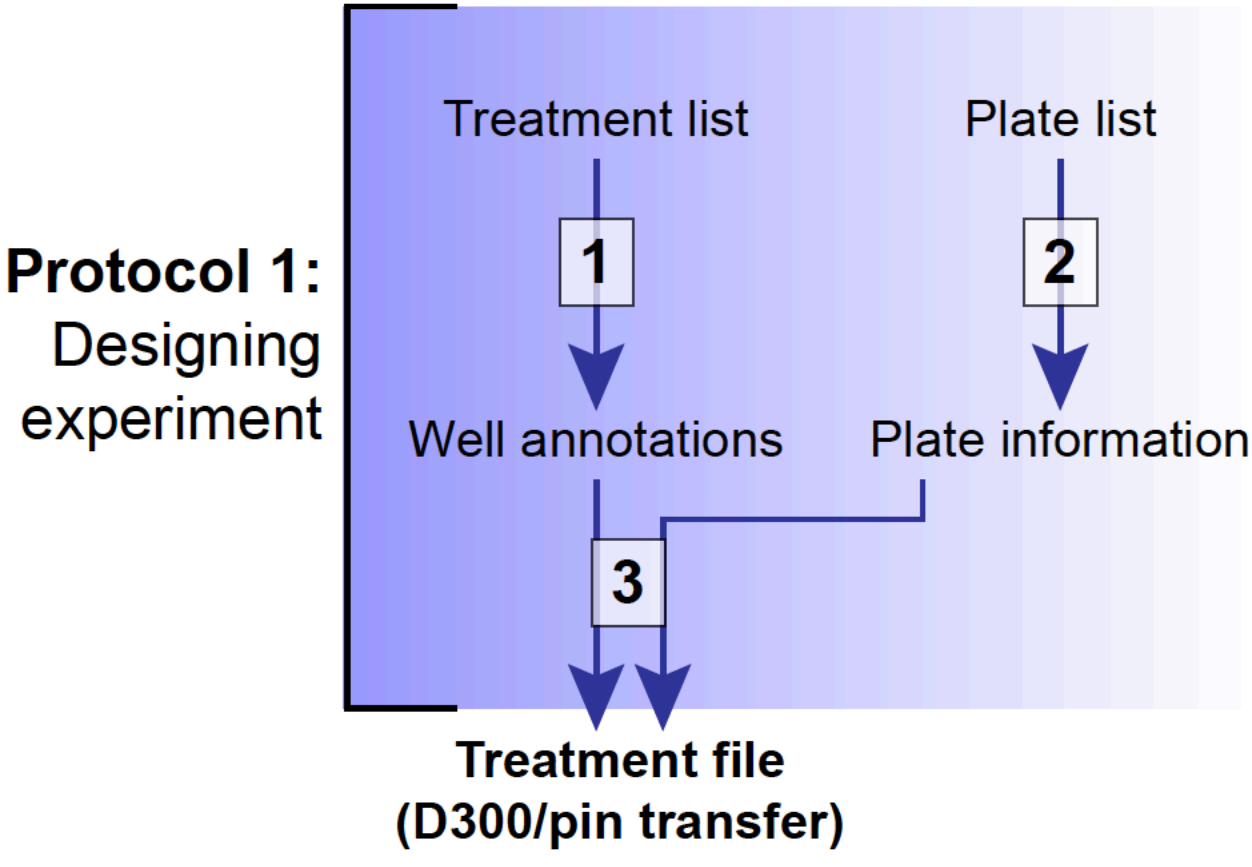
2. Processing data files

3. Evaluating sensitivity metrics



Hafner*, Niepel*, Subramanian*, Sorger
Curr Protoc Chem Biol, in press

Design


Protocol 1:
Designing
experiment
Treatment list
Plate list
1
2
Well annotations
Plate information
3
Treatment file
(D300/pin transfer)

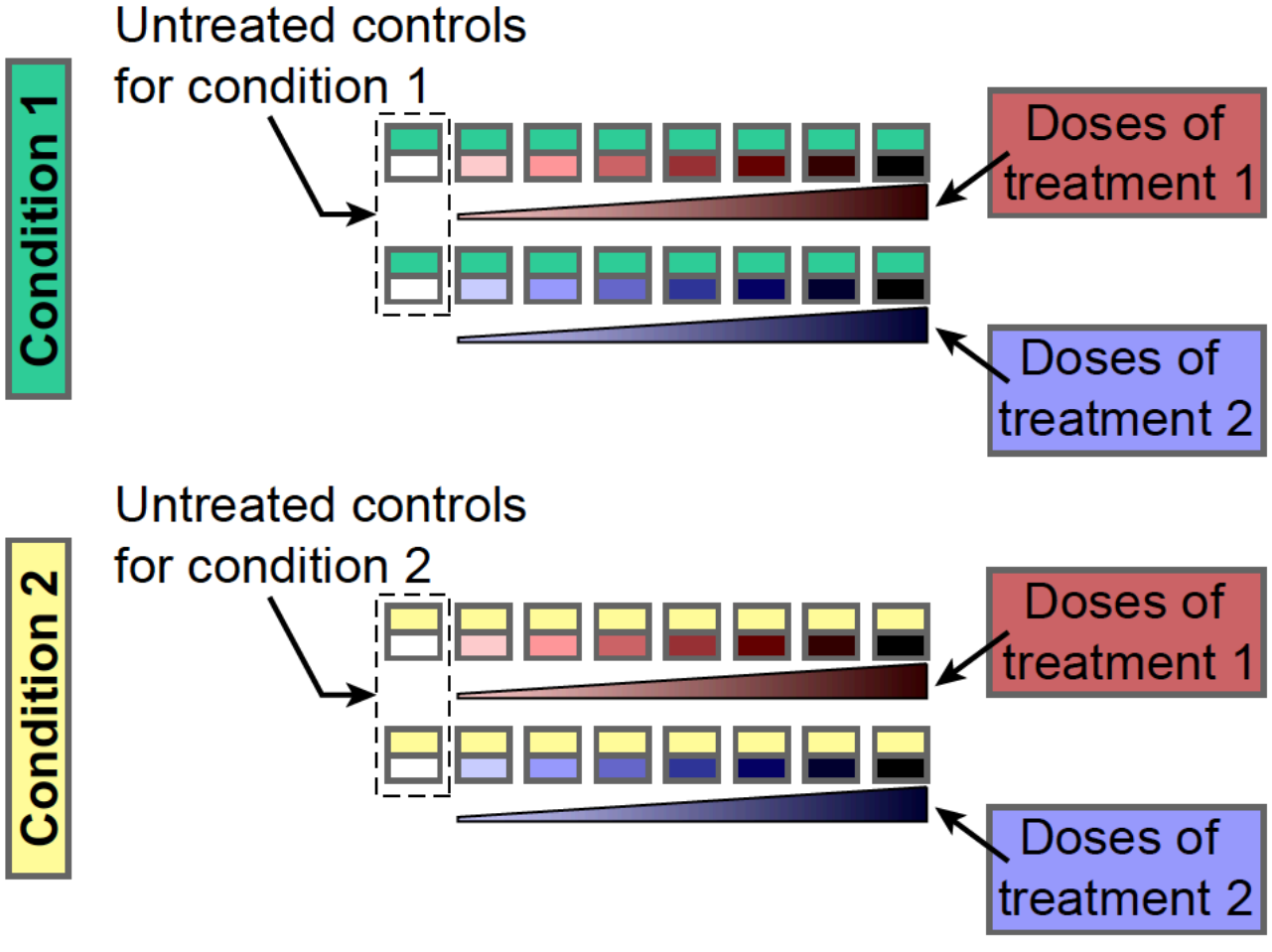
Additional notes: types of variables

- Model variables:
  - Treatment variables (drug, concentration, …)
  - Condition variables (growth media, seeding density, …)

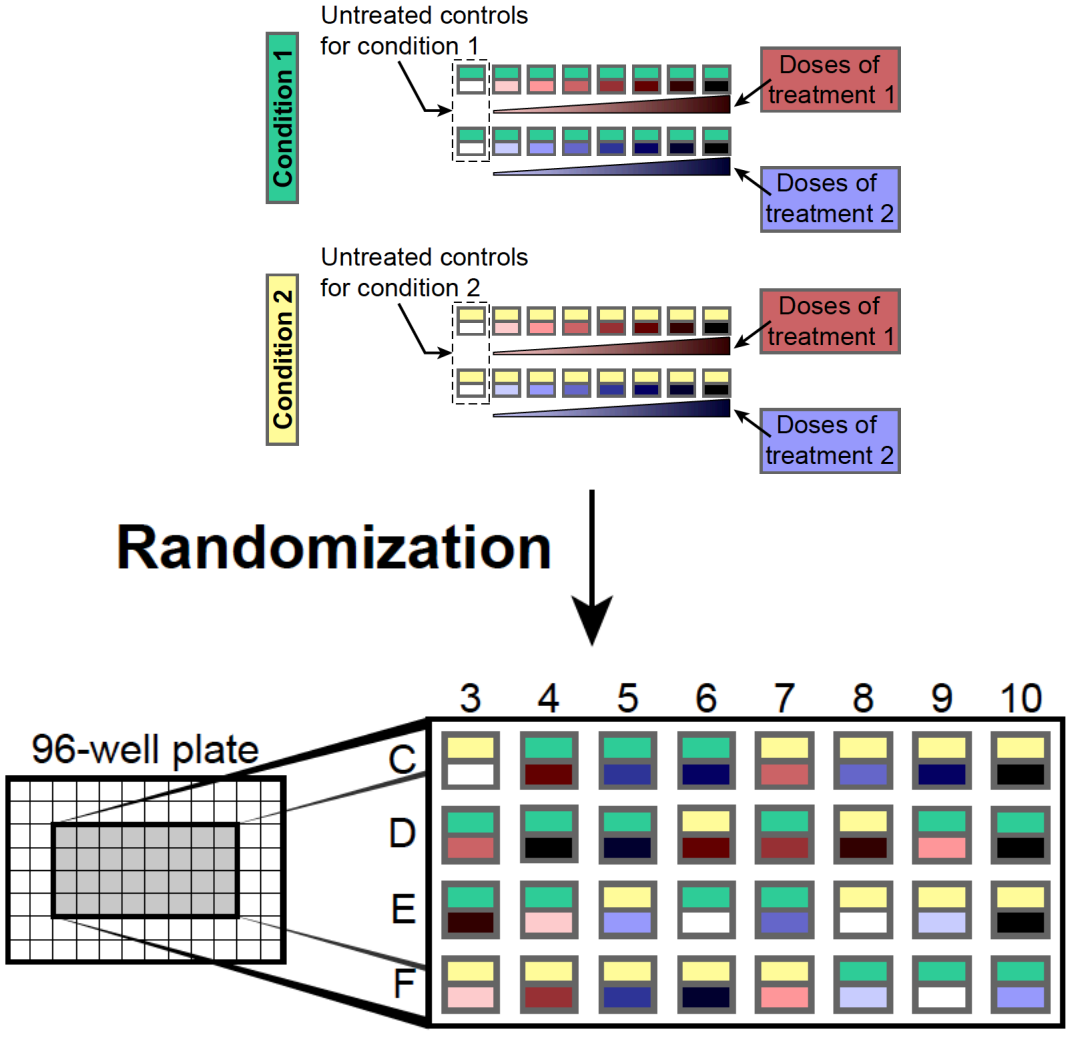
- Confounder variables:
  - Plate model
  - Assay date

- Readout variables

Design example: testing 2 drugs across multiple doses in 2 conditions



Randomizing the position on the plate avoids biases and artefacts



Use Python and Jupyter notebooks to produce the experimental design

Template for specifiying the experimental design.

The compounds, number of doses and information about the role of each compound (treatment, negative control etc) is defined in the file "compound_list.tsv". The scripts below take this tsv file as input in order to design the layout on the plate.

The size of the plate has to be provided as number of rows and columns. The number of replicates and the plate barcode are also provided in the block of code below.

Explanatory text

Design of the experiment and treatment layout (protocol 1)

```
import datarail.experimental_design.process_assay as process_assay
import datarail.experimental_design.designer as designer
import datarail.experimental_design.plot_panels as design_plot
import matplotlib.pyplot as plt
%matplotlib inline

input_file = 'INPUT/compound_list.tsv'
plate_dims = [16, 24]
fingerprint_prefix = 'DRUG_TRT_'
num_replicates = 3

treatment_dicts = process_assay.read_input(input_file, plate_dims,
                            fingerprint_prefix, encode_plate=True,
                            num_replicates=num_replicates)
```

User inputs

```
There are 20 untreated wells on the inner plate. Consider alloting more wells to negative con
trols
```

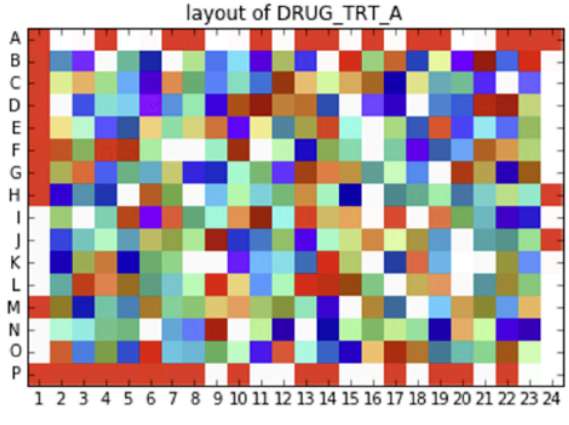
Warning messages

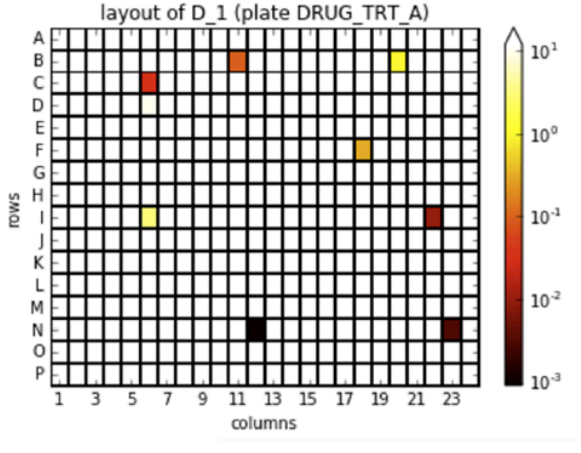
Use Jupyter notebooks to keep track of design steps and export drug layout

```
Designs = designer.make_layout(treatment_dicts, fingerprint_prefix,
                      encode_fingerprint=True,
                      plate_dims=plate_dims, num_replicates=num_replicates,
                      randomize=True, biased_randomization=True)

design_plot.plot_layout(Designs.sel(plates='DRUG_TRT_A'))
design_plot.plot_drug(Designs.sel(plates='DRUG_TRT_A'), 'D_1')
```

Optional inputs

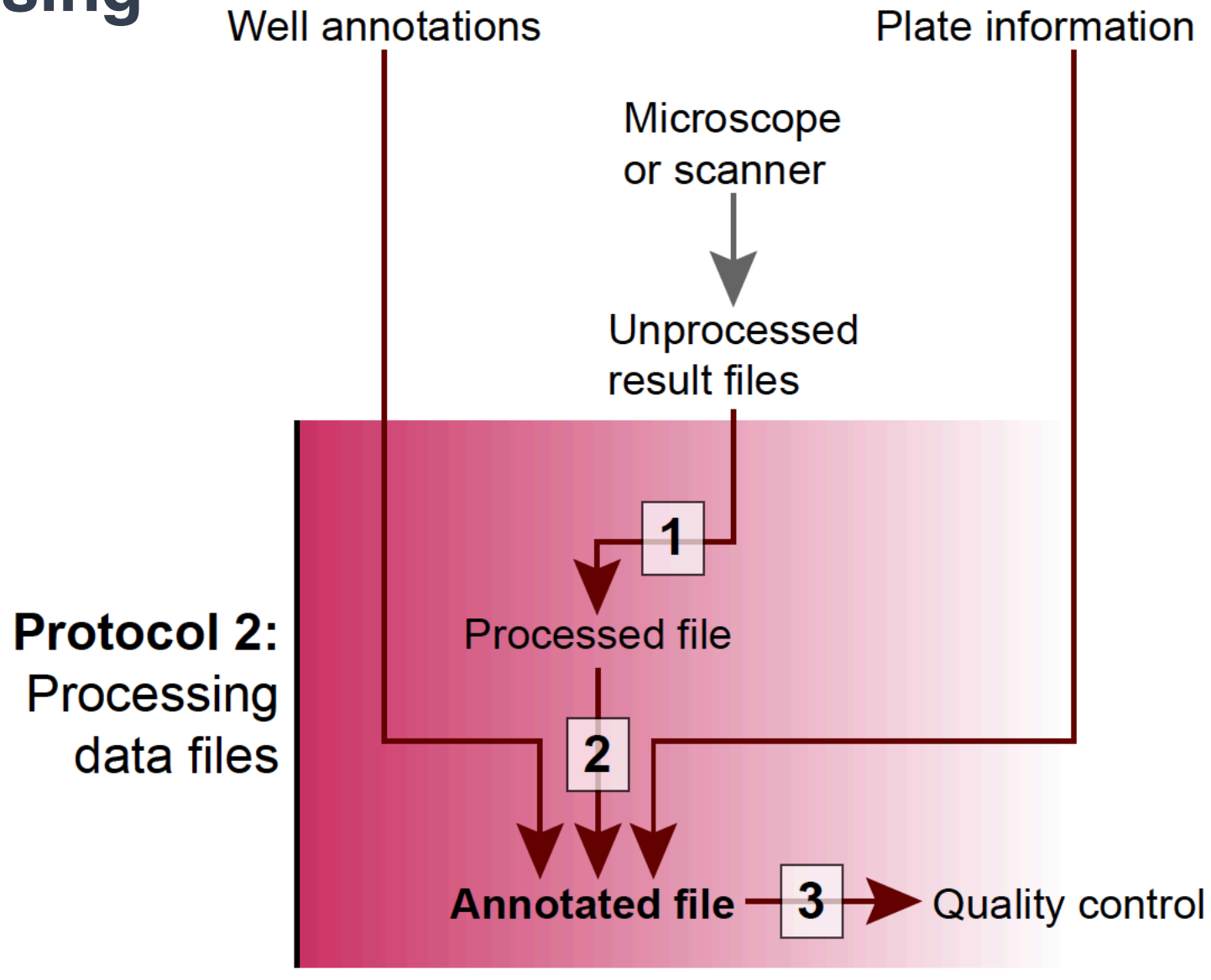




Limitations and space constraints in the design of plate-based experiments

- Control wells (both negative and positive)
- Number of concentrations for dose-response curves
- Number of replicates
- Edge and plate-based effects

Processing


Well annotations
Plate information
Microscope
or scanner
Unprocessed
result files
1
Processed file
2
Annotated file
3
Quality control
Protocol 2:
Processing
data files

Use Jupyter notebooks to import and annotate results from experiments

Data import and annotation (protocol 2, steps 1 and 2)

```
# load the synthetic data (Columbus output)
df=CoImp.Columbus_processing('../tests/drug_response_data/OUTPUT/Example1_Columbus_output.tsv',

                             (('Hoechst_pos', 'cell_count__total'),
                              ('LDR_pos_Hoechst_neg', 'corpse_count'),
                              ('Hoechst_LDR_pos', 'cell_count__dead')),
                             'cell_count__total - cell_count__dead')

# annotate the data
df_annotated = TrtAnnot.add_treatments(
    TrtAnnot.add_plate_info(df,
        pd.read_csv('../tests/drug_response_data/OUTPUT/Example1_plate_info.tsv', sep='\t')),
    '../tests/drug_response_data/OUTPUT/')

df_annotated.to_csv('OUTPUT/AnnotatedData_Example1.tsv', sep='\t', index=False)
```

```
Default number of fields: 6 ; 505 wells with missing field(s)
Concentrations rounded in the log domain
```

Data file

Readout variables

Folder containing well annotation files

Annotated file

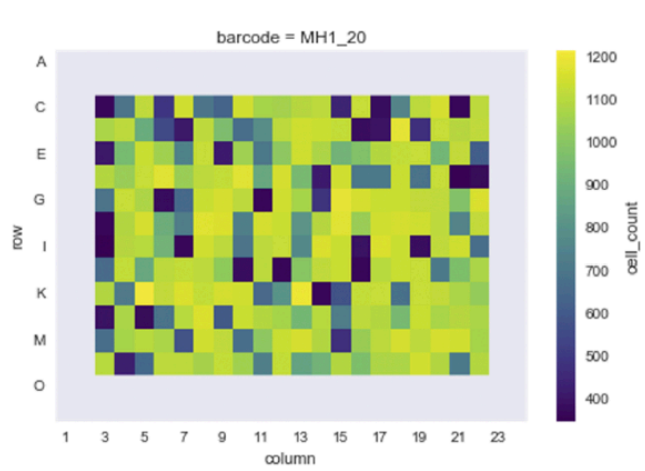
Check for unwanted biases using embedded functions

Quality control (protocol 2, step 3)

```
import datarail.data_processing.drug_response.qc_plate as qcfct

# use the raw data to perform the plate QC
qcfct.Plate_bias(pltfct.dfplate2xr(df), filename='OUTPUT/QC_report_Example1.pdf')
# use the annotated data to perform QC based on the negative controls
qcfct.Negative_control_bias(df_annotated)
```

QC report file



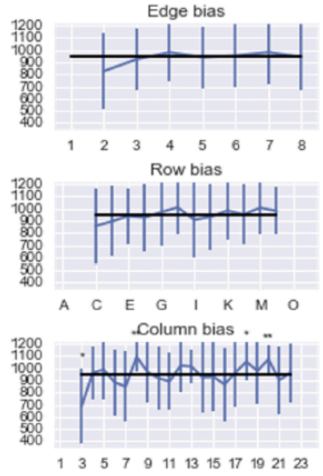
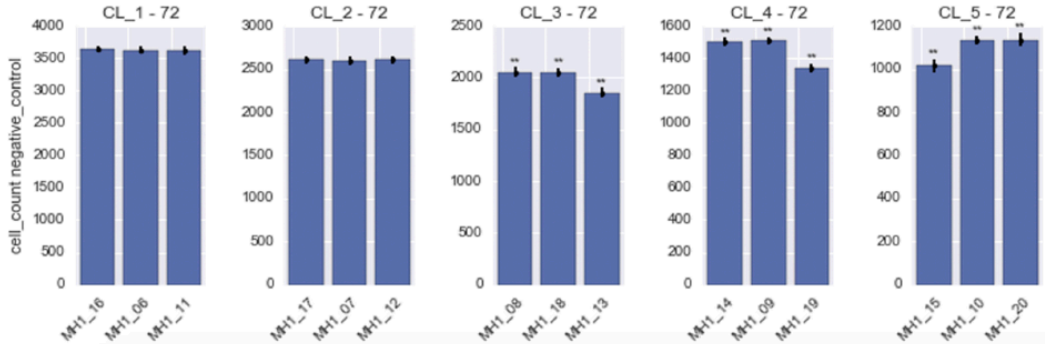
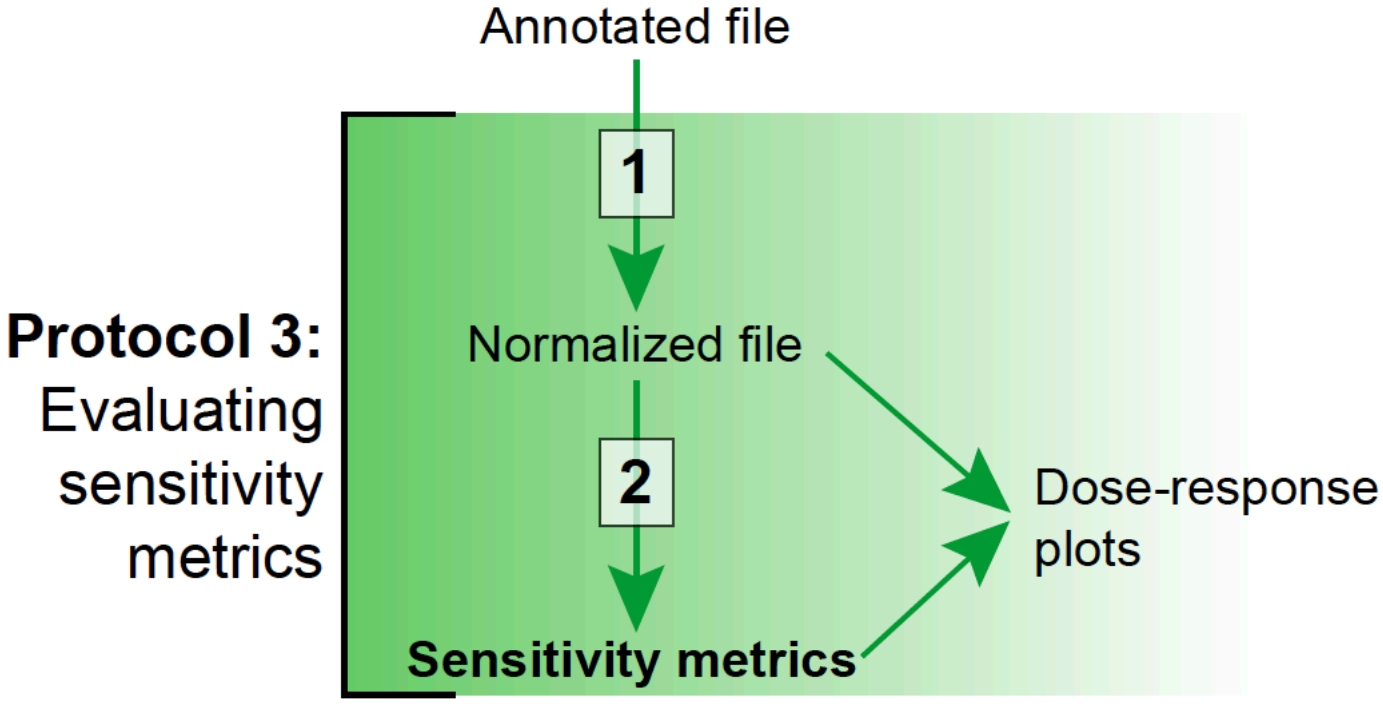


Plate bias QC



Negative control QC

Analysis: data normalization and dose-response curve parametrization



Normalize the data to obtain the GR values

Calculate the GR values (protocol 3, step 1)

```
# first calculate the GR values for each replicate then merge them
df_mean = TrtAnnot.average_replicates(
    gr50.compute_gr(
        gr50.assign_ctrls(df_annotated, ['cell_line'])))

df_gr = df_mean.drop(['cell_count__dead','corpse_count','role','cell_count__total'],axis=1)
df_gr.to_csv('OUTPUT/GRvalues_Example1.tsv', sep='\t', index=False)
df_gr.head()
```

```
Columns to average: "corpse_count" "cell_count__total" "cell_count" "cell_count__dead" "cell_
count__ctrl" "GRvalue" "cell_count__time0"
Columns added as annotations: "date"

-->Following columns are discarded:
 "treatment_file" "well" "barcode"
        (set as key if necessary)
```

	cell_line	treatment_duration	concentration	agent	date	cell_count	cell_count__ctrl	GRvalue	cell_count__t
0	CL_1	72.0	0.001	D_1	2016-06-06 12:34:56	3583.444444	3627.85	0.991393	491.525
1	CL_1	72.0	0.001	D_2	2016-06-06 12:34:56	3612.000000	3627.85	0.996945	491.525

Calculate GR values

Normalized file

Fit a dose-response curve to obtain sensitivity metrics

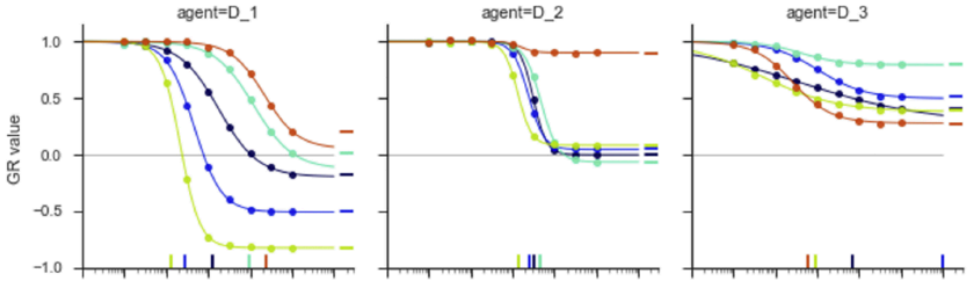
Evaluate the GR metrics and plot the dose-response curves (protocol 3, step 2)

```
df_grmetrics = gr50.gr_metrics(df_gr)
df_grmetrics.to_csv('OUTPUT/GRmetrics_Example1.tsv', sep='\t', index=False)
print df_grmetrics.head()
fig = gr50.plot.plot_curves(df_grmetrics, df_gr, colorvar='cell_line', colvar='agent')
fig.savefig('OUTPUT/GRcurves_Example1.pdf')
```

Evaluate and plot GR metrics

```
                 date cell_line agent  treatment_duration      GR50      GRmax  \
0 2016-06-06 12:34:56      CL_1   D_1                72.0  0.126677 -0.173488
1 2016-06-06 12:34:56      CL_1   D_2                72.0  0.311289 -0.004219
2 2016-06-06 12:34:56      CL_1   D_3                72.0  0.690251  0.401416
3 2016-06-06 12:34:56      CL_1   D_4                72.0  3.703113 -0.096859
4 2016-06-06 12:34:56      CL_1   D_5                72.0  0.668006 -0.094478

     GR_AOC     GEC50      GRinf      h_GR        r2          pval
0  0.521884  0.180590 -0.195387  0.930270  0.999900  9.992007e-15
1  0.375490  0.311573 -0.001281  2.805116  0.999949  8.881784e-16
2  0.403562  0.039185  0.276279  0.280334  0.999646  8.330003e-13
3  0.117477  4.170068 -0.194446  2.766167  0.999968  2.220446e-16
4  0.312724  0.739848 -0.107022  1.898776  0.999925  3.552714e-15
```



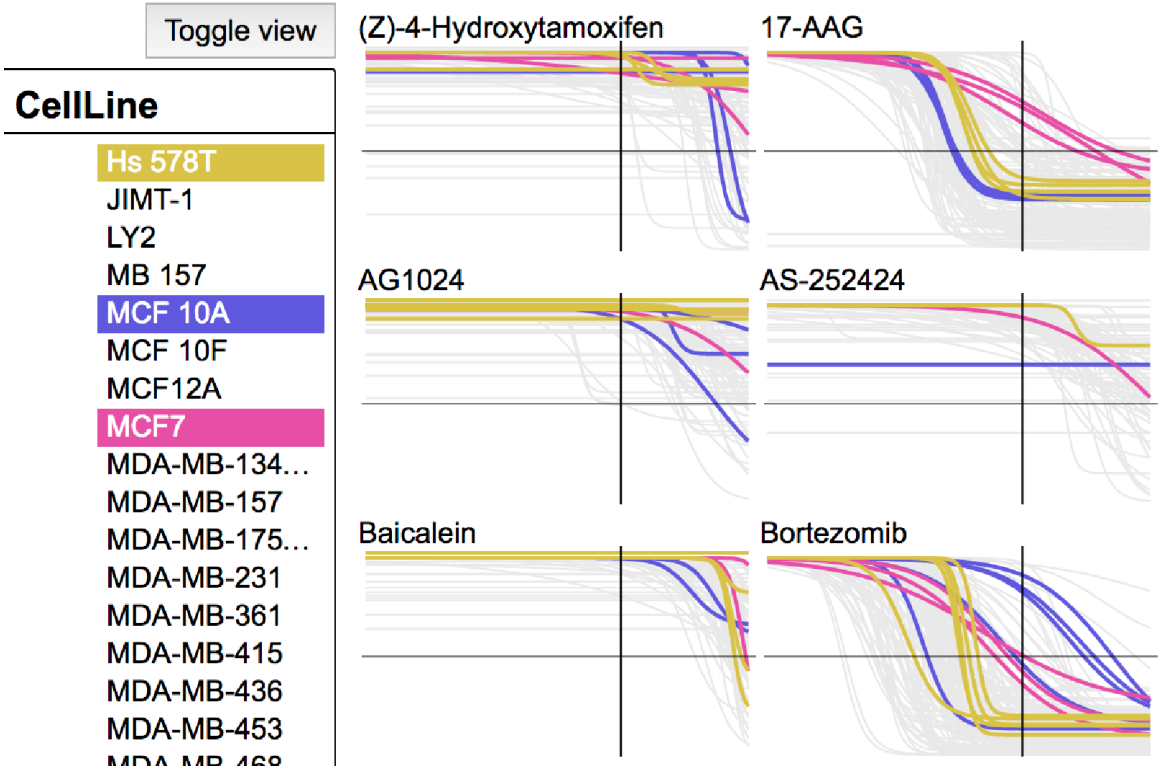
Dose-response plots

GRcalculator.org can replace the last part of the protocol

GRcalculator.org

Clark*, Hafner* et al.,
BMC Cancer, in review

Hafner*, Heiser* et al.,
Sci Data, in review



Note on the programmatic approach: advantages of scripts over UI-driven software

- Permanent record of the design and data processing
- Jupyter notebooks simplify use and allow reuse of scripts
- Less prone to unnoticed errors (e.g. excel spreadsheets)

1. Theory of drug response
2. Experimental setup
3. Designing and analyzing experiments
4. **Biological examples**
    - **Case study**
    - **Efficacy vs. potency**

Profiling the response of triple negative breast cancer models to kinase inhibitors

- Why study kinase inhibitors in TNBC?
  - Unmet clinical need
  - Patients have a poor prognosis, and no targeted therapy options
- GR metrics were used to enable comparisons across cell lines

Selection of cell lines and drug treatments

20 TNBC

6 HR+

4 Her2amp

2 NM

4 from PDX

Cell Line	Receptor Status	Molecular Subtype
BT-20	TNBC	Basal A
HCC1143	TNBC	Basal A
HCC1806	TNBC	Basal A
HCC1937	TNBC	Basal A
HCC70	TNBC	Basal A
MDA-MB-468	TNBC	Basal A
BT-549	TNBC	Basal B
CAL-51	TNBC	Basal B
HCC1395	TNBC	Basal B
HCC38	TNBC	Basal B
Hs 578T	TNBC	Basal B
MDA-MB-157	TNBC	Basal B
MDA-MB-231	TNBC	Basal B
MDA-MB-436	TNBC	Basal B
SUM1315	TNBC	Basal B
SUM149	TNBC	Basal B
SUM159	TNBC	Basal B
CAL-85-1	TNBC	Basal
CAL-120	TNBC	Luminal
MDA-MB-453	TNBC	Luminal
CAMA-1	HR+	Luminal
HCC1428	HR+	Luminal
HCC1500	HR+	Luminal
MCF7	HR+	Luminal
MDA-MB-134	HR+	Luminal
T47D	HR+	Luminal
HCC1954	HER2amp	Basal A
HCC1419	HER2amp	Luminal
MDA-MB-361	HER2amp	Luminal
SK-BR-3	HER2amp	Luminal
hTERT-hME1	NM	Basal
MCF 10A	NM	Basal
PDX-DFCI-1206	TNBC	N/A
PDX-DFCI-1258	TNBC	N/A
PDX-DFCI-1328	TNBC	N/A
PDX-HCI-002	TNBC	N/A

X

Drug Name	Primary Target	Clinical Status
Alpelisib/BYL719	PI3Ka	Phase 3
TGX221	PI3Kb	Preclinical
Taselisib/GDC0032	PI3Ka, g, d	Phase 1/2
Pictilisib/GDC0941	pan PI3K	Phase 2
Buparlisib/NVP-BKM120	pan PI3K	Phase 2
INK128/MLN0128	mTORC1/2	Phase 2
Torin2	mTOR/ATM/ATR	Tool
Everolimus	mTOR1	Approved
Ipatasertib/GDC0068	AKT	Phase 1/2
PF-4708671	p70S6K	Phase 1
Neratinib/HKI272	EGFR/HER2	Phase 3
Tivantinib/ARQ197	MET	Phase 3
Cabozantinib	VEGFR2/MET	Approved
Cediranib/AZD2171	VEGFR/cKIT	Phase 3
Ceritinib/LDK378	ALK	Phase 2/3
Saracatinib/AZD0530	SRC	Phase 2/3
Dasatinib	BCR/ABL	Approved
Trametinib/GSK1120212	MEK	Phase 2
Luminespib/NVP-AUY922	HSP90	Phase 2
Palbociclib/PD0332991	CDK4/6	Phase 3
Dinaciclib/SCH727965	pan CDK	Phase 1
Abemaciclib/LY2835219	CDK4/6	Phase 3
Volasertib/BI6727	PLK	Phase 2/3
AZD7762	CHK1/2	Phase 1
Olaparib/AZD2281	PARP	Phase 3
ABT-737	Bcl2/XL	Tool
A-1210477	Mcl-1	Tool
Vorinostat	HDAC	Phase 2
Paclitaxel	Chemotherapy	Approved
Doxorubicin	Chemotherapy	Approved
Cisplatin	Chemotherapy	Approved
Etoposide	Topoisomerase II	Approved
Topotecan	Topoisomerase I	Approved
Bleomycin	Radiomimetic	Approved
Ionizing radiation	DNA damage	Approved

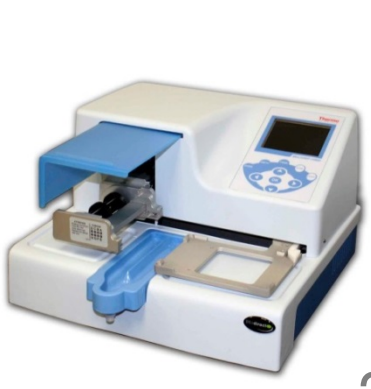
24 kinase inhibitors

4 misc inhibitors

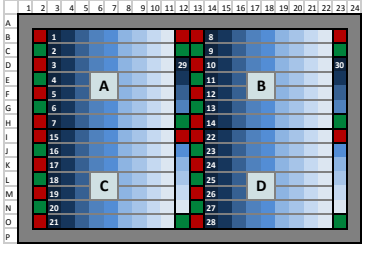
3 chemo

4 DNA damage

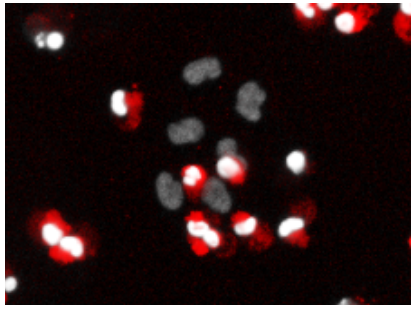
Data collection workflow



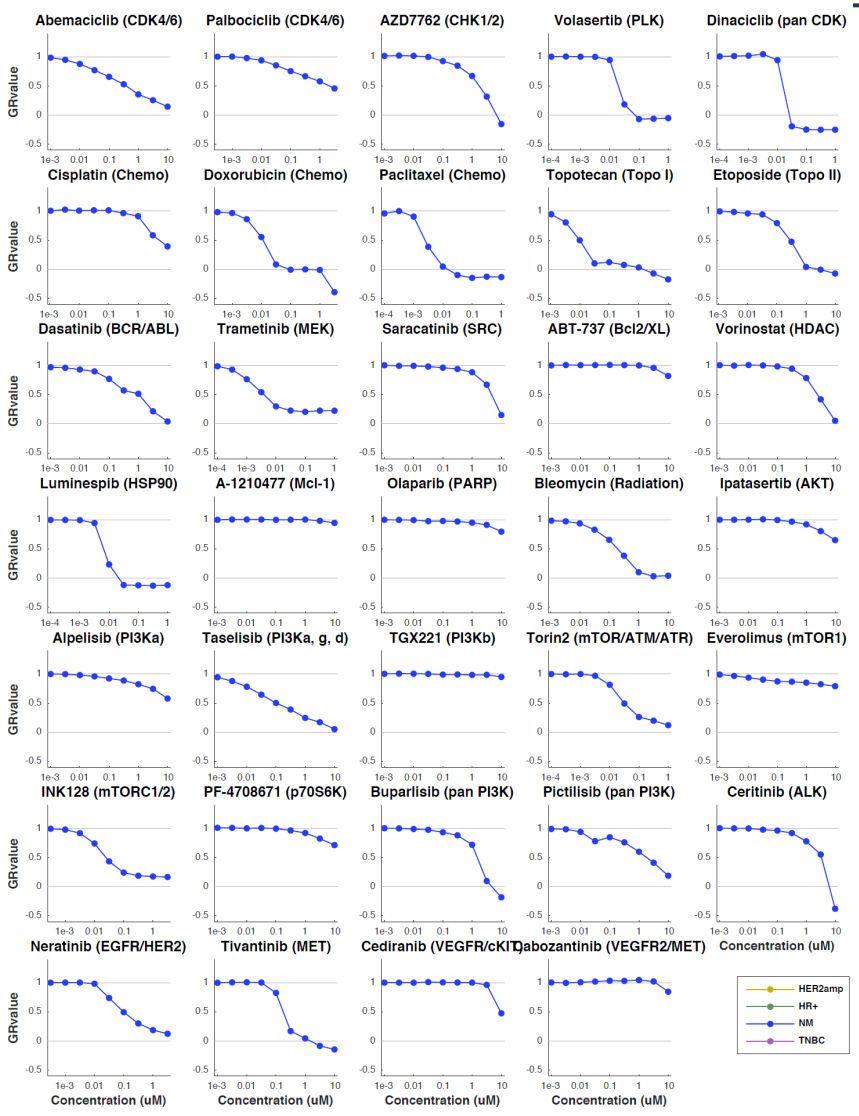
36 hrs

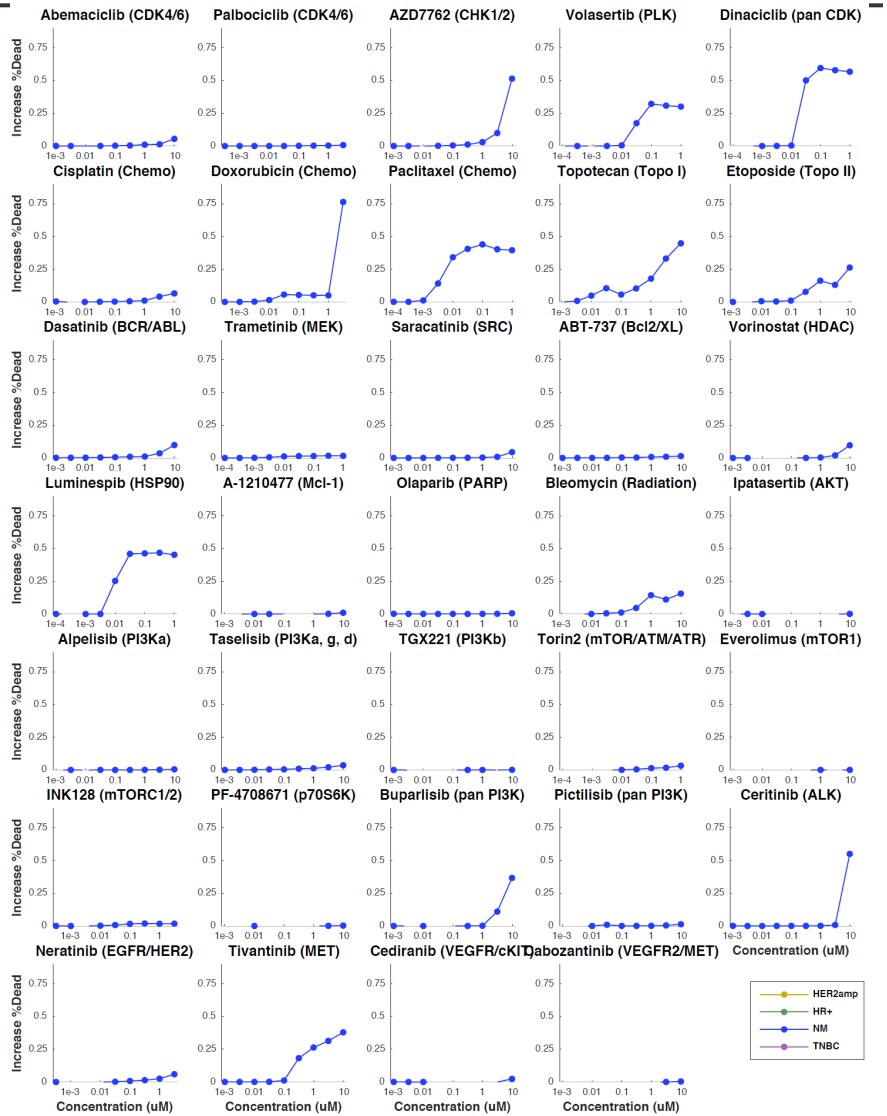


72 hrs

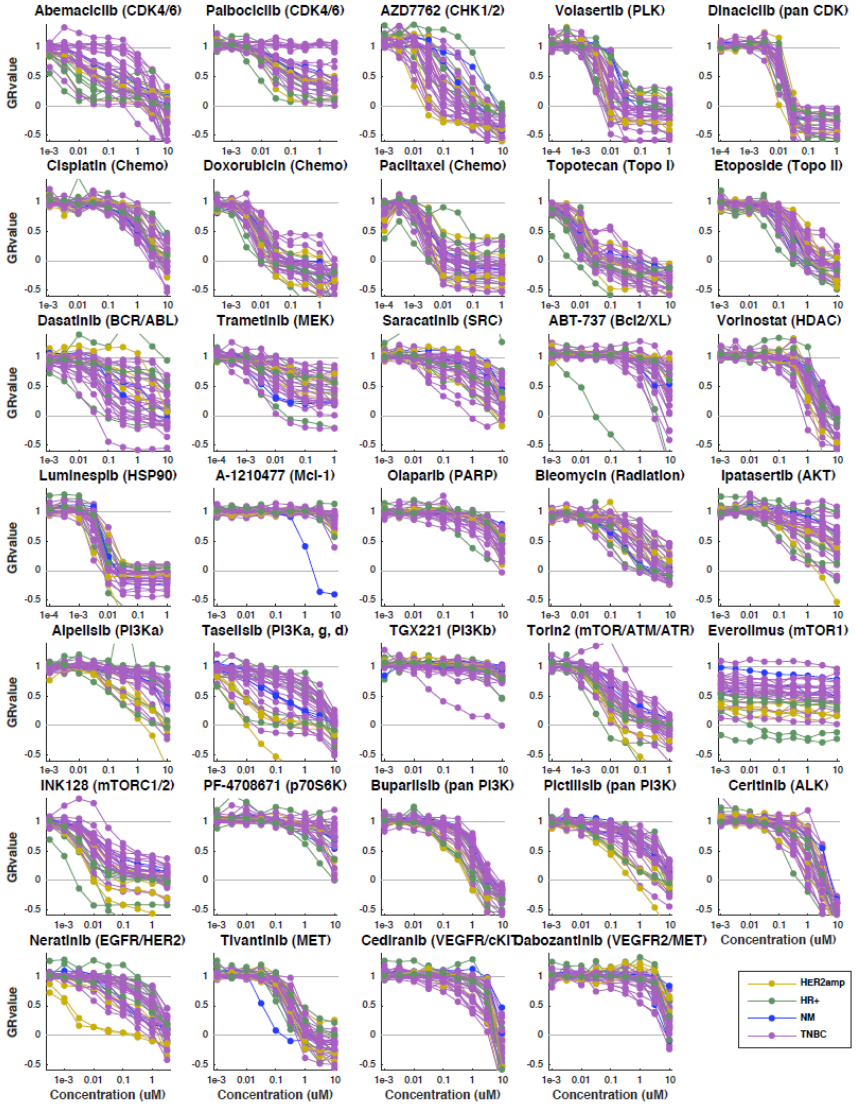


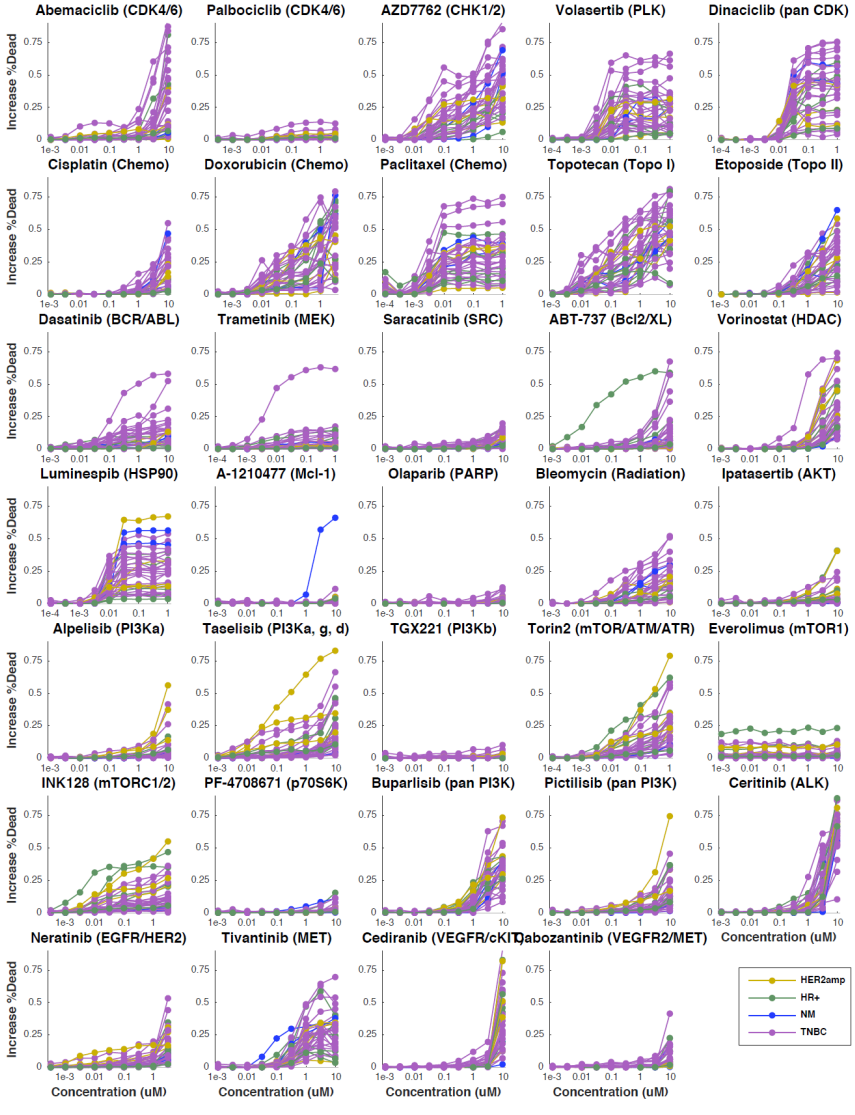
Dose response results for one cell line



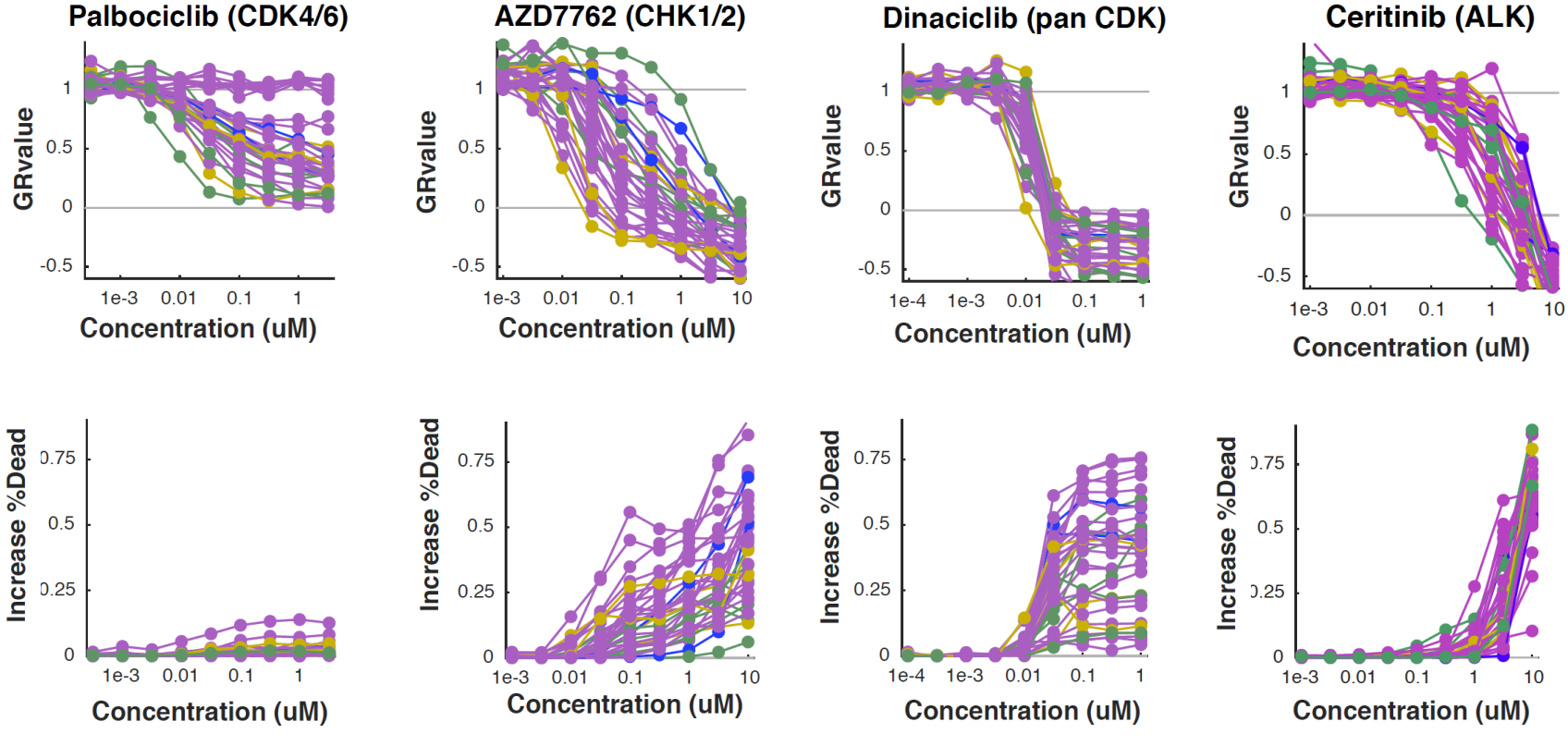


Dose response results for all cell lines

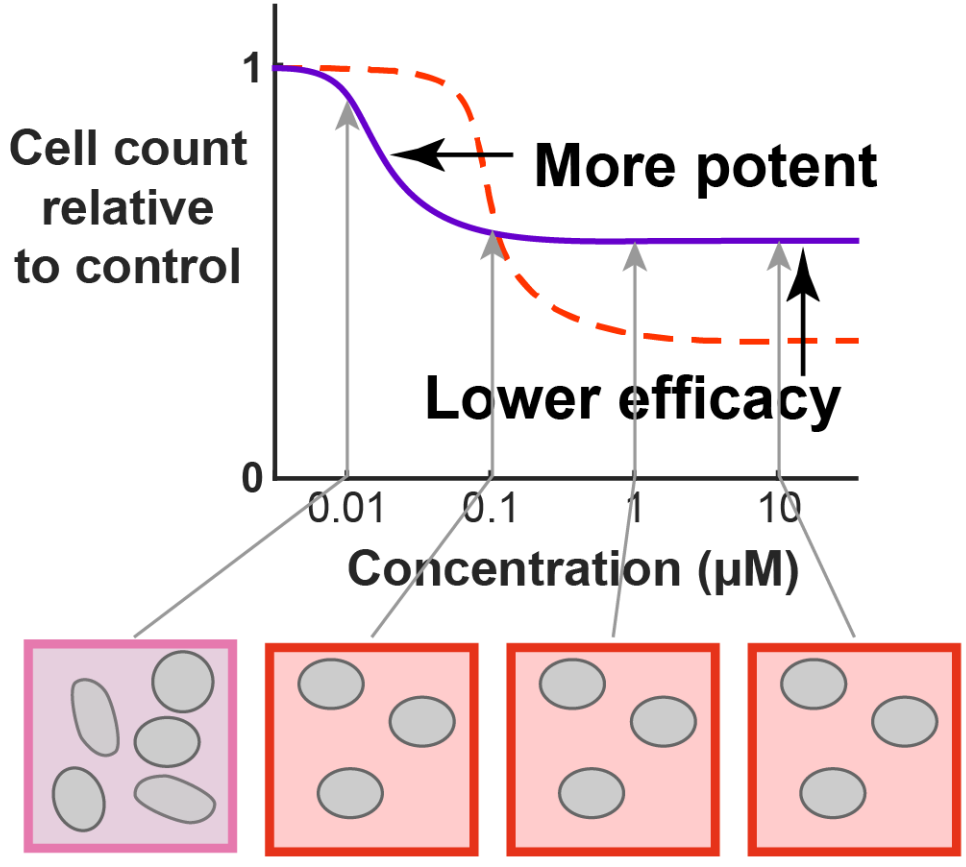




Diversity in response profiles…



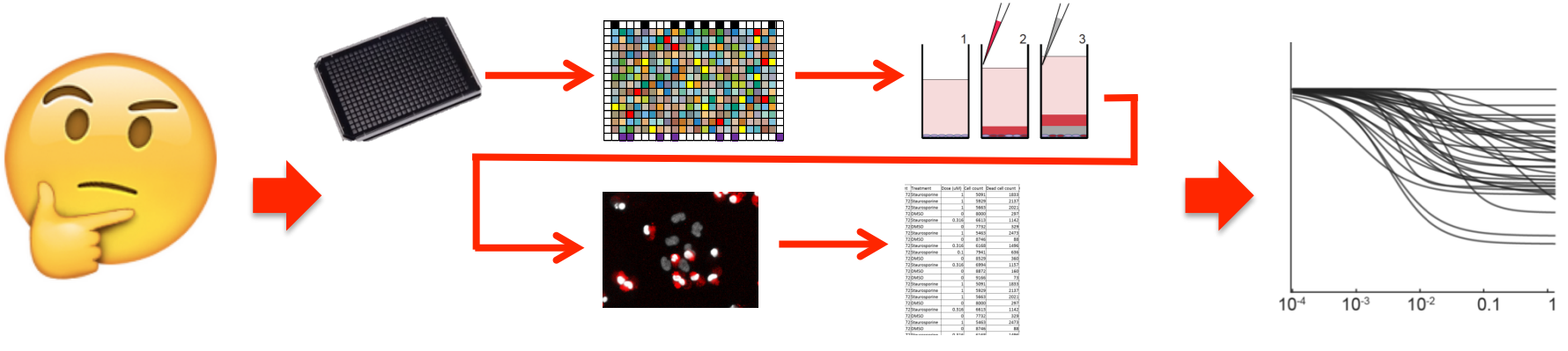
…occurs in both potency and efficacy across cell lines and drugs



We aim to understand the biology underlying these differences with the goal of being able to predict the response of a cell line to a perturbation.

What about reproducibility?

- Planning, and optimization
- Automate as much as possible, know how it works
- Script the experimental design and analysis
- Use appropriate metrics for your experiment



References

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- Hafner, et al., Nat Biotech, 2017, accepted

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- Niepel*, Hafner* et al., Curr Protoc Chem Biol, in press (June 2017)

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http://github.com/datarail

GRcalculator.org

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