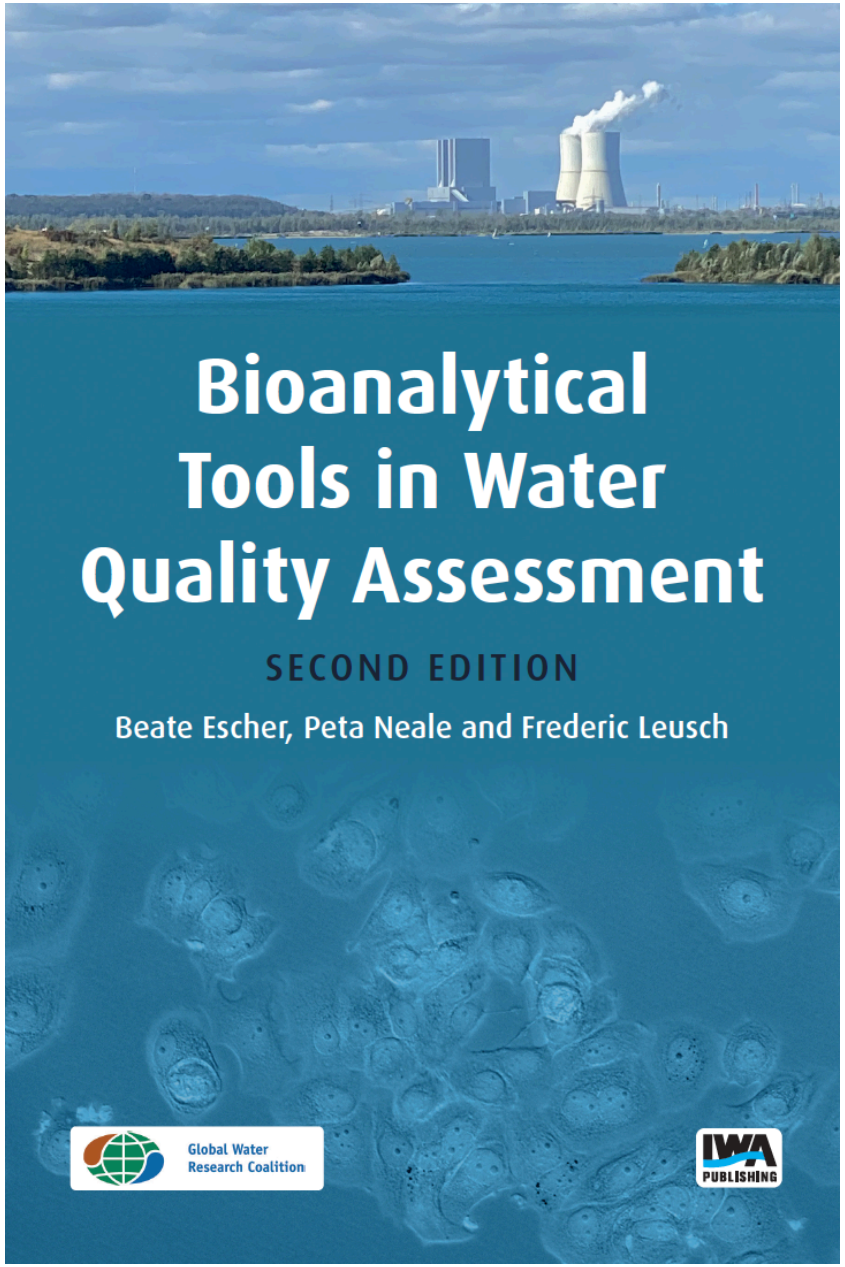
Chapter 7
Dose-response assessment

This presentation accompanies Chapter 7 of
“Bioanalytical Tools in Water Quality Assessment”
https://www.iwapublishing.com/books/9781789061970/bioanalytical-tools-water-quality-assessment-2nd-edition

Chapter 7 describes dose-response assessments, data reporting and derivation of benchmark values. It also gives the mathematical background for calculating bioanalytical equivalent concentrations (BEQ) from bioassay data as a measure of mixture effects.

Exercises and more material can be found at
www.ufz.de/bioanalytical-tools

Questions? please send an e-mail to bioanalytical-tools@ufz.de

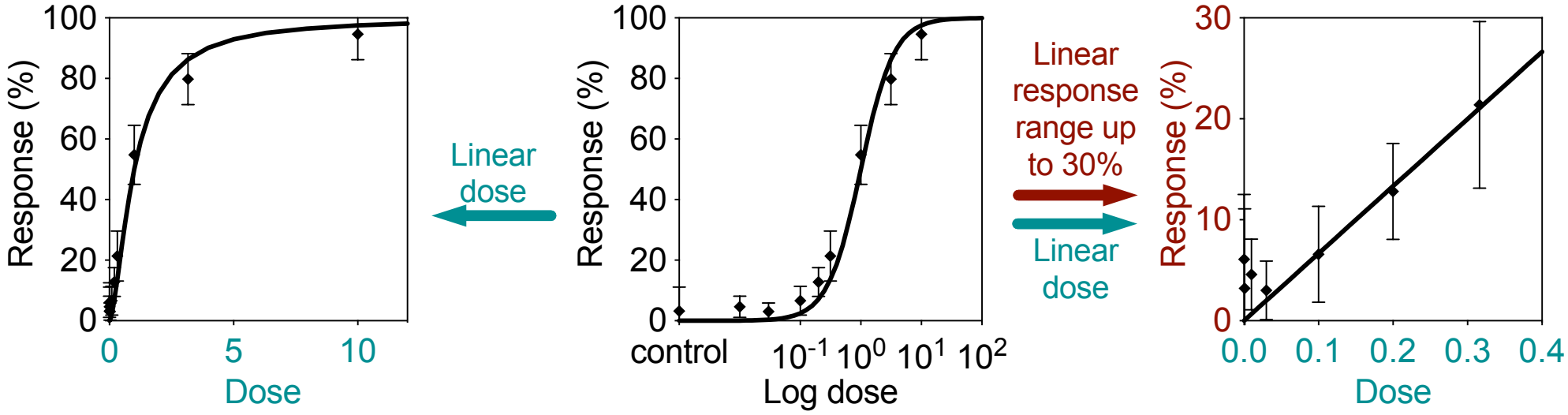


Learning goals

- You can analyse concentration-response data with mathematical models and derive benchmark concentrations
- You can translate benchmark concentrations into bioanalytical equivalency to express the effect of a complex mixture in equivalent concentration of a reference compound

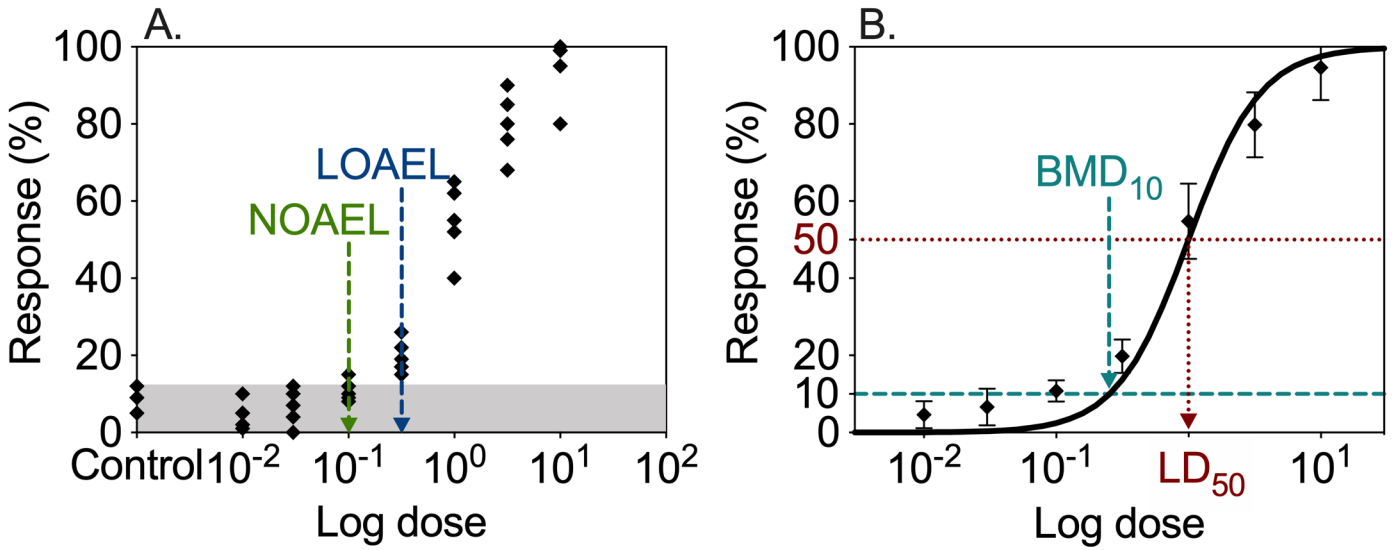
Biological life (and toxicity) is log-normally distributed

- Dose– response curve looks like a saturation curve on a linear dose scale but is typically sigmoidal on a logarithmic scale
- At the low effect level- the dose– response curve becomes linear on a non-log dose scale



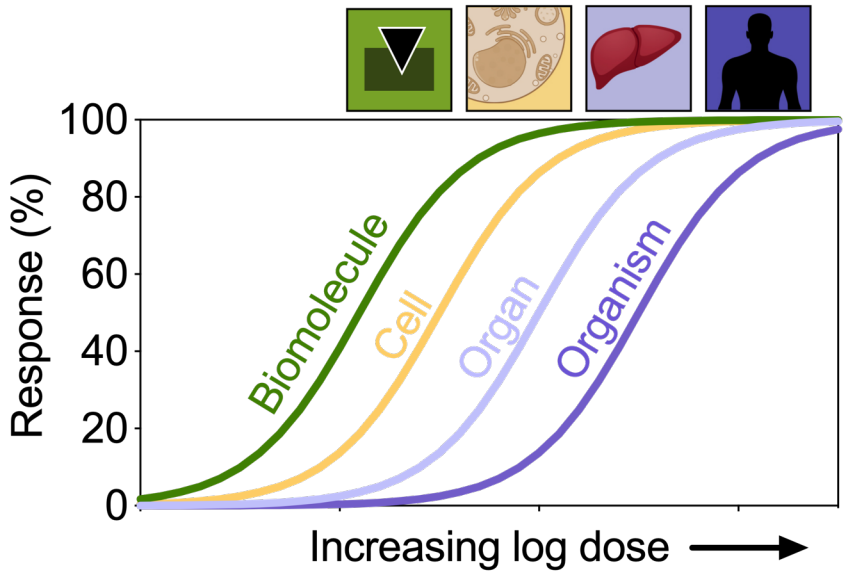
Dose benchmark values (animal testing)

- NOAEL ‘no observed adverse effect level ’
- LOAEL ‘lowest observed adverse effect level ’
- BMD_{10}: dose calculated from the dose–response curve triggering 10% of the maximum response
- LD_{50} : lethal dose killing 50% of the test animals



Continuum of toxicity

- As the dose increases so does the level of biological complexity affected
- Measurements on the cellular level may serve as sensitive measures for toxicity
- Higher biological level: defence mechanism -> lower sensitivity
- Any biological effect caused by a chemical must be linked to a molecular and cellular effect



The more specific we are, the more universal something can become. Life is in the details. If you generalize, it doesn't resonate. The specificity of it is what resonates.
Jacqueline Woodson

Bench Marks!

Chemical Concentrations in Cell Culture Compartments (C5) – Concentration Definitions

Jaffar Kisitu [1,2], Susanne Hougaard Bennekou [3] and Marcel Leist [1,2,4]

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- A dose is a measured quantity of a chemical delivered to a system.
  - *a normalized doses* can be indicated in amount of chemical per kg body weight
- a "concentration" is considered to be *the ratio of (i) chemical and (ii) the surrounding system*.
  - expressed in terms of its volume, its mass, the number of its molecules/particles, or in terms of physical or biological properties such as its radioactivity.
  - The system may be expressed as weight or volume. Alternatively, it may be quantified by the number or mass of certain molecules in the system (e.g., water, water-free substance, protein, DNA, or lipid)

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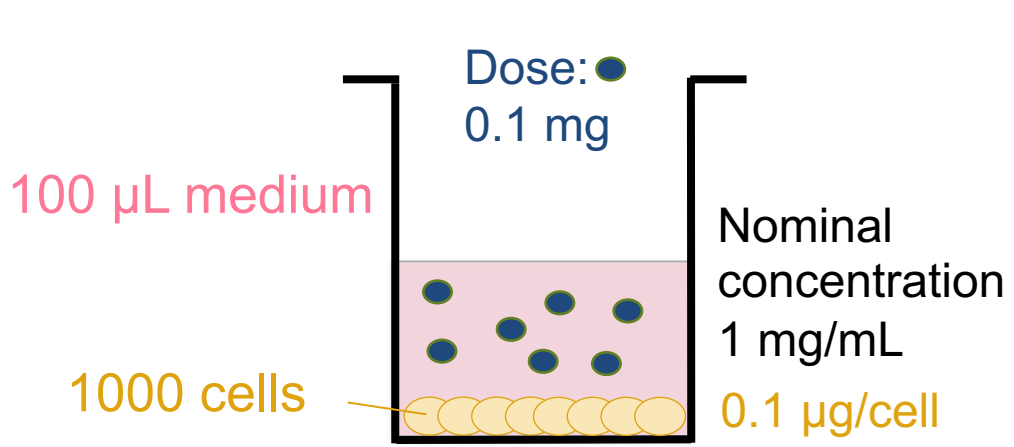
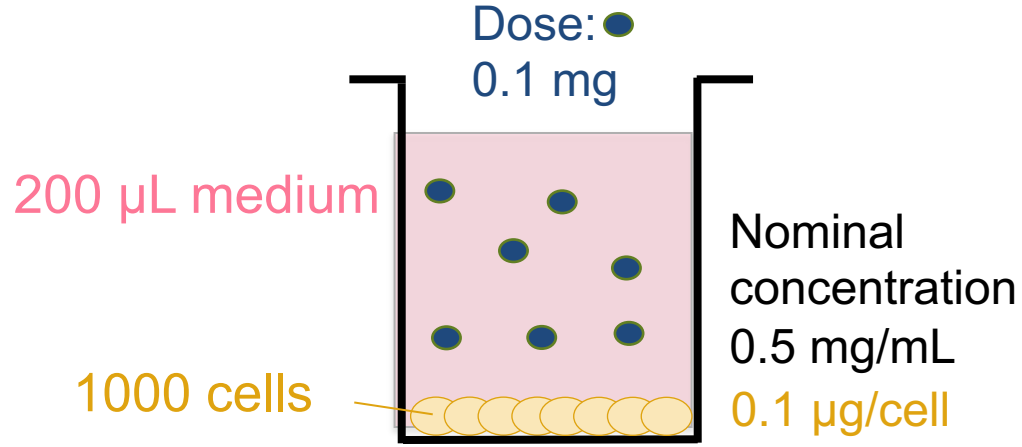
Chemical Concentrations in Cell Culture Compartments (C^5) – Concentration Definitions

If 1000 cells are placed in a well plate in 100 µL of medium, and 0.1 mg chemical is added, then the dose is 0.1 mg/ well or 0.1µg/cell; the concentration is 1 mg/mL. If the same cells are cultured in 200 µL medium, and the same amount of compound is added (0.1 mg), then the dose is still 0.1 mg/well and 0.1 µg/cell; however, the concentration is 0.5 mg/mL.





ALTEX 36(1), 155-161.doi:10.14573/altex.1901031

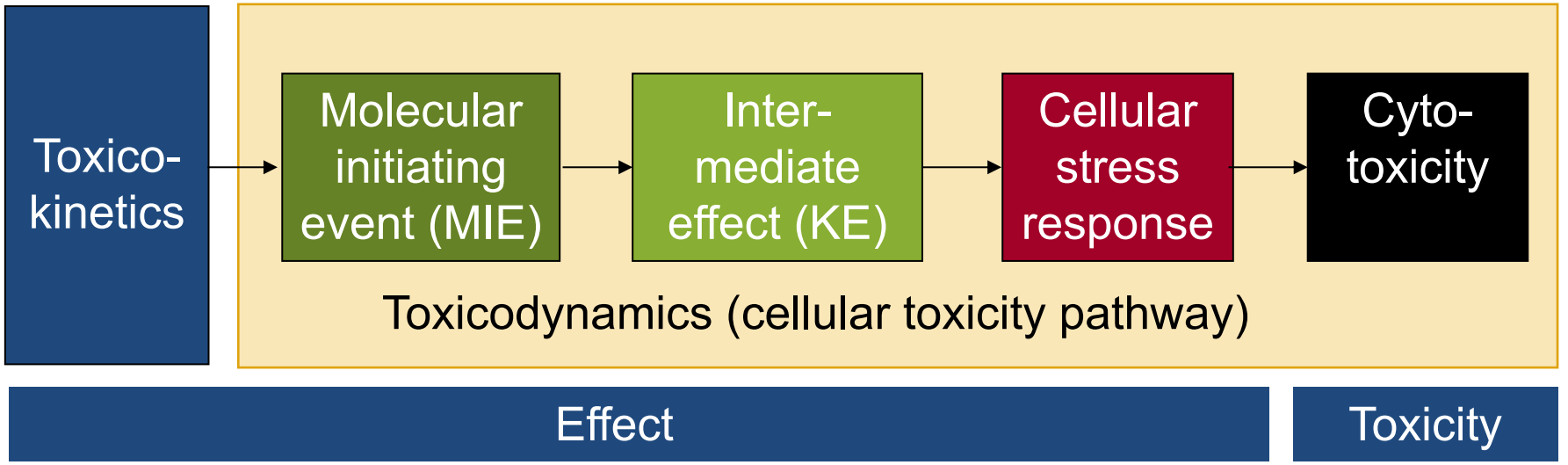
Response

Toxicity = adverse effect

- lethality, inhibition of growth, inhibition of cell viability

Effect = any response – adverse or not

- binding to nuclear receptors, induction or inhibition of metabolic enzymes or activation of adaptive stress response



There are many more mathematical models for sigmoidal dose-response curves

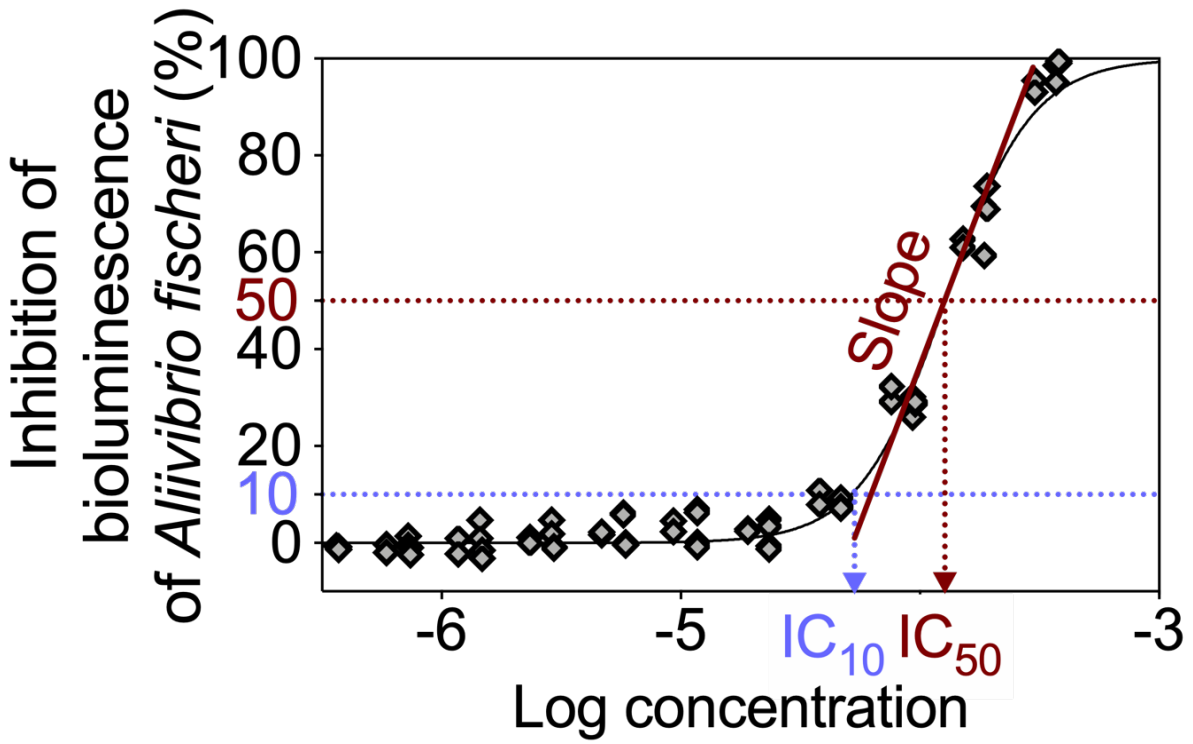
Table 1. Sigmoidal regression function $f(x)$ and the corresponding inverse formulae $x = f^{-1}(Y)$

Model	Function	Reference	Inverse[a]	
Probit	$f(x) = \frac{1}{\sqrt{2\pi}}\int_{-\infty}^{\beta_1+\beta_2\log_{10}(x)} \exp(-u^2/2)\,du = \Phi(\eta)$	[16,18]	$x = \mathrm{POW}\left(\frac{\Phi^{-1}(Y) - \hat{\beta}_1}{\hat{\beta}_2}\right)$	(1)
Logit	$f(x) = \frac{1}{(1 + \exp[-\eta])}$	[16,18]	$x = \mathrm{POW}\left(\frac{\log_e(K) - \hat{\beta}_1}{\hat{\beta}_2}\right)$ with $K = \left(\frac{Y}{1-Y}\right)$	(2)
Weibull	$f(x) = 1 - \exp(-\exp[\eta])$	[8,10]	$x = \mathrm{POW}\left(\frac{\log_e(K) - \hat{\beta}_1}{\hat{\beta}_2}\right)$ with $K = -\log_e(1-Y)$	(3)
MMF	$f(x) = 1 - \frac{1}{1 - (\beta_1 x)^{\beta_2}}$	[9,10]	$x = \frac{1}{\hat{\beta}_1}\cdot\left(\frac{Y}{1-Y}\right)^{1/\hat{\beta}_2}$	(4)
Generalized Logit I	$f(x) = \frac{1}{(1 + \exp[-\eta])^{\beta_3}}$	[11–13]	$x = \mathrm{POW}\left(\frac{-\log_e(K) - \hat{\beta}_1}{\hat{\beta}_2}\right)$ with $K = \left(\frac{1}{Y}\right)^{1/\hat{\beta}_3} - 1$	(5)
Generalized Logit II	$f(x) = 1 - \frac{1}{(1 + \exp[\eta])^{\beta_3}}$	[11–13]	$x = \mathrm{POW}\left(\frac{\log_e(K) - \hat{\beta}_1}{\hat{\beta}_2}\right)$ with $K = \left(\frac{1}{1-Y}\right)^{1/\hat{\beta}_3} - 1$	(6)
Aranda-Ordaz	$f(x) = 1 - \frac{1}{(1 + \exp[\eta]/\beta_3)^{\beta_3}}$	[14,16]	$x = \mathrm{POW}\left(\frac{\log_e(K) - \hat{\beta}_1}{\hat{\beta}_2}\right)$ with $K = \hat{\beta}_3\cdot\left(\left[\frac{1}{1-Y}\right]^{1/\hat{\beta}_3} - 1\right)$	(7)
Box-Cox Logit	$f(x) = \left(1 + \exp\left[-\beta_1 - \beta_2\frac{x^{\beta_3} - 1}{\beta_3}\right]\right)^{-1}$	[15,16]	$x = \left\{\frac{\hat{\beta}_3}{\hat{\beta}_2}\cdot\left(-\log_e\left[\frac{1-Y}{Y}\right] - \hat{\beta}_1\right) + 1\right\}^{1/\hat{\beta}_3}$	(8)
Box-Cox Weibull	$f(x) = 1 - \exp\left(-\exp\left[\beta_1 + \beta_2\frac{x^{\beta_3} - 1}{\beta_3}\right]\right)$	[15,16]	$x = \left[\frac{\hat{\beta}_3}{\hat{\beta}_2}\cdot(\log_e[-\log_e(1-Y)] - \hat{\beta}_1) + 1\right]^{1/\hat{\beta}_3}$	(9)
Box-Cox Probit	$f(x) = \Phi\left(\beta_1 + \beta_2\frac{x^{\beta_3} - 1}{\beta_3}\right)$	[15,16]	$x = \left[\frac{\hat{\beta}_3}{\hat{\beta}_2}\cdot(\Phi^{-1}(Y) - \hat{\beta}_1) + 1\right]^{1/\hat{\beta}_3}$	(10)

[a] $\Phi(y)$ is the cumulative normal (Gauss) distribution, meaning that the probability of a standard normal random variable is less than y, and Φ^{-1} is the corresponding inverse. POW(t) means raise the value of 10 to the power t, and the linear predictor term η is defined as $\eta = \beta_1 + \beta_2\log_{10}(x)$. $\hat{\beta}_1$, $\hat{\beta}_2$, and $\hat{\beta}_3$ are the estimates of the unknown model parameter β_1, β_2, and β_3.

Concentration–response modelling

- Median response concentration RC_{50}
- Inhibition: IC_{50}, IC_{10}; effect: EC_{50}, EC_{10}



$$\text{Response (\%)} = \text{min} + \frac{\text{max} - \text{min}}{1 + 10^{\text{slope} \cdot (\log RC_{50} - \log C)}}$$

If max = 100%, min = 0%:

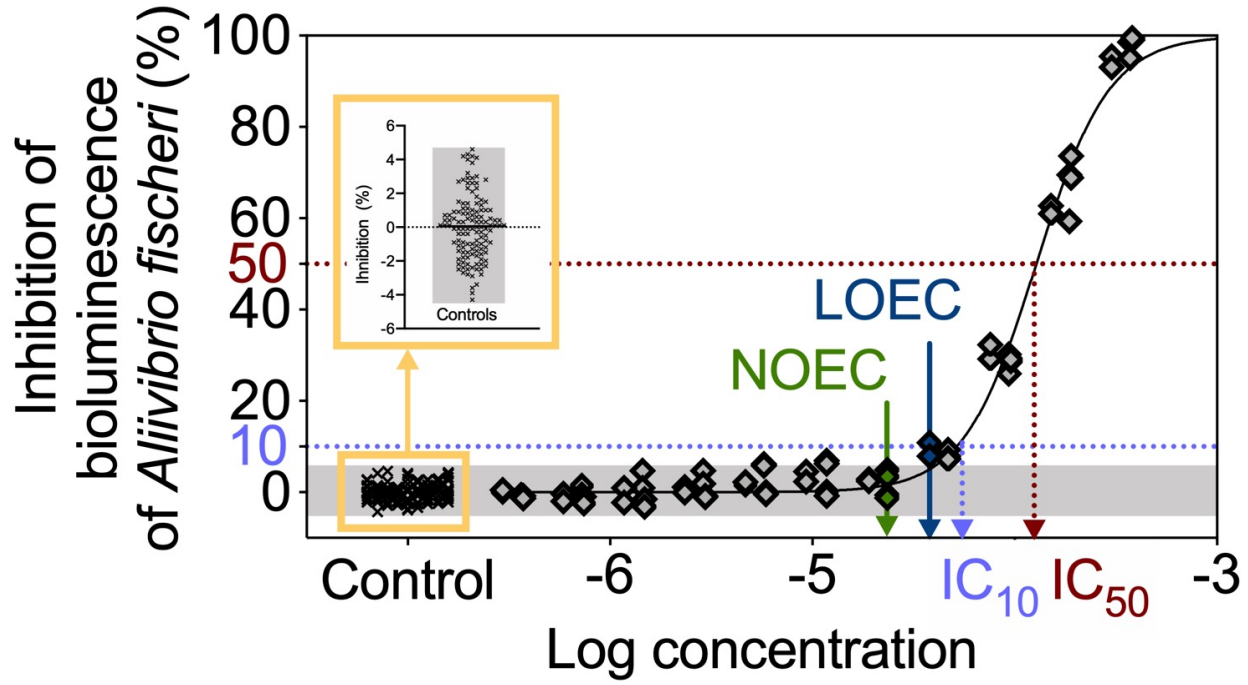
$$\text{Response (\%)} = \frac{1}{1 + 10^{\text{slope} \cdot (\log RC_{50} - \log C)}}$$

For any response level y:

$$\log RC_y = \log RC_{50} - \frac{1}{\text{slope}} \cdot \log\left(\frac{\text{max} - \text{min}}{y - \text{min}} - 1\right)$$

Concentration benchmark values

- ‘lowest observed effect concentration ’ (LOEC): lowest concentration tested that produce a significant deviation from the control
- ‘no observed effect concentration ’ (NOEC): highest tested concentration that does not produce an effect significantly different from the control

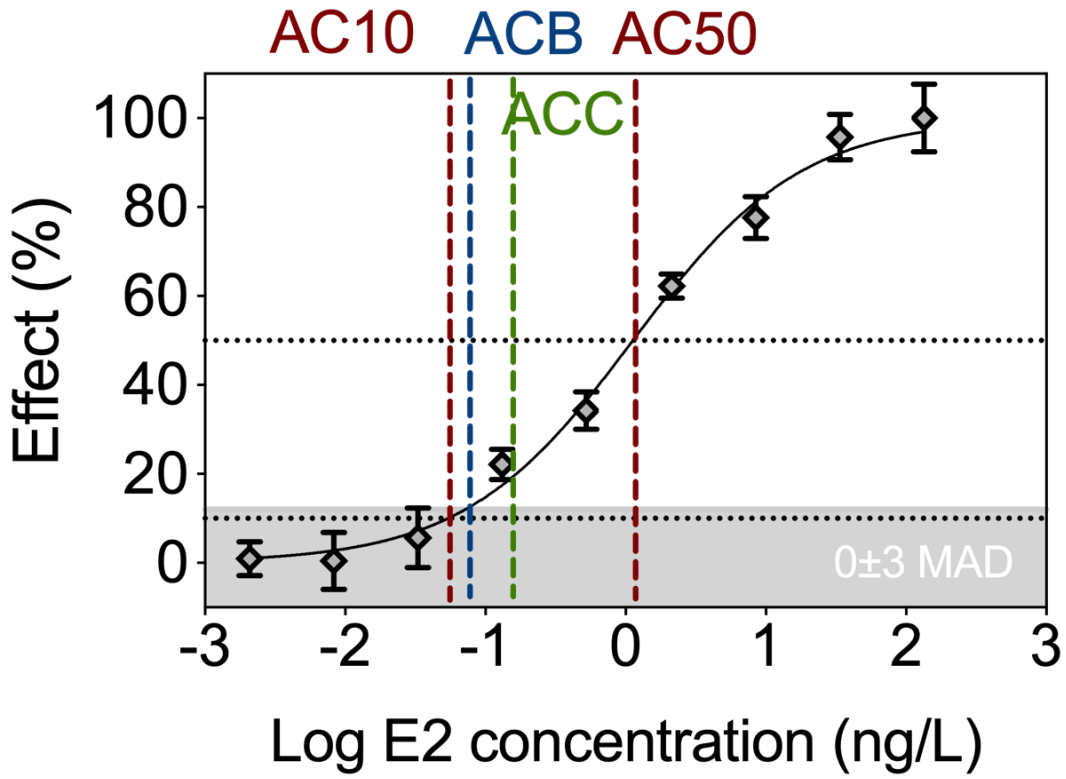


- PC_{50}: concentration of a test chemical, at which the measured activity is 50% of the maximum activity induced by the positive control (PC)

Concentration–response modelling in Tox21

- Data evaluation pipeline in R called ToxCast Analysis Pipeline (tcpl) for high-throughput screening data

- AC50 and AC10 are equivalent to EC_{50} and EC_{10}
- AC: activity concentration at cut-off (ACC) set at a user-defined cut-off (usually 15-20%).
- ACB: activity concentration at baseline: the two lowest concentration are used to derive the baseline band (BMAD) accounting for the noise of the bioassay by using three times Median Absolute Deviation (MAD) over all responses of the two lowest concentrations around the zero effect



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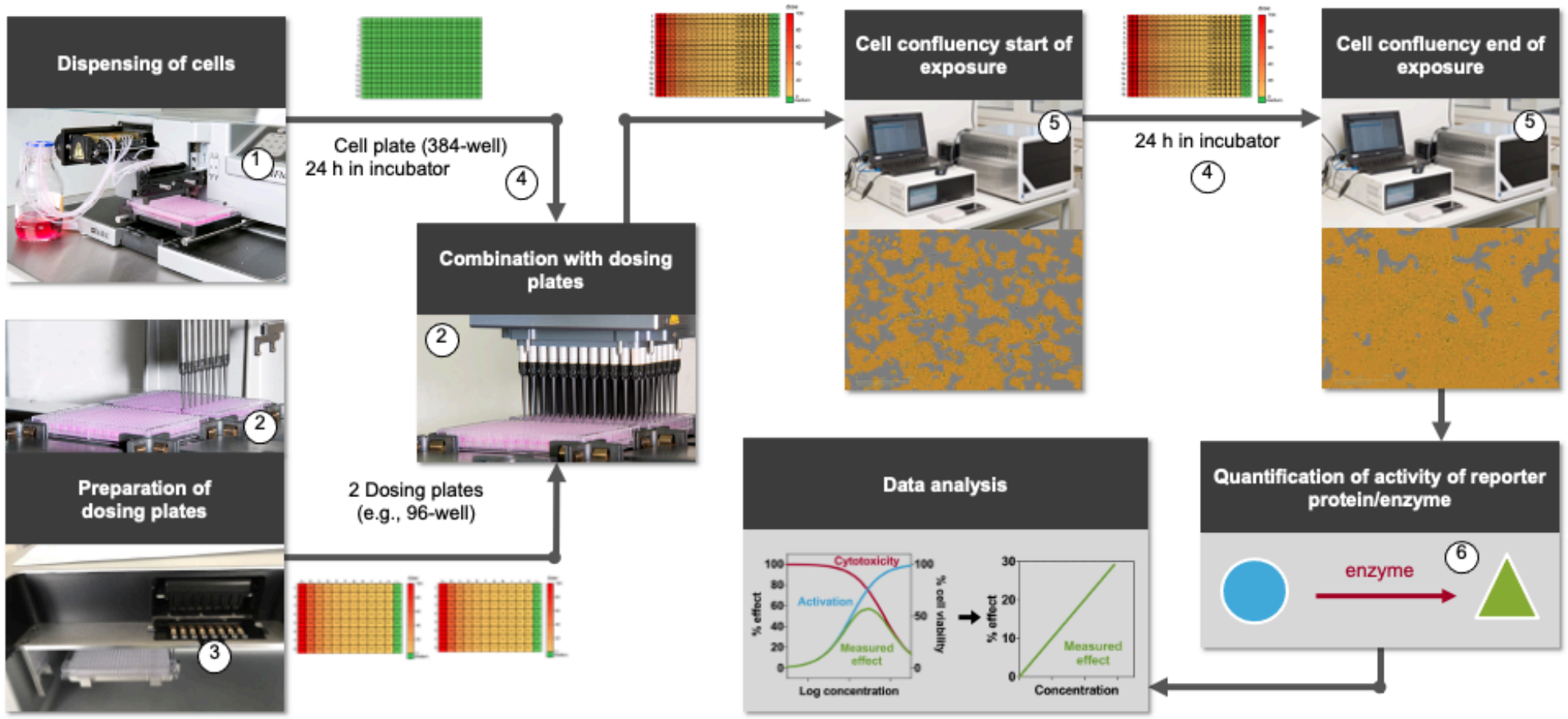
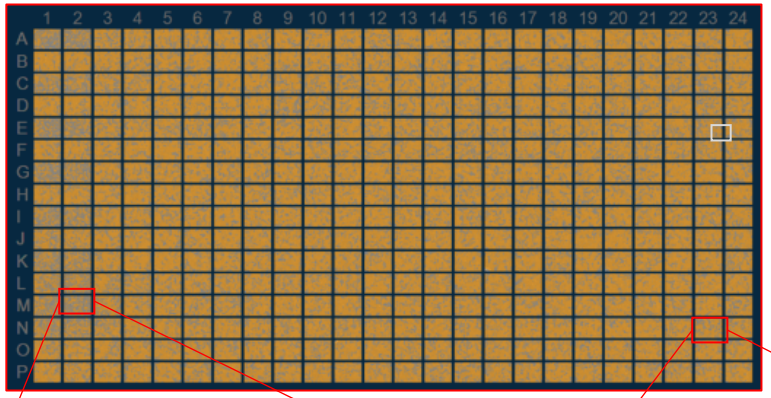
How is an *in vitro* assay run in practise?



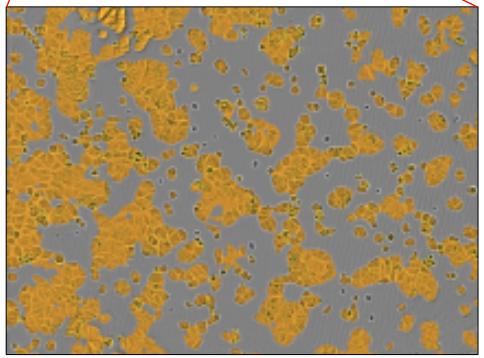
Cytotoxicity

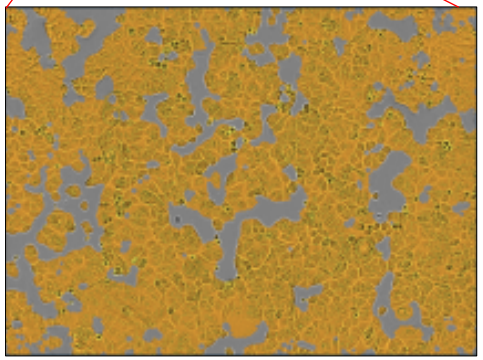
Incucyte: measurement of % confluency after 24 h of exposure



$$\text{Cell viability} = \frac{\%\ \text{confluency (exposed cells)}}{\%\ \text{confluency (unexposed cells)}}$$

$$\text{Cytotoxicity} = 1 - \frac{\%\ \text{confluency (exposed cells)}}{\%\ \text{confluency (unexposed cells)}}$$



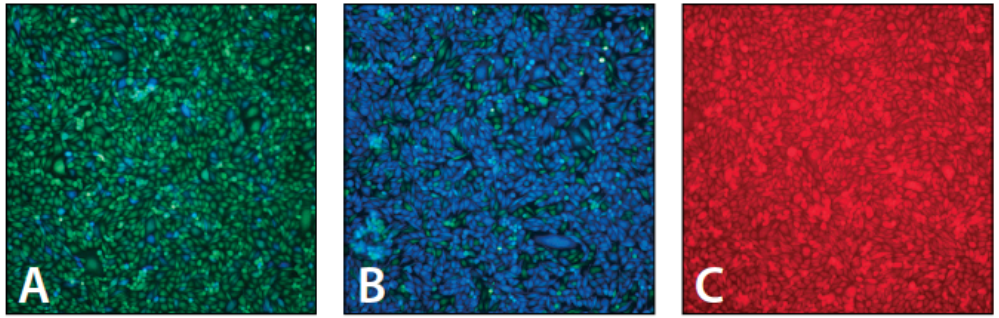


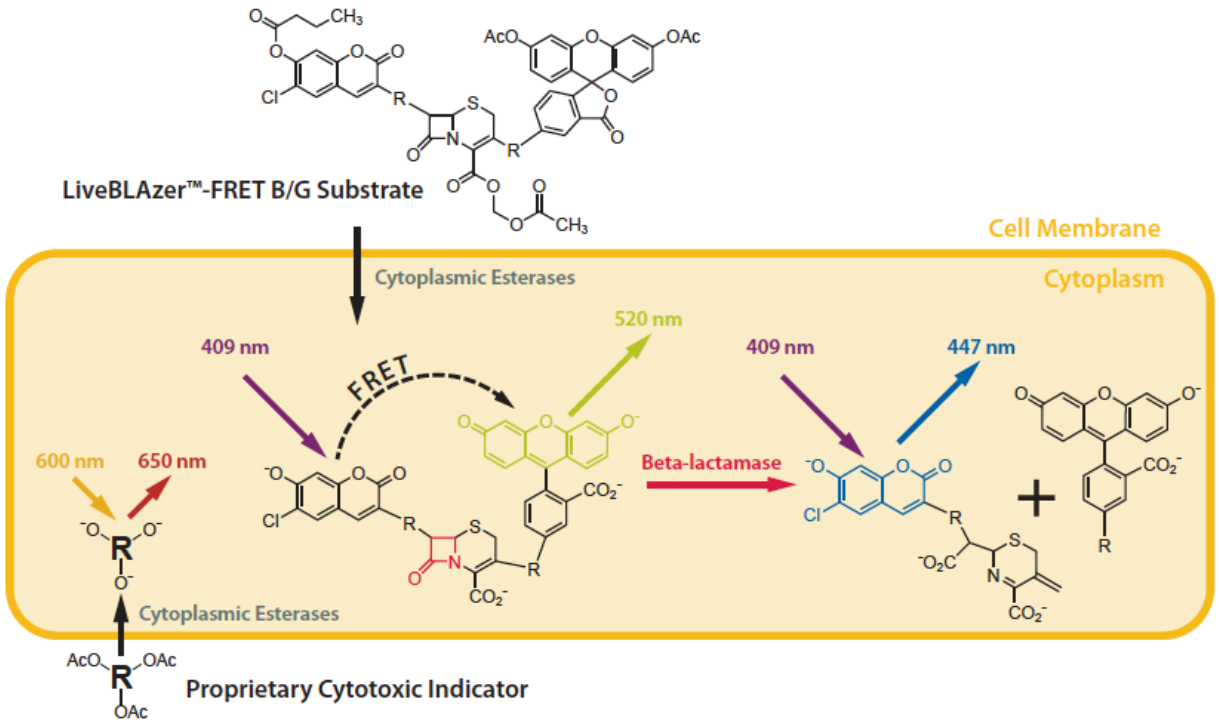
exposed cells:

- reduced cell numbers
- reduced confluency

unexposed cells

Cytotoxicity: ToxBLAzer and cell viability dyes





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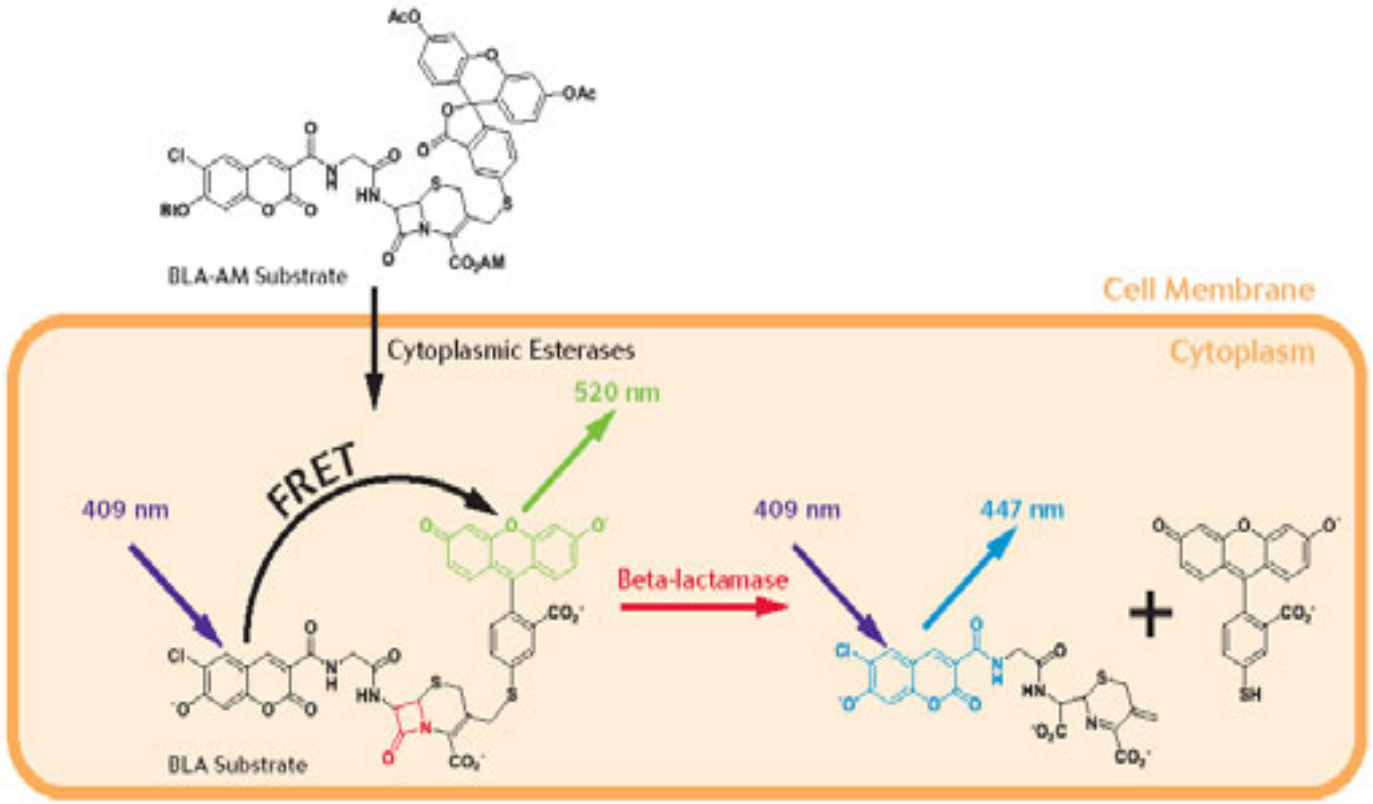
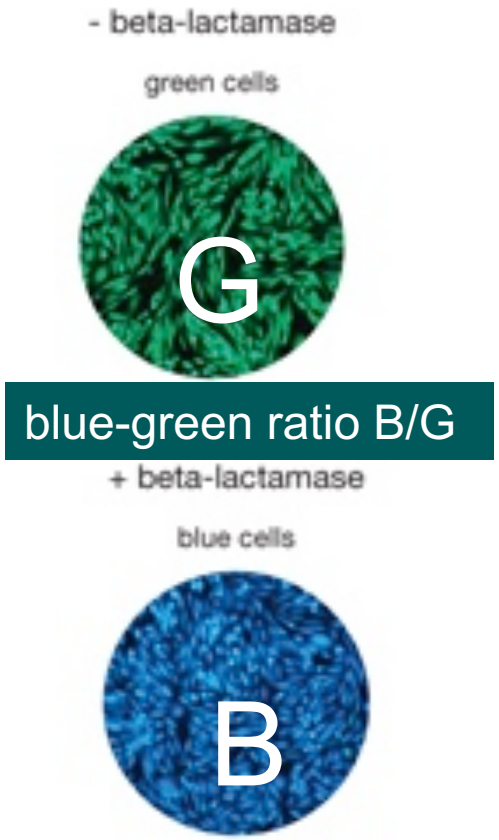
GeneBLAzer Assay – Fluorescence Measurement



http://www.biotek.com/assets/tech_resources/185/gb1.jpg

GeneBLAzer Assay – Fluorescence Measurement

Activation of the reporter gene, or more precisely production of β-lactamase, is measured by the FRET fluorescence signal, excitation at 409 nm and emission at 460 (for activated response element) and 530 (for inactive response element).
The Invitrogen protocol recommends the subtraction of cell free blanks:
For the raw data of both the measured wavelengths (460 and 530 nm) the average background (no cells, only medium and substrates) value is subtracted from each raw data point:



$$signal_{460nm} = F_{460nm}(2h) - F_{460nm}(2h, cell - free control)$$

$$signal_{530nm} = F_{530nm}(2h) - F_{530nm}(2h, cell - free control)$$

The blue-green ratio B/G is then a measure of the production of β-lactamase because the enzyme reaction product fluoresces at 460 nm and the educt produces the FRET signal at 530 nm

$$B / G = \frac{F_{460nm}(2h) - F_{460nm}(2h, cell - free control)}{F_{530nm}(2h) - F_{530nm}(2h, cell - free control)}$$

GeneBLAzer Assay – fluorescence measurement in the presence of autofluorescent chemicals

To correct for autofluorescence of the dosed chemicals (or environmental sample) the fluorescence is measured right after addition of the FRET reagent (t0)) and after the 2h incubation time and the measurement of t0 is used to make the autofluorescence correction. The term $F_{460nm}(0h, sample) - F_{460nm}(0h, average\ unexposed\ cell)$

refers to the sample's autofluorescence signal, which is the difference between the fluorescence directly after adding the FRET reagent and the additional signal caused by the autofluorescence of the sample. We have to therefore subtract the fluorescence signal of the sample with that of the unexposed cells measured at the same time, so we capture as difference only the contribution of the chemical, which is not present in the average unexposed cells. The autofluorescence signal (in square brackets []) is then subtracted from the $F_{460nm}(2h)$ and likewise for $F_{530nm}((2h)$

$$F_{530nm}(autofluorescence-corrected) = F_{530nm}(2h) - [F_{530nm}(0h, sample) - F_{530nm}(0h, average\ unexposed\ cell)]$$

$$F_{460nm}(autofluorescence-corrected) = F_{460nm}(2h) - [F_{460nm}(0h, sample) - F_{460nm}(0h, average\ unexposed\ cell)]$$

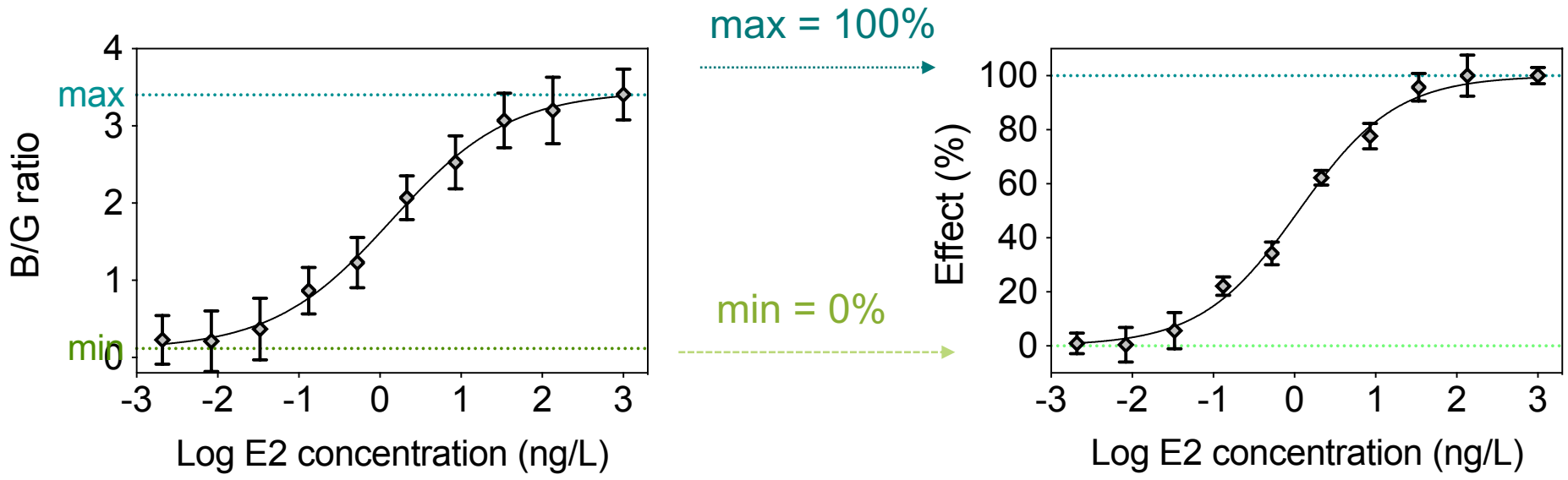
The B/G ratio is then defined as:

$$B/G = \frac{F_{460nm}(2h)_{sample} - [(F_{460nm}(0h, sample) - F_{460nm}(0h, average\ unexposed\ cell)] - F_{460nm}(2h, cell-free)}{F_{530nm}(2h) - [(F_{530nm}(0h, sample) - F_{530nm}(0h, average\ unexposed\ cell)] - F_{530nm}(2h, cell-free)}$$

This equation will become equal to equation 6 if there is no autofluorescence. In practise the value is small or close to zero if there is no autofluorescence and therefore the correction can be made routinely in all data evaluation sheets.

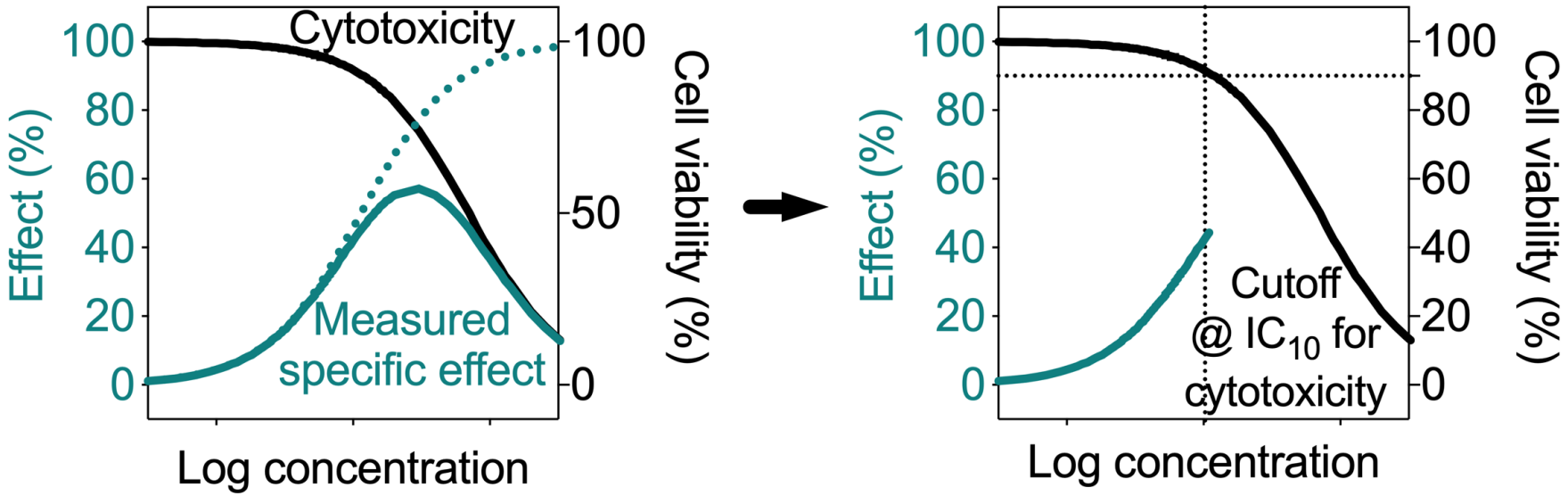
From B/G to % effect

- Set min B/G ratio as 0%
- Set max B/G ratio (max of positive control E2) as 100%



Bioassay response with cytotoxicity interference

- Activation of the reporter gene or the transactivation decreases when cells are dying
- Potentially even artifact close to cytotoxic concentrations: “cytotoxicity burst”
- Warning: Do NOT normalise responses to cell numbers!



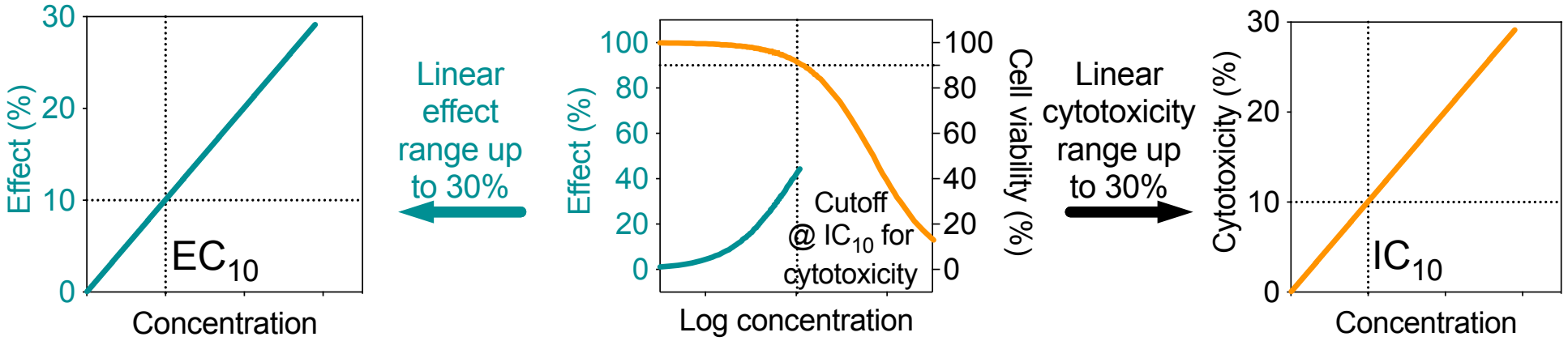
Evaluation of the linear portion of the concentration–response curve

2 Effect y = slope · concentration

$$\text{Effect concentration } EC_y = \frac{y}{\text{slope}}$$

1 Cytotoxicity (%) = slope · concentration

$$\text{Inhibitory concentration } IC_{10} = \frac{10\%}{\text{slope}}$$

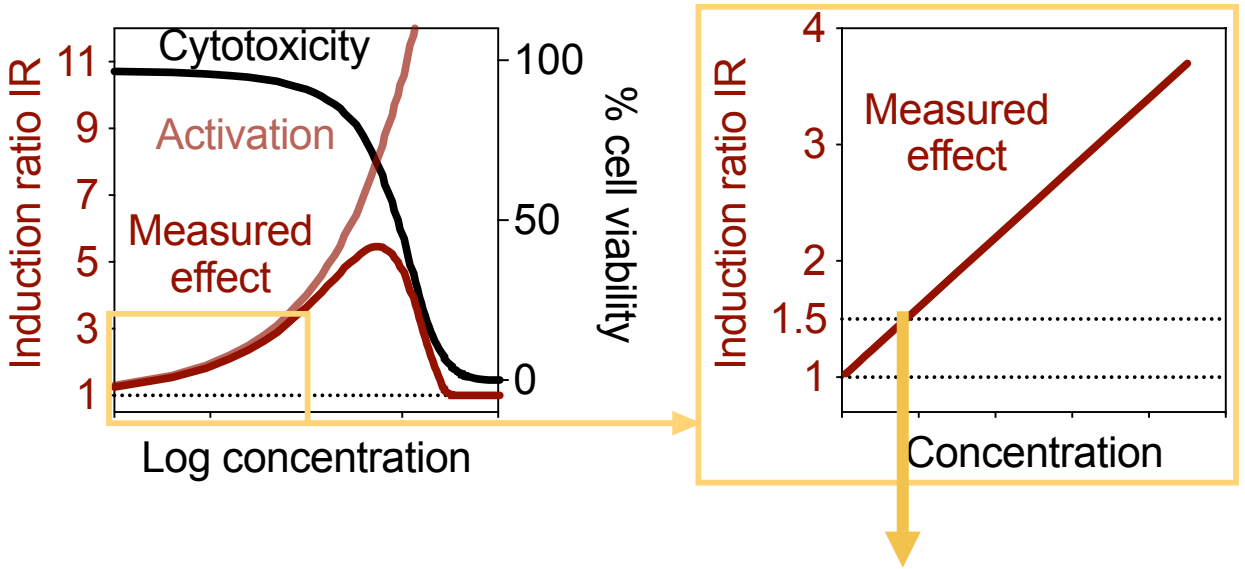


% cytotoxicity = 100% – % cell viability

Evaluation of the linear portion of the concentration–response curve: adaptive stress responses

$$IR = \frac{\text{signal}}{\text{signal (unexposed cell)}}$$

Induction ratio IR = 1 + slope · concentration



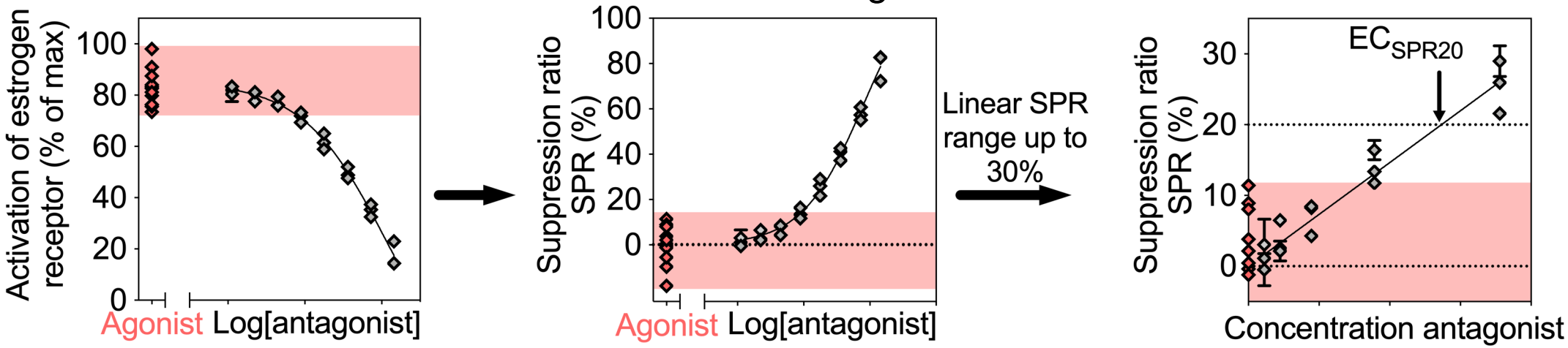
Effect concentration $EC_{IR1.5} = \frac{0.5}{\text{slope}}$

Antagonism

- All wells contain agonist in concentrations that trigger ±80% activation of receptor
- Suppression of the agonist signal by antagonist or sample is detected

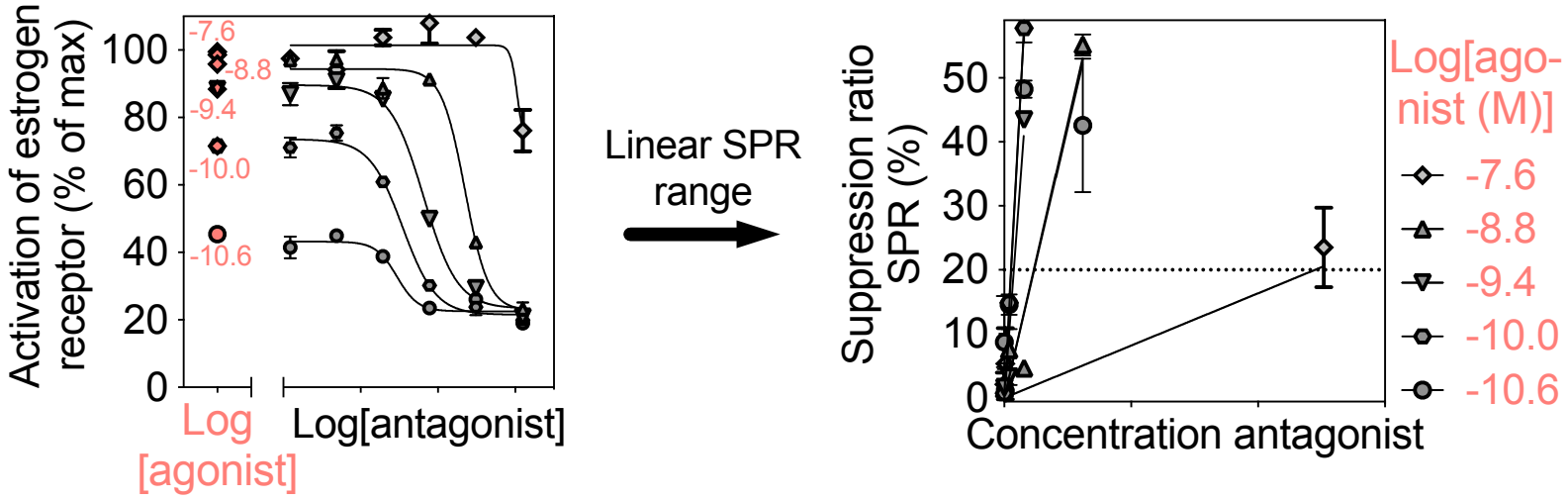
$$SPR = 1 - \frac{activation_{sample}}{activation_{agonist}}$$

$$EC_{SPR20} = \frac{20\%}{slope}$$



Antagonism: watch out for caveats

- Suppression ratio SPR is dependent on initial agonist activation (recommended 80%)
- More variable antagonistic CRCs than agonistic CRCs (hence EC_{SPR20})
- Cytotoxicity also decreases the %response relative to agonist and that cannot be differentiated from the cytotoxicity → stricter cytotoxicity cutoff (e.g., 1%)
- Coextracted natural organic matter may bind agonist and reduces its availability to receptor → apparent but false positive antagonism



$$SPR = 1 - \frac{activation_{sample}}{activation_{agonist}}$$

$$EC_{SPR20} = \frac{20\%}{slope}$$

Concentration–response curves of water samples

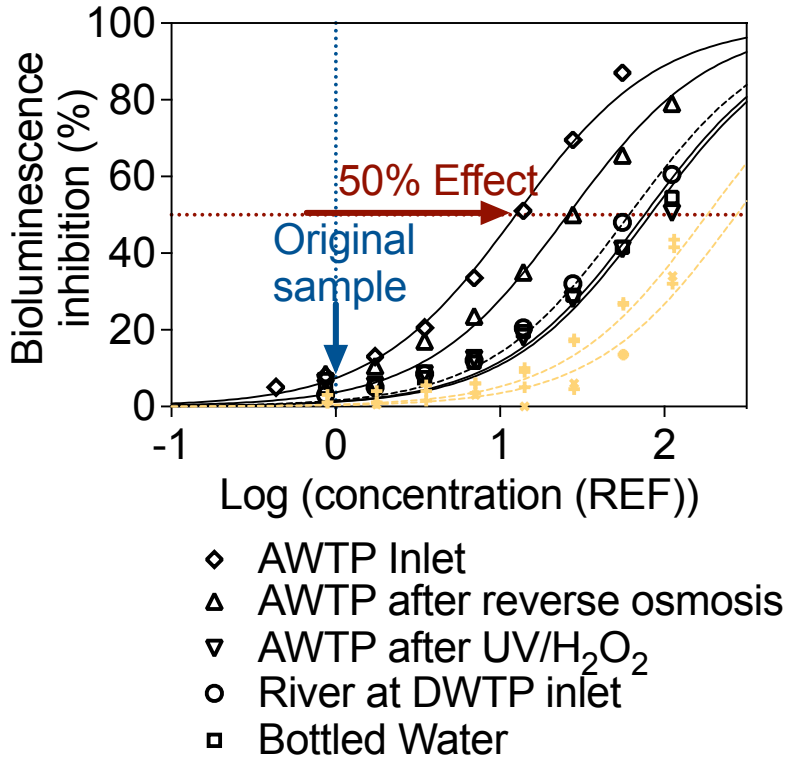
Dose-metric:

Relative extraction factor (or relative enrichment factor)

$$REF = \frac{\text{volume water extracted}}{\text{final volume in bioassay}} = EF \cdot DF \left[\frac{L_{water}}{L_{bioassay}}\right]$$

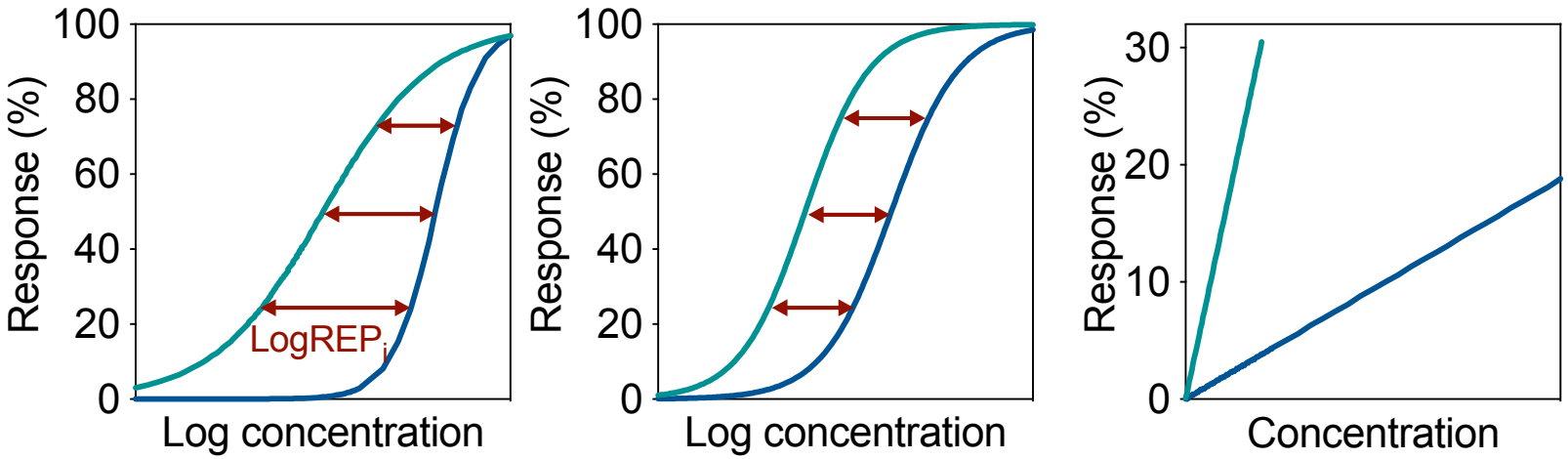
$$\text{Extraction factor } EF = \frac{\text{volume water extracted}}{\text{final volume of extract}} \left[\frac{L_{water}}{L_{extract}}\right]$$

$$\text{Dosing factor } DF = \frac{\text{volume of extract dosed}}{\text{final volume in bioassay}} \left[\frac{L_{extract}}{L_{bioassay}}\right]$$



Relative effect potency REP

$$REP_i = \frac{EC_y(reference)}{EC_y(i)}$$



$logREP_i = \log EC_y(reference) - \log EC_y(i)$

$$REP_i = \frac{slope_i}{slope_{reference}}$$

Toxic equivalency concept

Applies to cytotoxicity

- Directly derived from bioassay response

Toxic unit $TU_{bio} = \frac{1}{IC_y(sample)}$

Toxic equivalent concentration

$TEQ_{bio} = \frac{IC_y(reference)}{IC_y(sample)}$

Compare with TU from chemical analysis of detected chemicals (Chapter 8)

$TU_{chem} = \sum_{i=1}^{n} TU_i = \sum_{i=1}^{n} \frac{C_i}{IC_{y,i}}$

Bioanalytical equivalency concept

Applies to effects

- Effect unit $EU_{bio} = \frac{1}{EC_y(sample)}$
- Bioanalytical equivalent concentration

$$BEQ_{bio} = \frac{EC_y(reference)}{EC_y(sample)}$$

Optimal reference compound should be

- a chemical linked to the mode of action of the bioassay
- one of the most potent compounds in the bioassay
- likely to be present in water samples

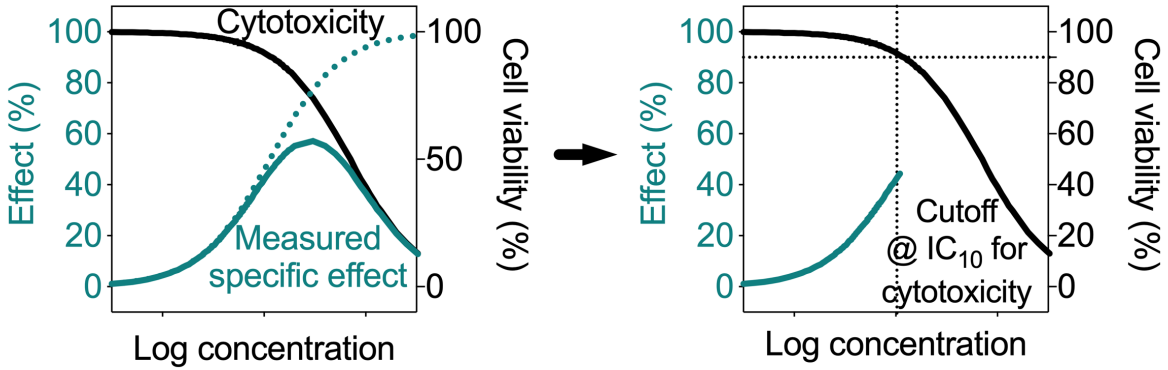
Compare with EU from chemical analysis of detected chemicals (Chapter 8)

$$EU_{chem} = \sum_{i=1}^{n} EU_i = \sum_{i=1}^{n} \frac{C_i}{EC_{y,i}}$$

And BEQ from chemical analysis (Chapter 13)

$$BEQ_{chem} = \sum_{i=1}^{n} REP_i \, C_i$$

Chapter 7
Dose-response assessment



Exercises and more material can be found at
www.ufz.de/bioanalytical-tools

Questions? please send an e-mail to bioanalytical-tools@ufz.de