Cell-based assays for protein interaction detection & quantification

Protein Interaction Biochemistry – Michael Jeltsch

Topics

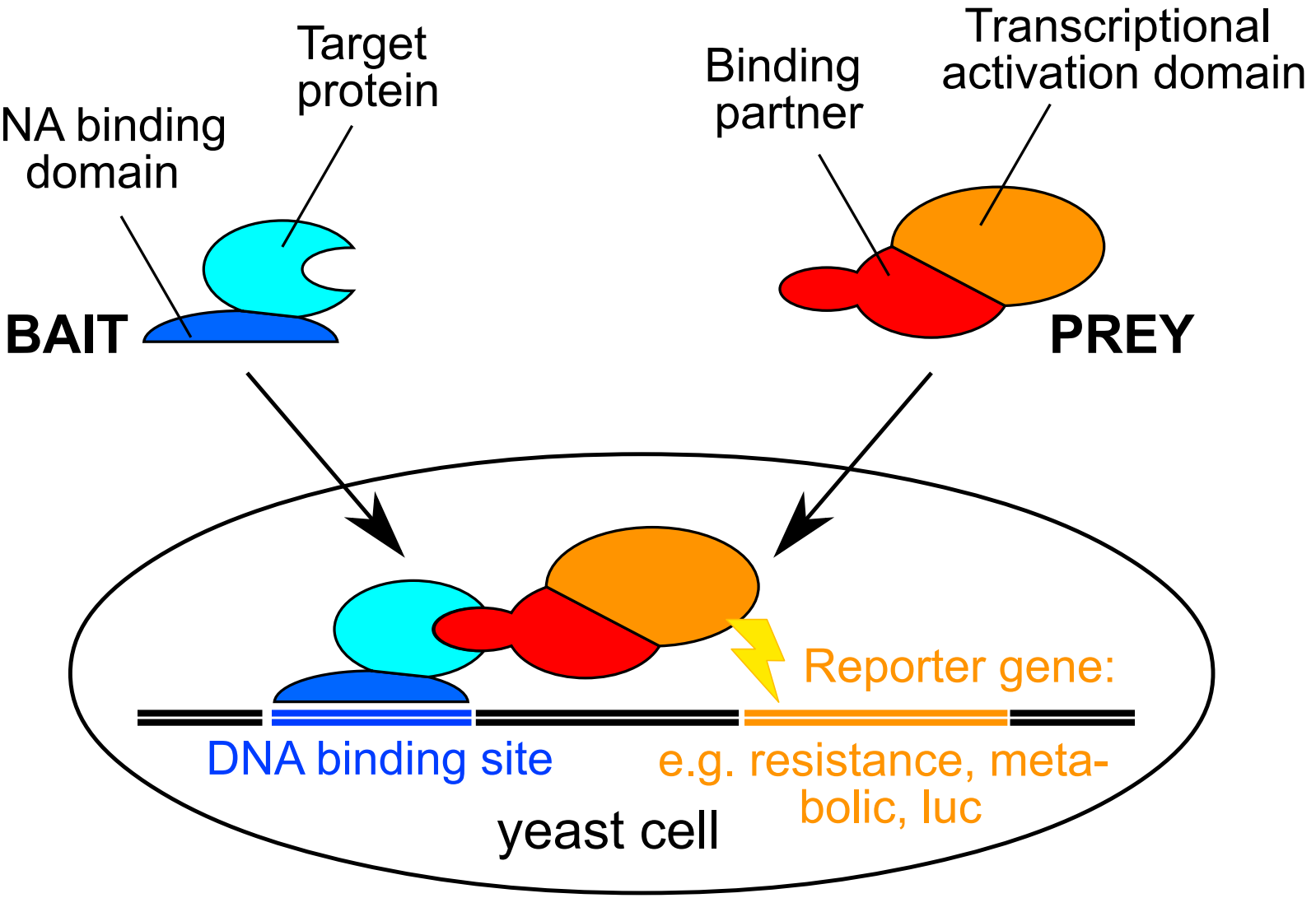
- (Yeast Two Hybrid (Y2H) System)
- PathHunter
- Ba/F3 cell line
- Illusion System

not cell-based:

- Isothermal calorimetry (ITC)

Yeast Two Hybrid (Y2H) System

- **Classic method of choice for protein interaction discovery**
- **Limitations: Interaction in yeast nucleus, false positives**



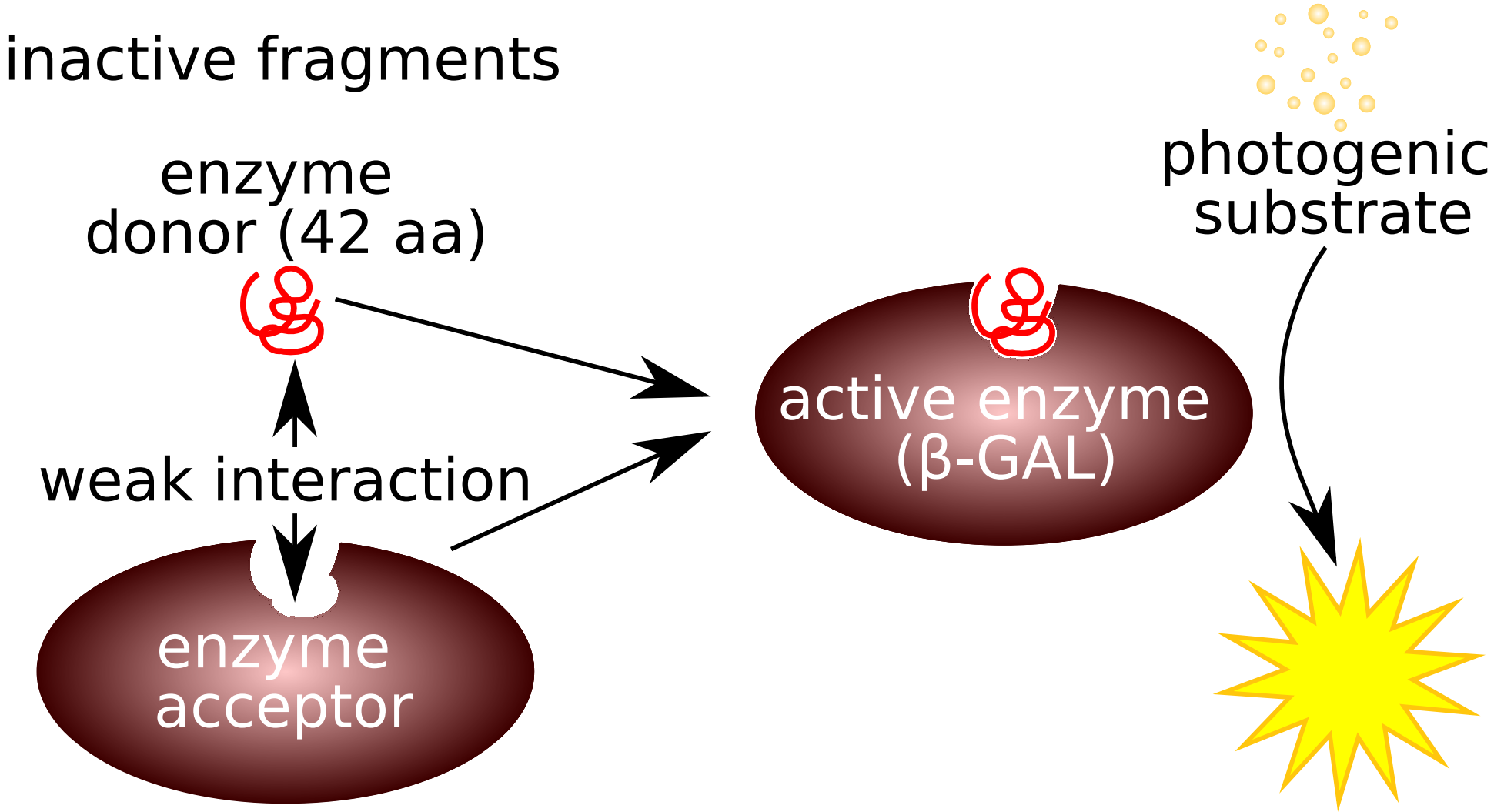
Yeast Two Hybrid (Y2H) System Developments

Year	Y2H method	Possible baits	Response	Cellular compartment *	Screen compatibility #
1989	Classic Y2H system [17]	Non-transactivating proteins capable of entering nucleus	Transcriptional activation	Nucleus	Yes [17]
1994	SOS recruitment system (SRS) [51]	Transactivating, cytosolic proteins	Ras signalling	Membrane periphery	Yes [52]
1994	Split-ubiquitin system [53]	Nuclear, membrane and cytosolic proteins	Uracil auxotrophy and 5-FoA resistance	Cytosol	Yes [54]
1998	Membrane split-ubiquitin system (MbY2H) [55]	Membrane proteins	Transcriptional activation	Membrane periphery	Yes [56]
1998	Ras recruitment system (RRS) [57]	Transactivating, cytosolic proteins	Ras signalling	Membrane periphery	Yes [57]
1999	Dual bait system [49]	Two non-transactivating proteins capable of entering nucleus	Transcriptional activation	Nucleus	Yes [49]
2000	G-protein fusion system [58]	Membrane proteins	Inhibition of protein G signalling	Membrane periphery	No
2001	RNA polymerase III based two-hybrid (Pol III) [59]	Transactivating proteins (in the RNA polymerase II pathway)	Transcriptional activation	Nucleus	Yes [59]
2001	Repressed transactivator system (RTA) [60]	Transactivating proteins capable of entering nucleus	Inhibition of transcriptional activation	Nucleus	Yes [60–62]
2001	Reverse Ras recruitment system (rRRS) [63]	Membrane proteins	Ras signalling	Membrane periphery	Yes [63]
2003	SCINEX-P system [64]	Extracellular and transmembrane proteins	Downstream signalling & transcriptional activation	Endoplasmic reticulum (ER)	No
2004	Split-Trp system [65]	Cytosolic, membrane proteins	Trp1p activity	Cytosol	Yes (Lentze & Auerbach, unpubl.)
2007	Cytosolic split-ubiquitin system (cytoY2H) [66]	Transactivating, cytosolic proteins	Transcriptional activation	ER membrane periphery	Yes [66]

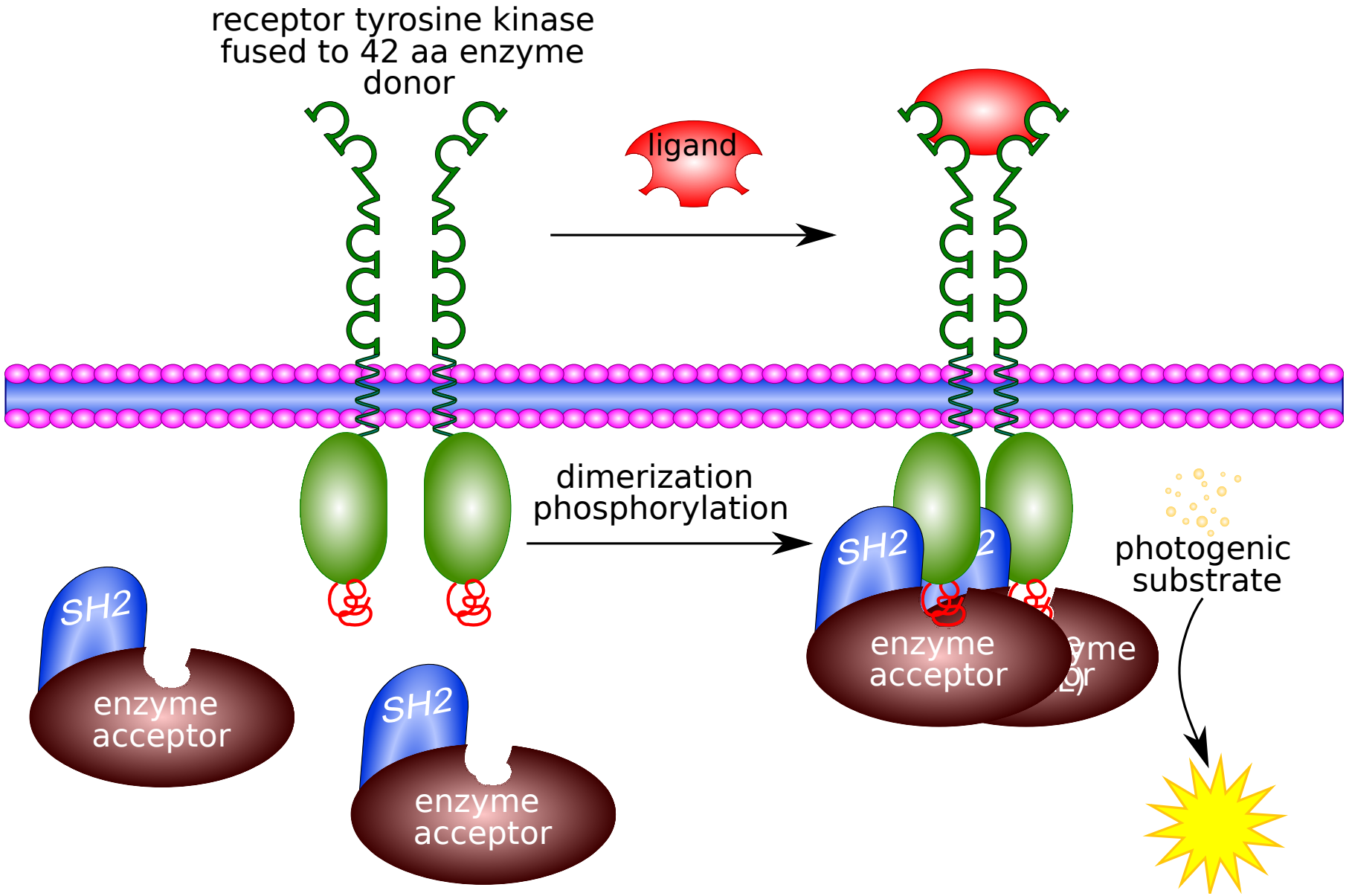
*Cellular compartment where the interaction occurs.
#Indicates whether a given Y2H system has been used for cDNA-library screening.

Table from: Brückner et al. 2009

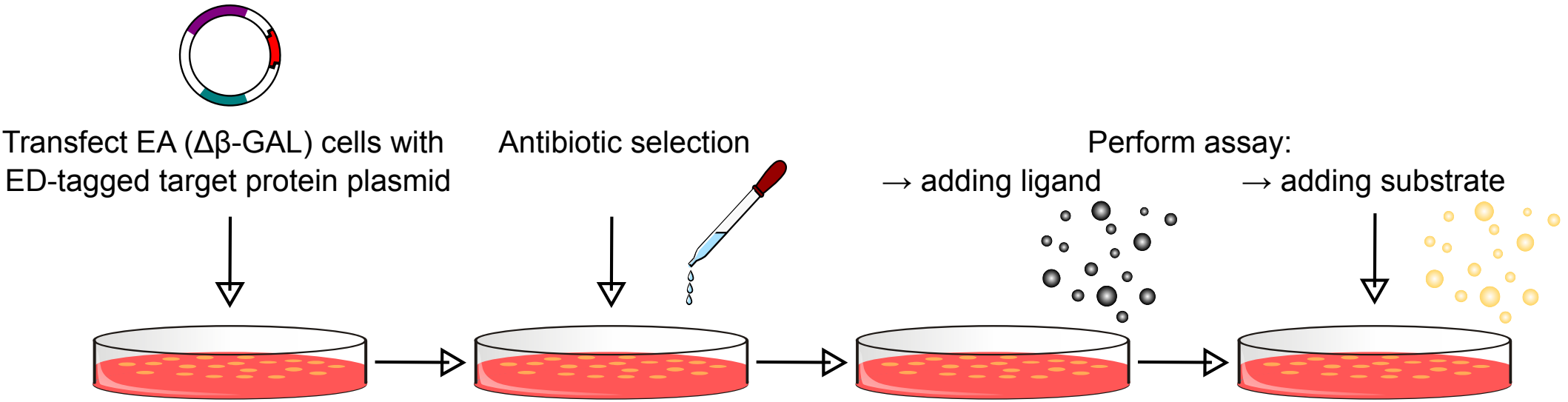
Path Hunter (by DiscoverX)

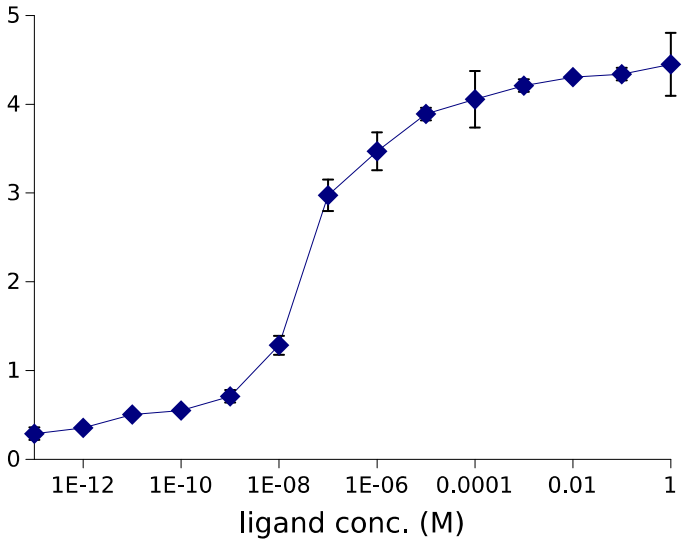


Path Hunter Example: Receptor Tyrosine Kinase



Path Hunter Workflow





Advantages of cell-based assays

Mimicking the physiological signalling...

- Low cost

Grow cells, add sample

- High-throughput

96 or 384 well plates

- Sensitive in the biologically relevant concentration range

Because they use the same or similar receptors that mediate the physiological function.

- Measures function,

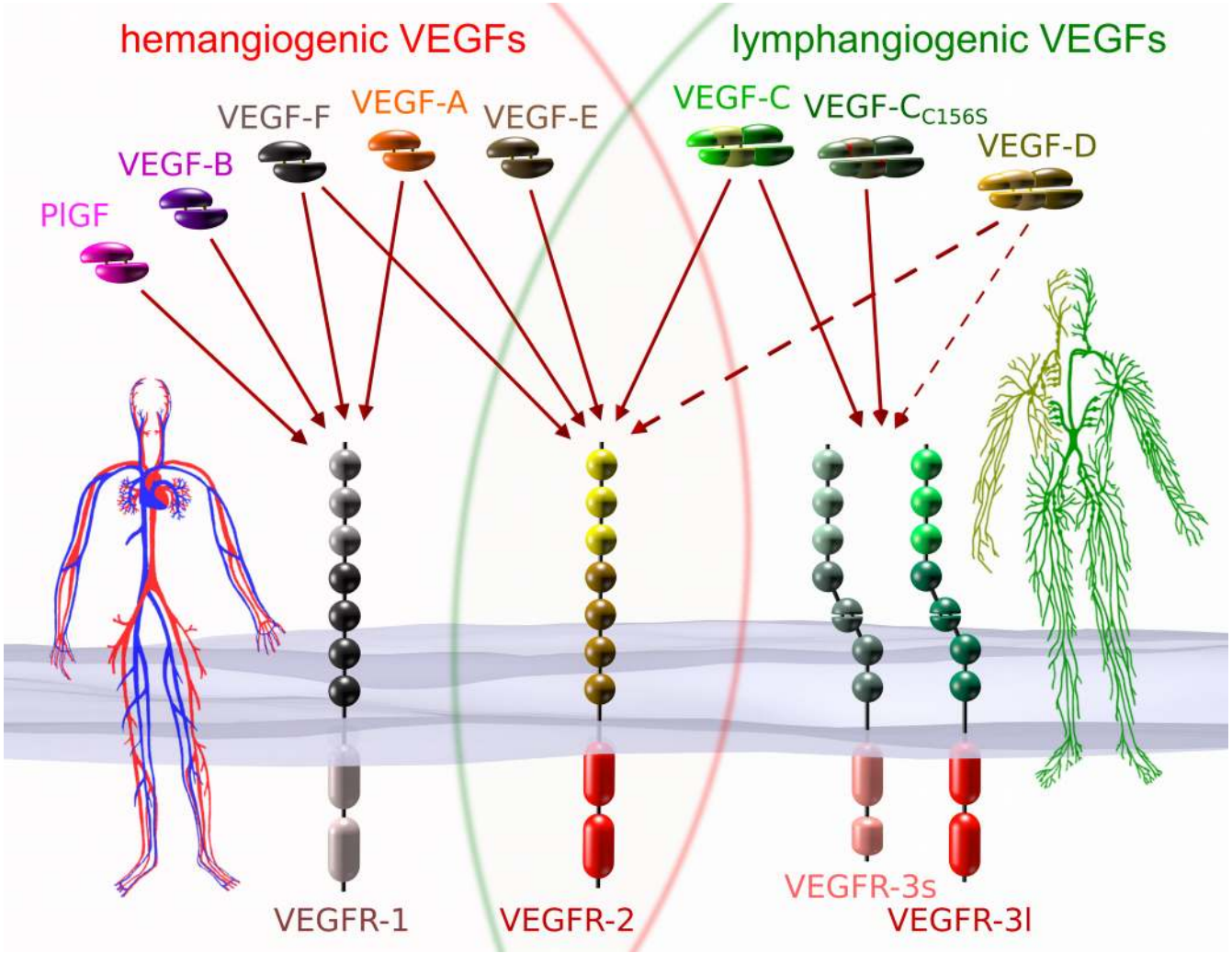
not only concentration

- High specificity despite complex protein mixtures

Similar or more specific as the physiologic molecule they are measuring

Advantages of cell-based assays

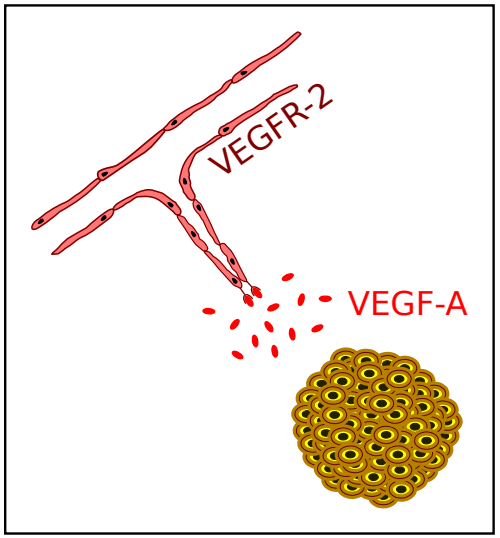
Proteins with multiple interaction partners (rather the rule than the exception)

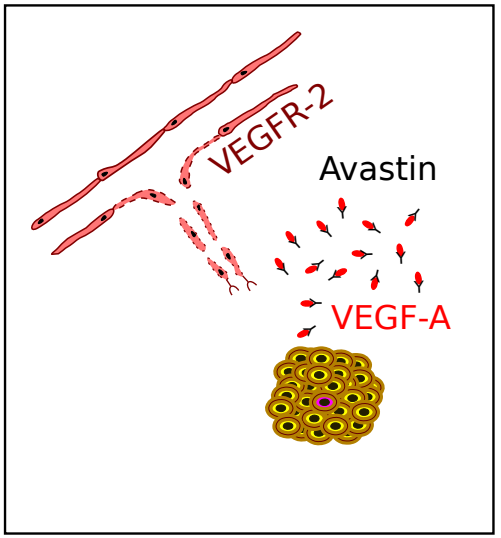


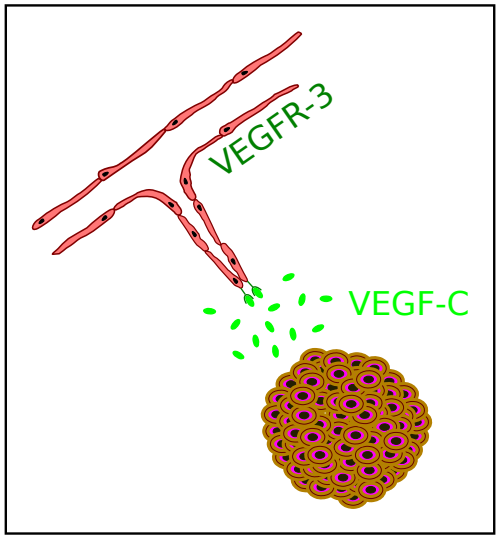
Rauniyar et al., 2018

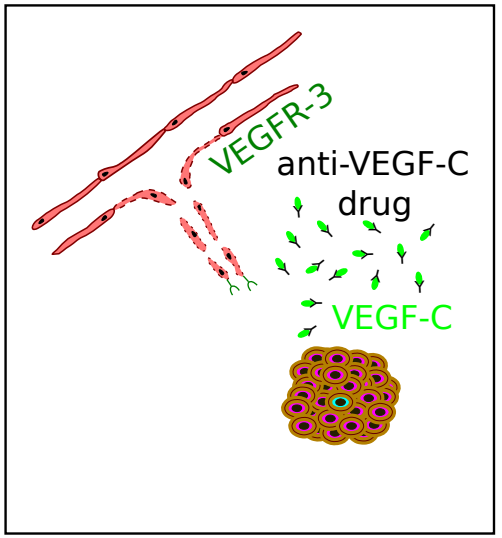
Applications of cell-based assays

- A major method to monitor biological drugs
- Measuring therapeutic antibodies
- Measuring effective concentrations









Ba/F3 cell line

Immortalized murine bone marrow-derived pro-B-cell

Proliferation depends on the presence of IL-3

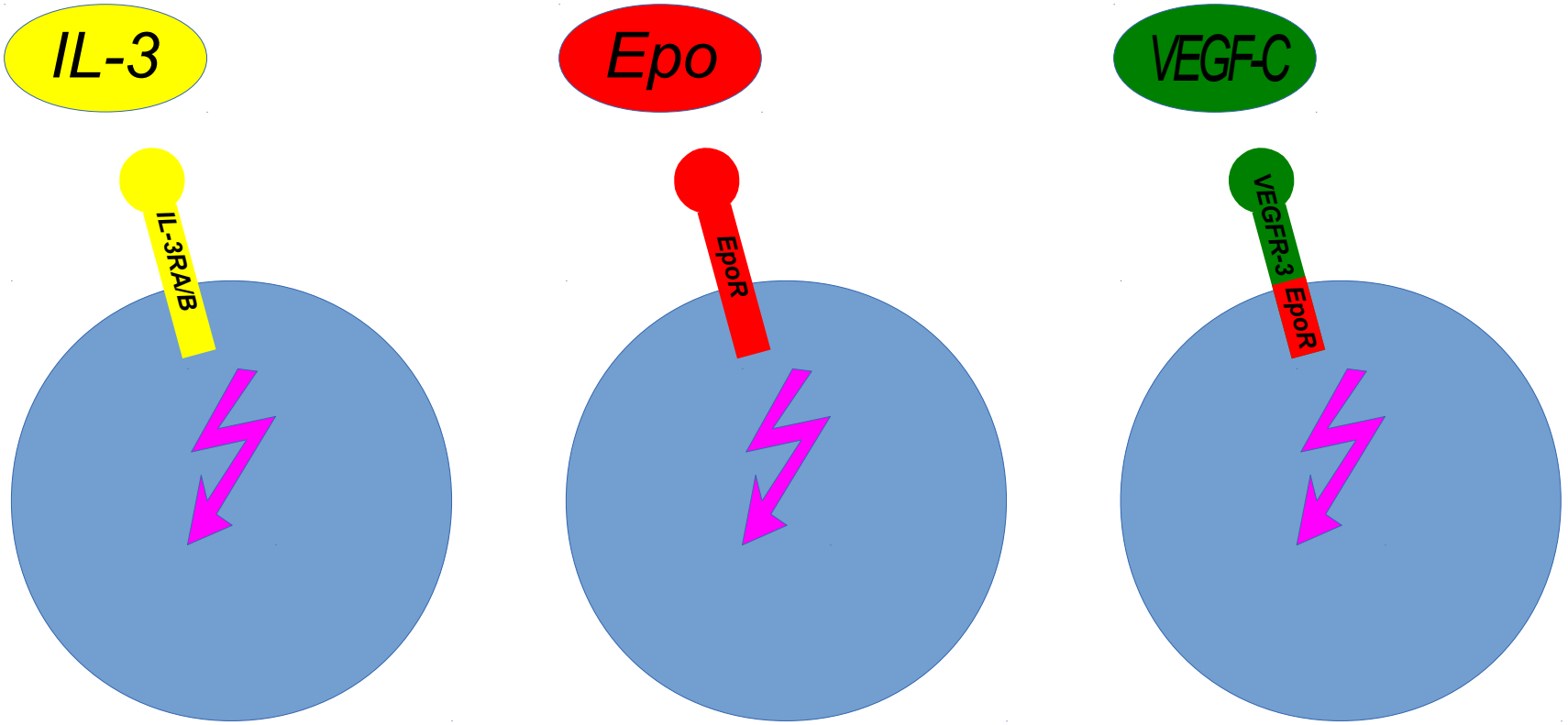
EpoR, G-CSFR, FGFR-1, c-fms can confer IL-3 independency (JAK/STAT pathway)*

Difficult to transfect: transfectants by electroporation, stable transduction with retrovirus is possible, integration is a very rare event

*Ohashi et al. PNAS 1994, 91: 158-162; Kawahara et al. J Biotechnol 2008, 133:154-61.

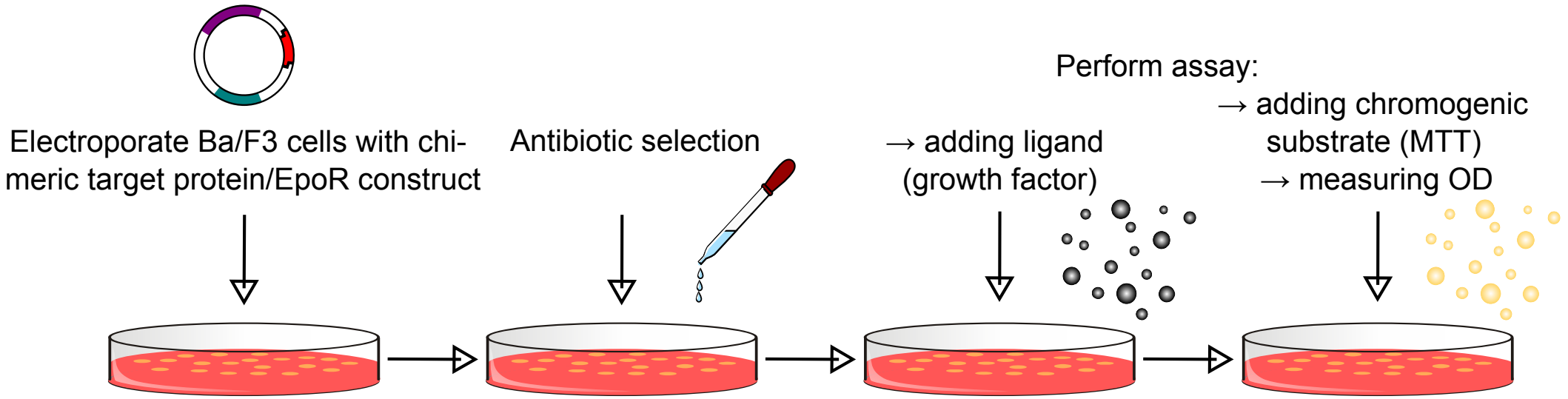
Ba/F3 cell line

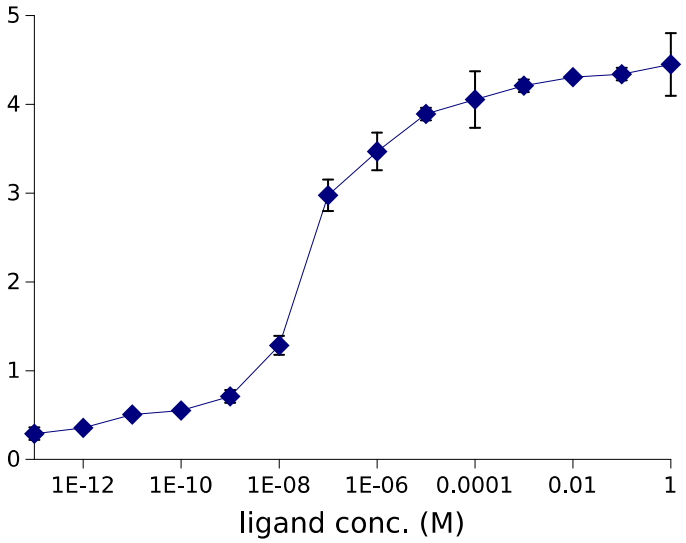
Bioassays for arbitrary growth factora are established by generating stable Ba/F3 cell lines expressing chimeric receptors



R. Palacios, M. Steinmetz, Cell 41 (1985) 727–734.
R.E. Pacifici, A.R. Thomason, J. Biol. Chem. 269 (1994) 1571–1574.

Ba/F3 assay workflow





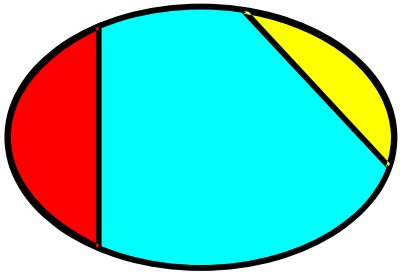
Ba/F3 assay problems #1

After prolonged culture, IL-3 independent cells will take over

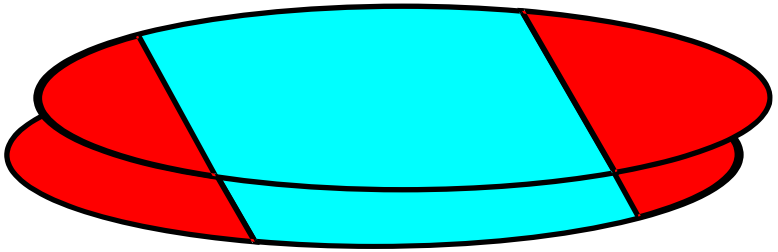
ATCC: "[...] split saturated culture 1:10 every 3 days; seed out at about 1-3 x 10^5 cells/ml; at high cell density (>2 x 10^6 cells/ml), a cytokine-independent subclone may grow out relatively quickly. CAVEAT: by favoring selective growth, suboptimal culture of cytokine-dependent cell lines may promote outgrowth of factor-independent subclones. Selective conditions include cytokine insufficiency and cell density."

Ba/F3 assay problems #2

IL-3 and erythropoietin are monomeric 4-α-helix bundle cytokines: two structurally completely different binding (non-symmetric) epitopes on the monomeric molecule.

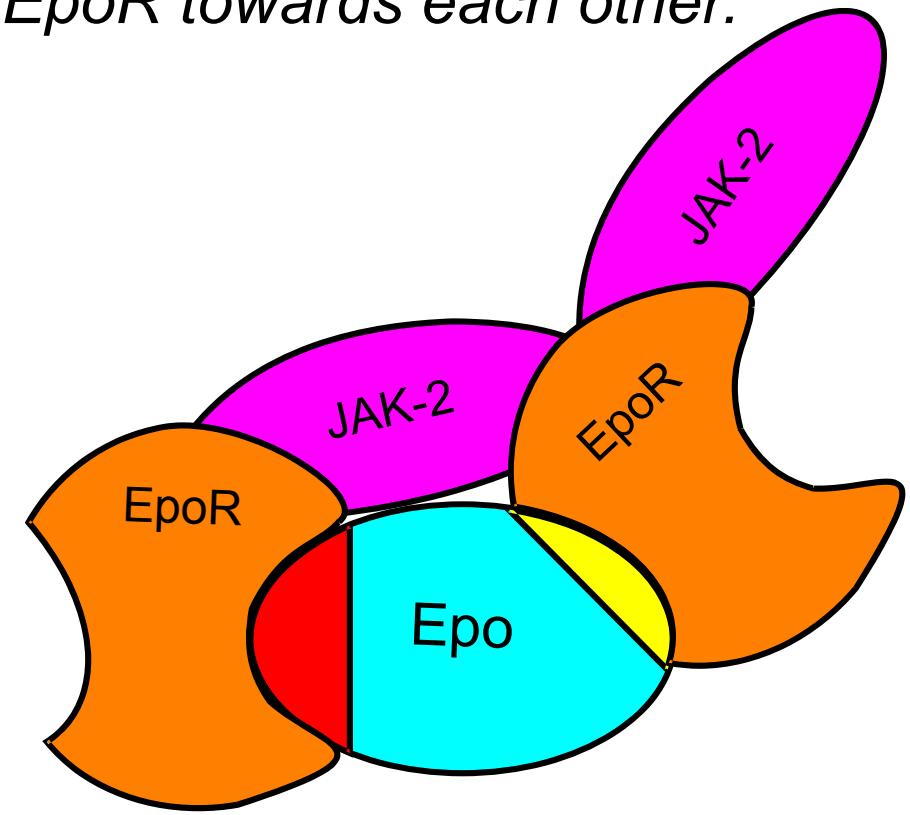


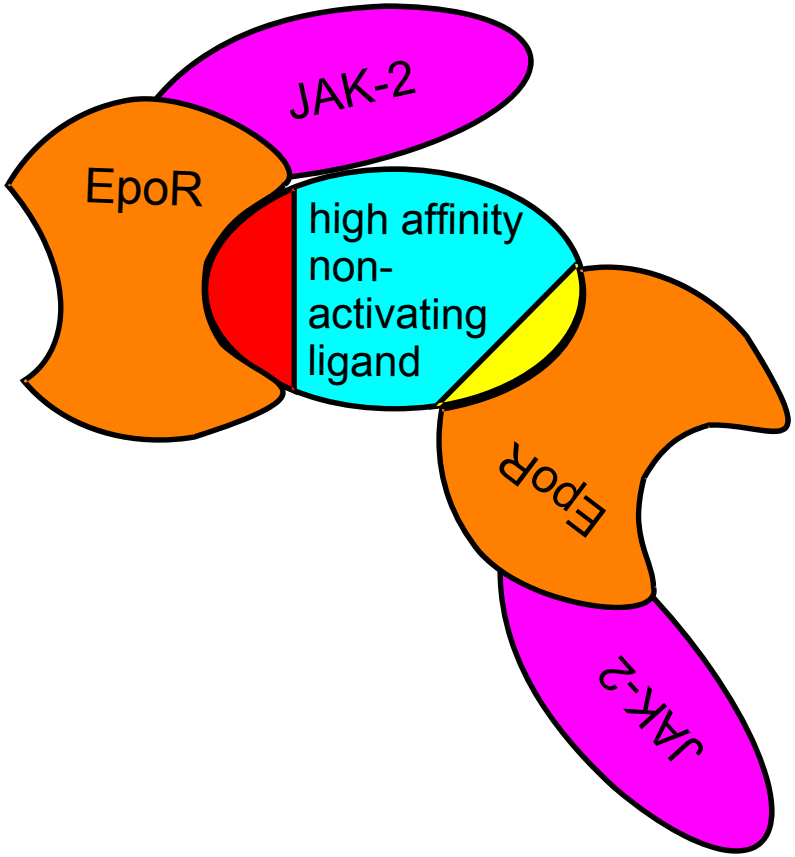
VEGFs are dimeric molecules with two structurally similar binding epitopes, each of which is composed of parts from both monomers.



Ba/F3 assay problems #2

- *EpoR are also dimeric in the absence of ligand.*
- *Dimerisation without ligand results in a distance between the associated JAK-2 molecules that doesn't allow cross-phosphorylation*
- *STAT2 cross-phosphorylation depends on the orientation of the two EpoR towards each other.*





Ba/F3 assay problems #2

Existing junctions between VEGFR(D7) and EpoR(TMD)

```
hVEGFR-2/EpoRv1  FFIIEGAQEKT--NGSLILTLSLILVLISLLLTVLALLSHRRTLQQKIWPGIP  non-functional
hVEGFR-3/EpoR    SVAVEGSEDKGSMEGSLILTLSLILVLISLLLTVLALLSHRRTLQQKIWPGIP  functional
hVEGFR-1/EpoR    YLTVQGTSDKS--NRSLILTLSLILVLISLLLTVLALLSHRRTLQQKIWPGIP  functional
```

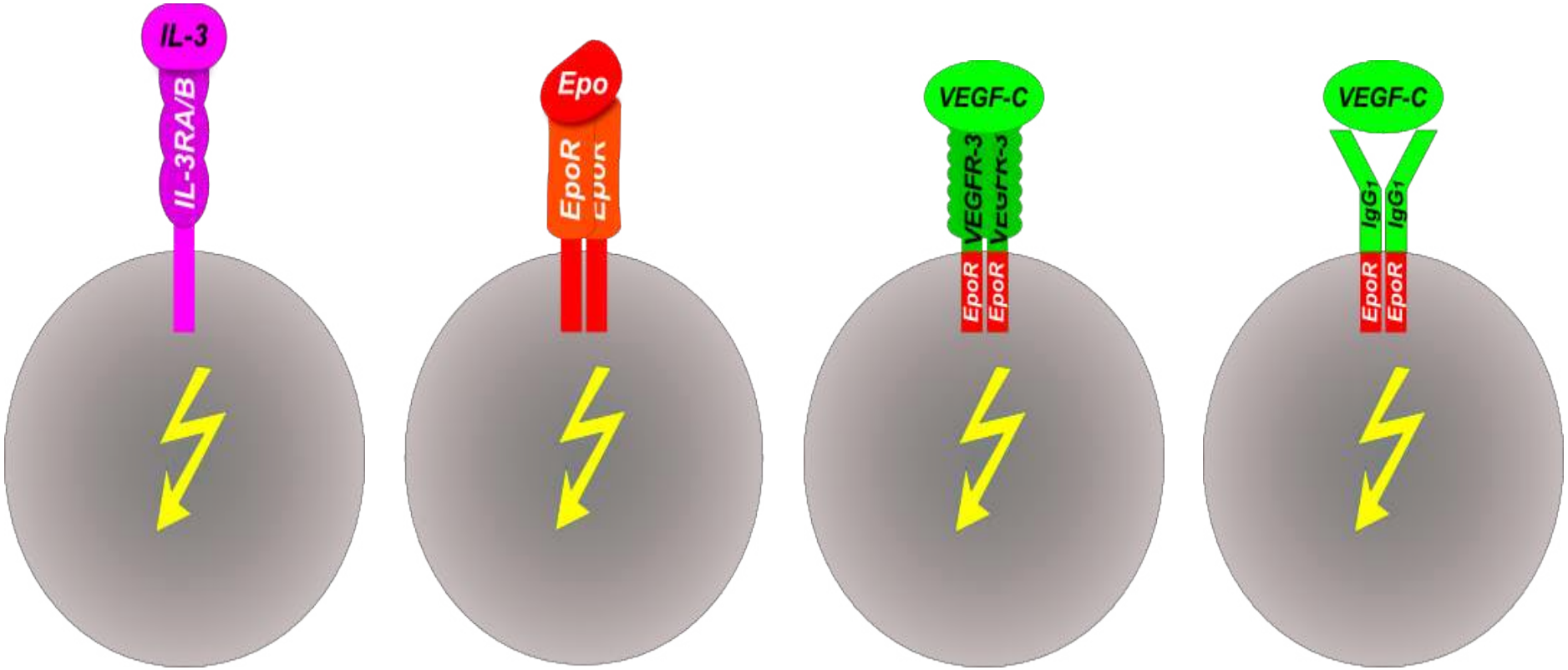

VEGFR (extracellular domain) | EpoR TMS | EpoR (intracellular domain)

Redesigned junctions

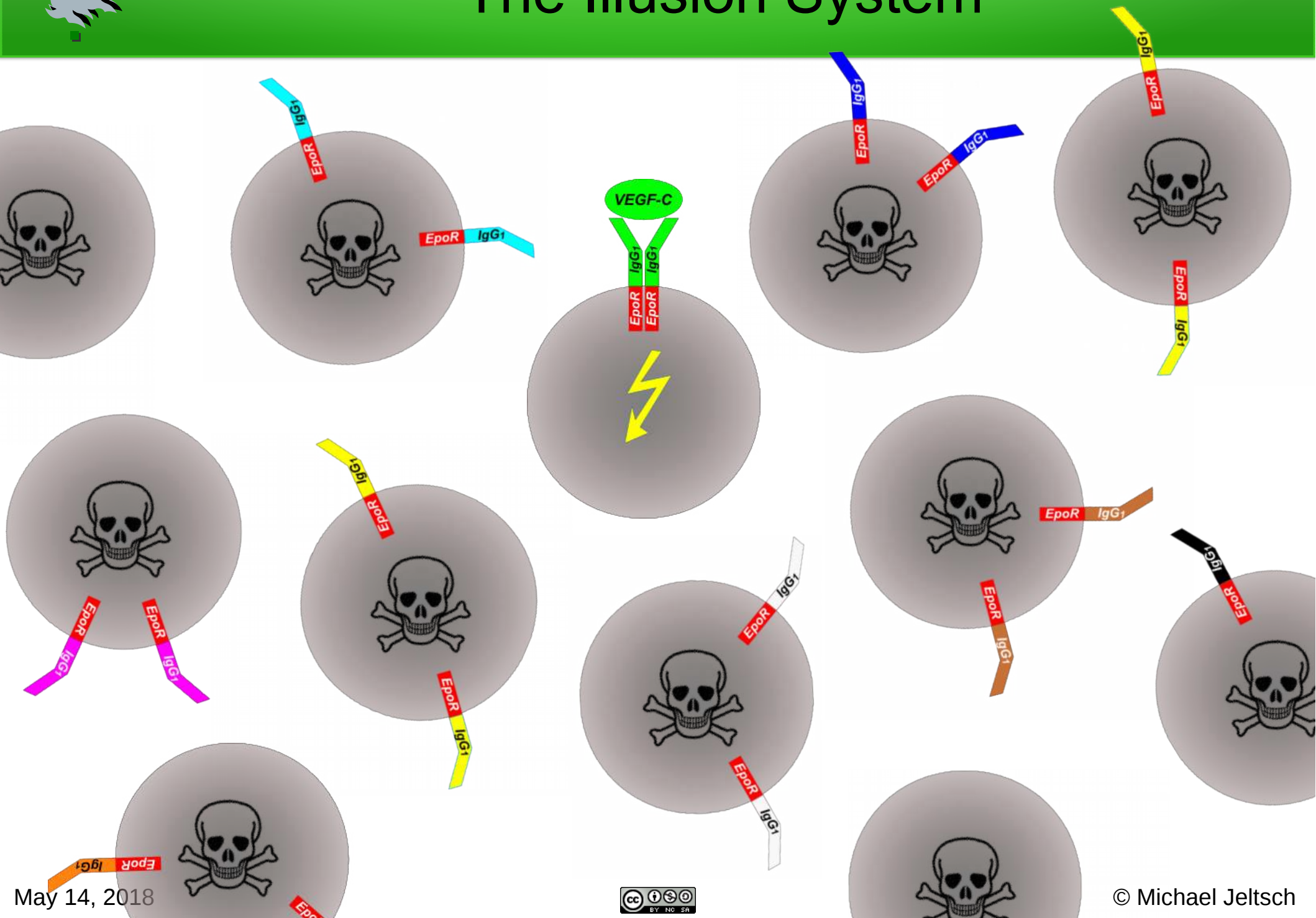
```
hVEGFR-2/EpoRv2  FFIIEGAQEKT--NRSLILTLSLILVLISLLLTVLALLSHRRTLQQKIWPGIP  non-functional
hVEGFR-2/EpoRv3  FFIIEGAQEKTNLEGSLILTLSLILVLISLLLTVLALLSHRRTLQQKIWPGIP  functional
```

The Illusion System

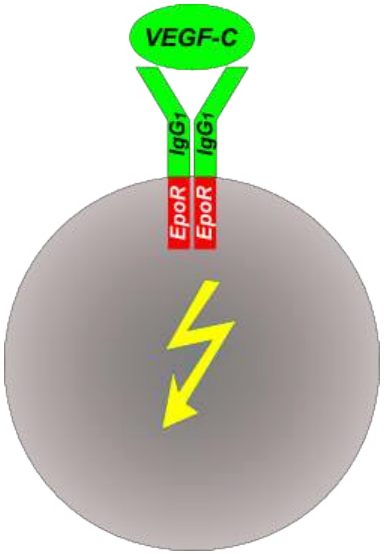
The Illusion system: Using a cell-based assay to screen antibody libraries



The Illusion System



The Illusion System



The Illusion System

Mammalian cells (Ba/F3)

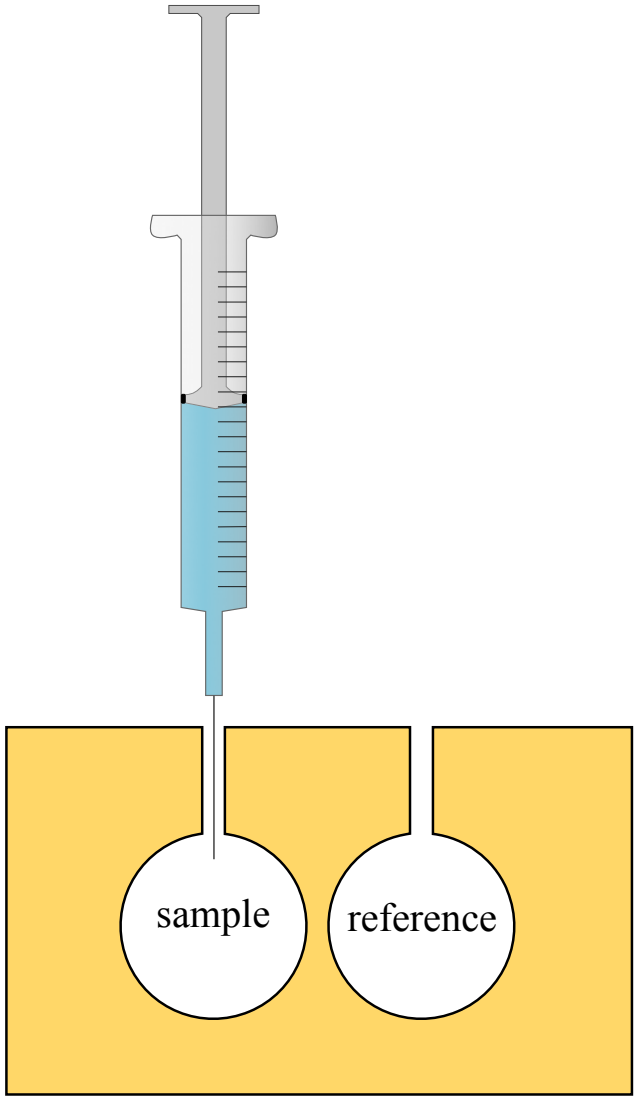
E. coli

Doubling time	1 day	25 min
Time to clonality	~6 weeks	2 days
Cell size	~10µm	~1µm
Library size (½l culture)	~5 · 10^8	~1 · 10^{11}
Transfectability	none	easy
Escape mutants	frequent	none

Isothermal calorimetry (ITC)

- Principle: Measuring the released heat when mixing two reactants
- One of the few “true” label-free systems (in most other “label-free” systems, a surface replaces the label)
- “Gold standard”
- Practical Course: Purification and Characterization of Recombinant Proteins (DPBM-135): https://jeltsch.org/flpc_itc

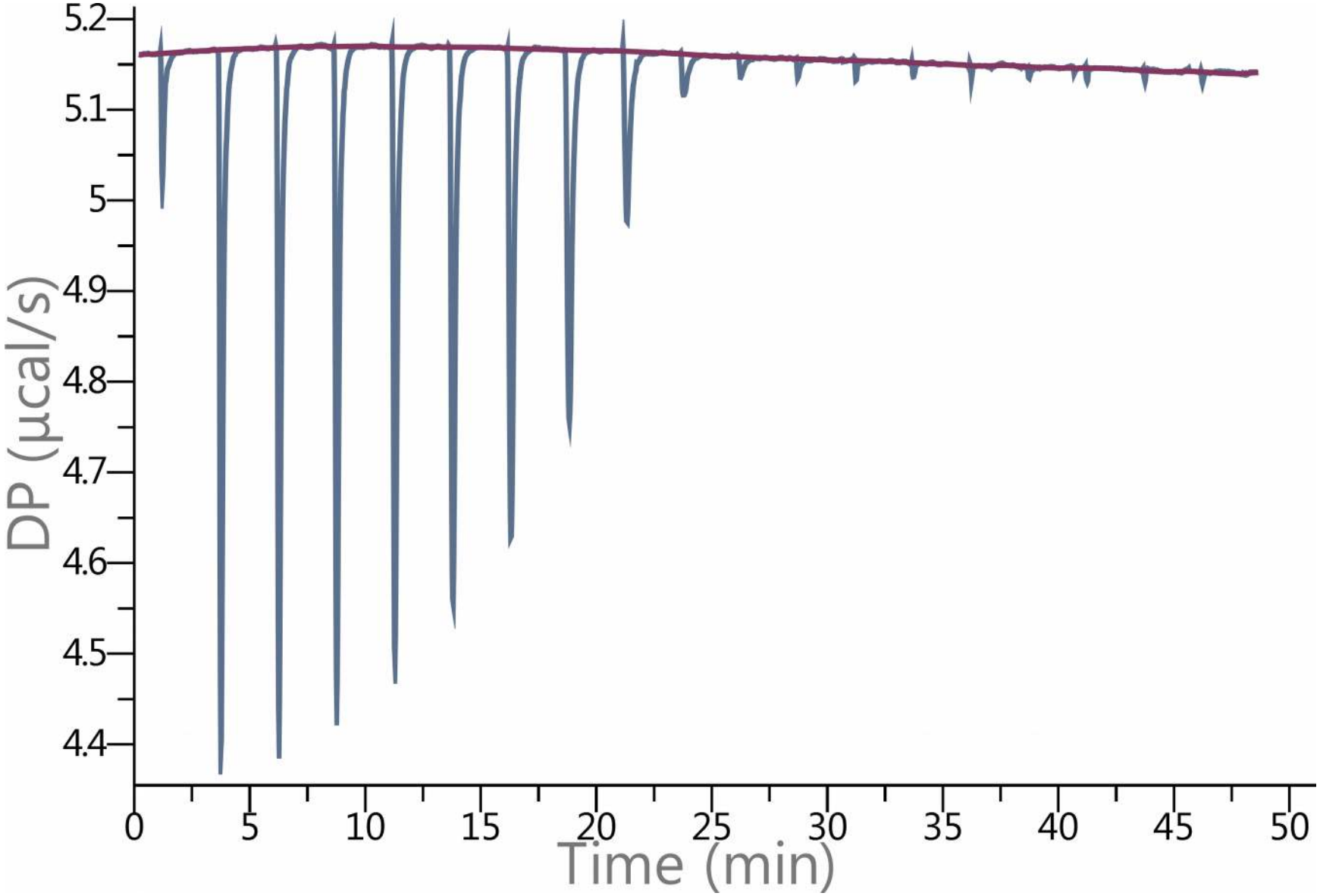
Isothermal calorimetry (ITC)



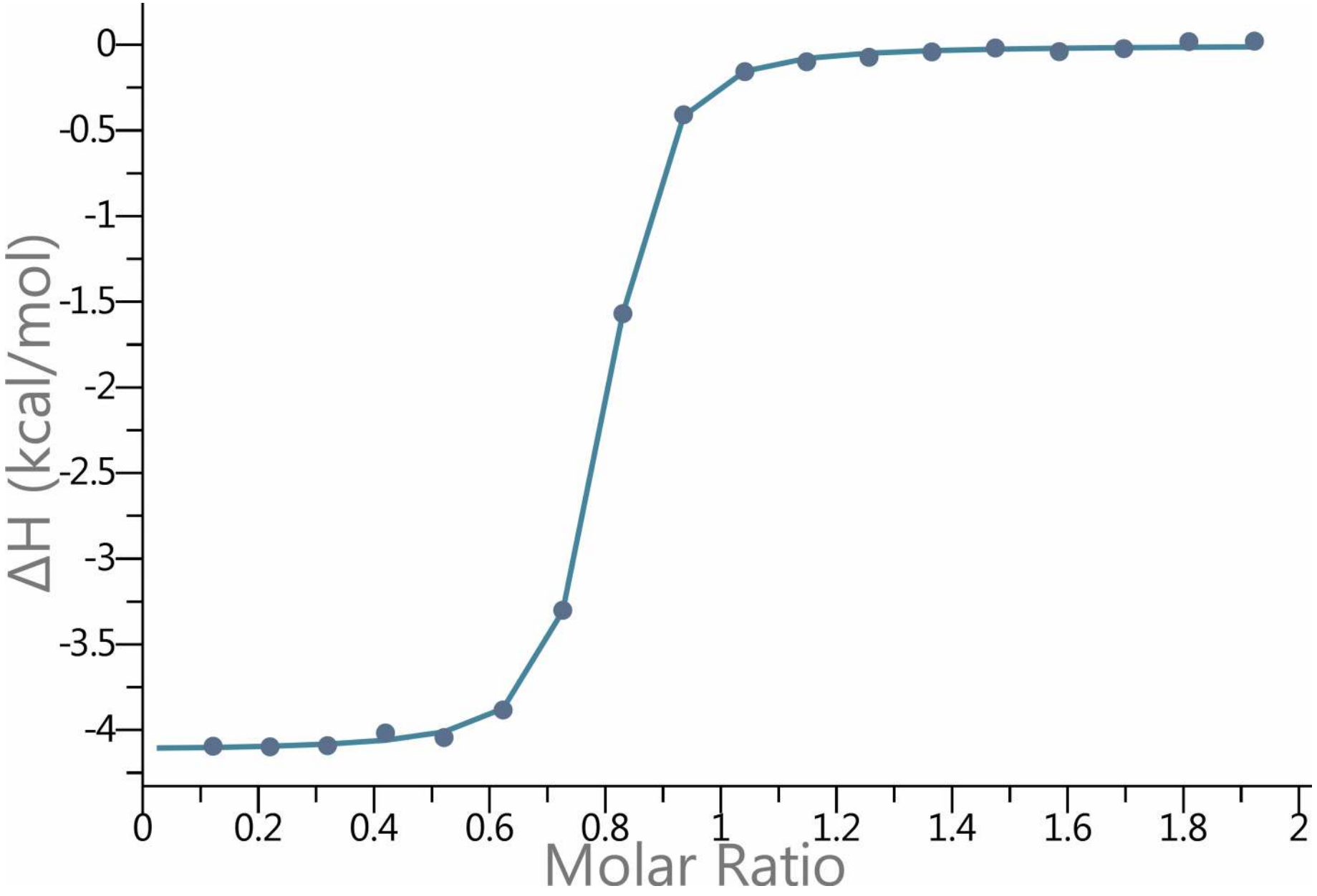
Main components:

- Syringe
- Sample cell
- Reference cell
- Insulation

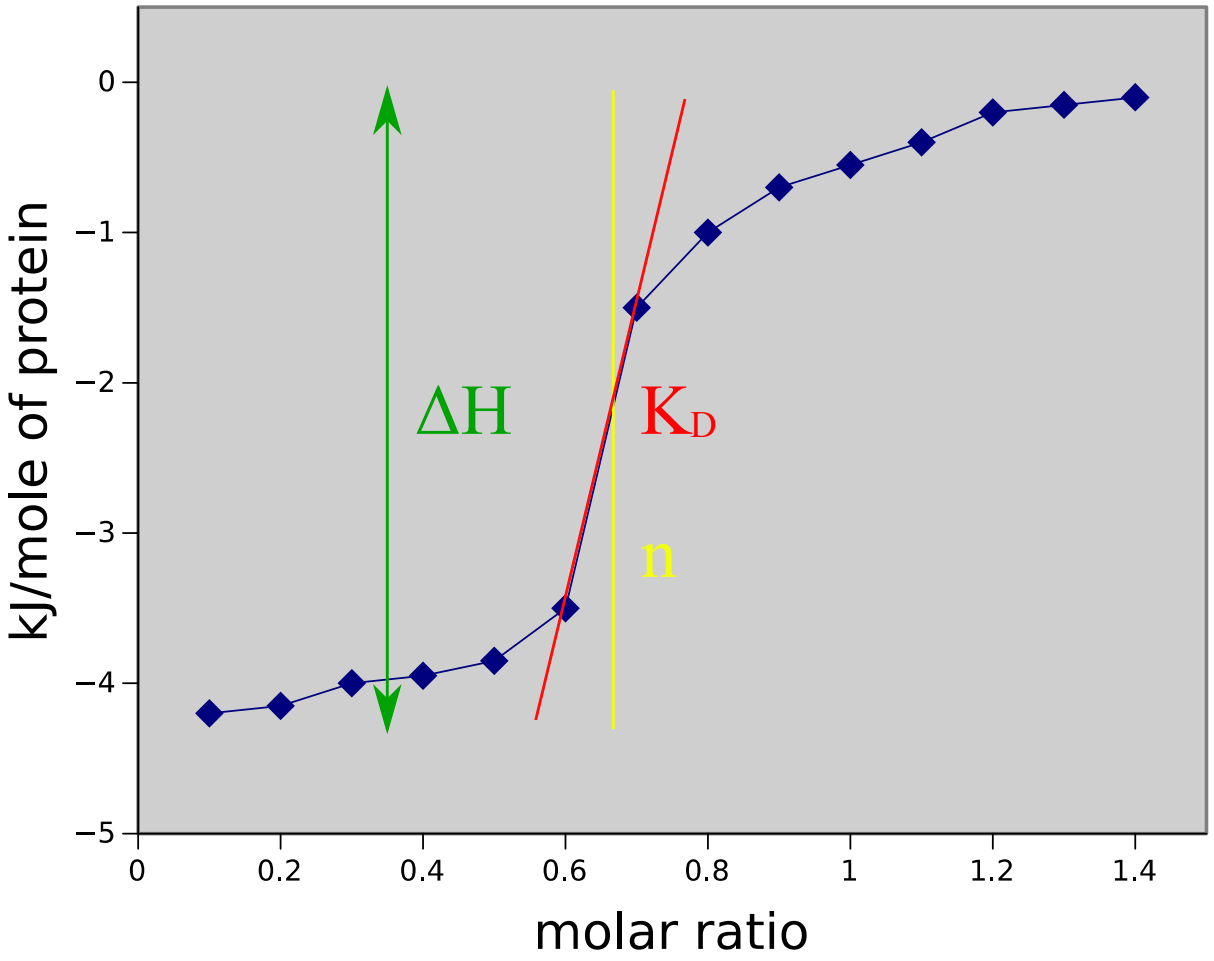
Isothermal calorimetry (ITC)



Isothermal calorimetry (ITC)



Isothermal calorimetry (ITC)



$\Delta G = \Delta H - T\Delta S$

$\Delta G = RT\ln K_D$

ΔG	Gibbs Free Energy
ΔH	Enthalpy change
T	Temperature (in Kelvin)
ΔS	Entropy change
R	Gas constant ($8.314 JK^{-1}mol^{-1}$)
K_D	Affinity constant

Isothermal calorimetry (ITC)

Limitations

- Typically, large amounts of “pure” protein are required (e.g. 200 µl, conc. > 1mg/ml)
- Troughput
- Replicability (buffer composition and pH need to be very exact)

The last slide

Questions?

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