Fast establishment of insect and mammalian production cell lines for structural and functional analysis by site-specific recombination

Helmholtz Centre for Infection Research
Protein Sample Production Facility
Braunschweig

Protein Sample Production Facility (PSPF)

New platform supported by the Helmholtz Association

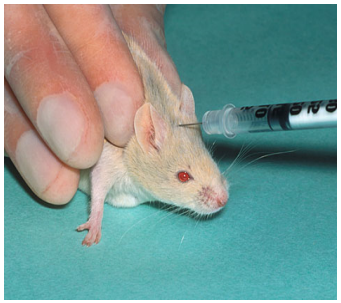
Core facilities for protein production for structural biology

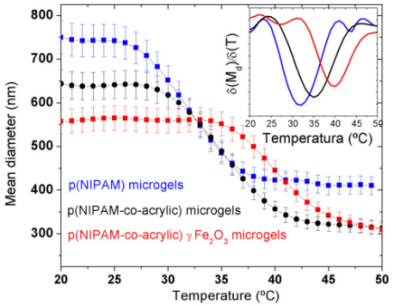
- Helmholtz Centre for Infection Research (HZI) in Braunschweig
- Max Delbrueck Centre for Molecular Medicin (MDC) in Berlin
- Helmholtz Centre Munich (HMGU) new partner

- Start of the facility in May 2007
- Online Request and Evaluation procedure (*www.pspf.de*)
- Training and technology transfer
- Free laboratory space available for visiting scientists

Recombinant Protein Expression

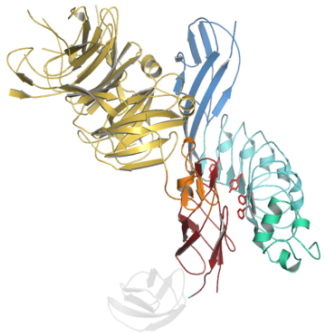
- Production of soluble mammalian receptor proteins
  - Structural analysis of receptor-ligand complexes
  - Functional proteomic studies
  - Analyzing therapeutics / drug targets interactions





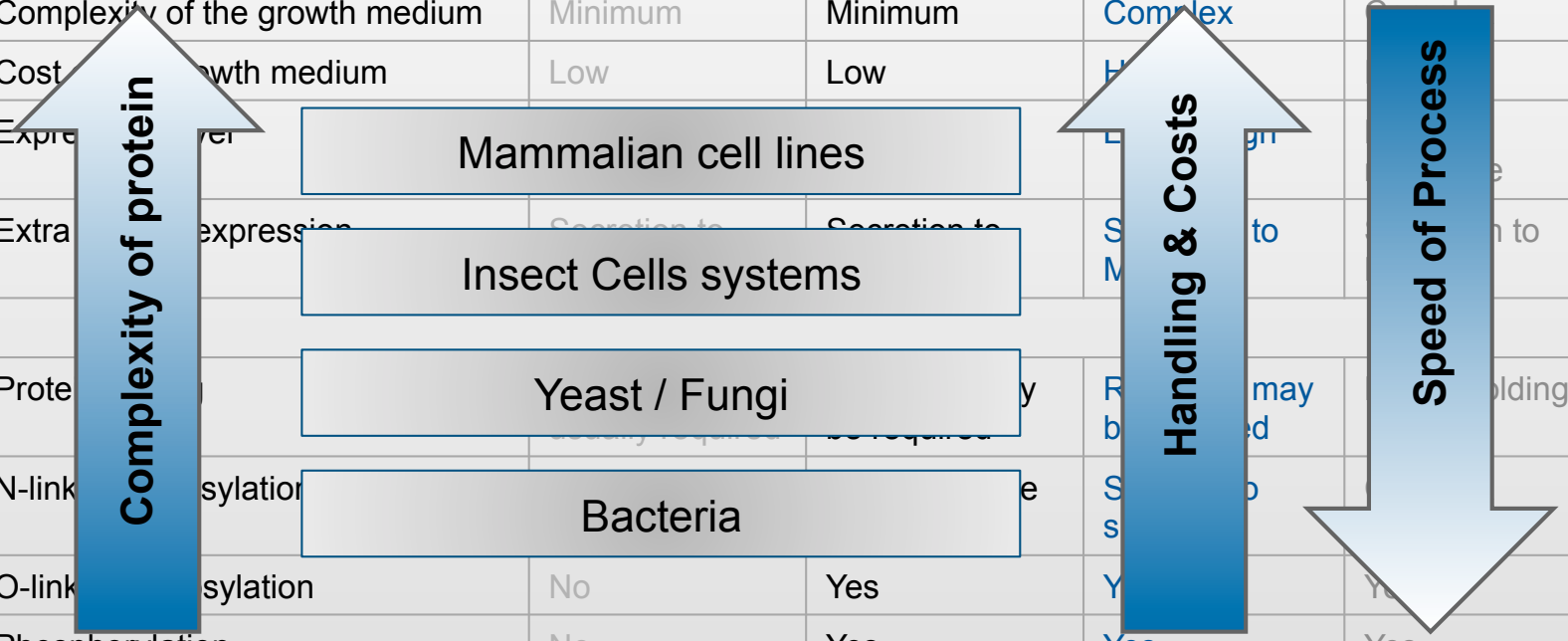
Aim

→ Large quantity with invariable quality



Expression Systems

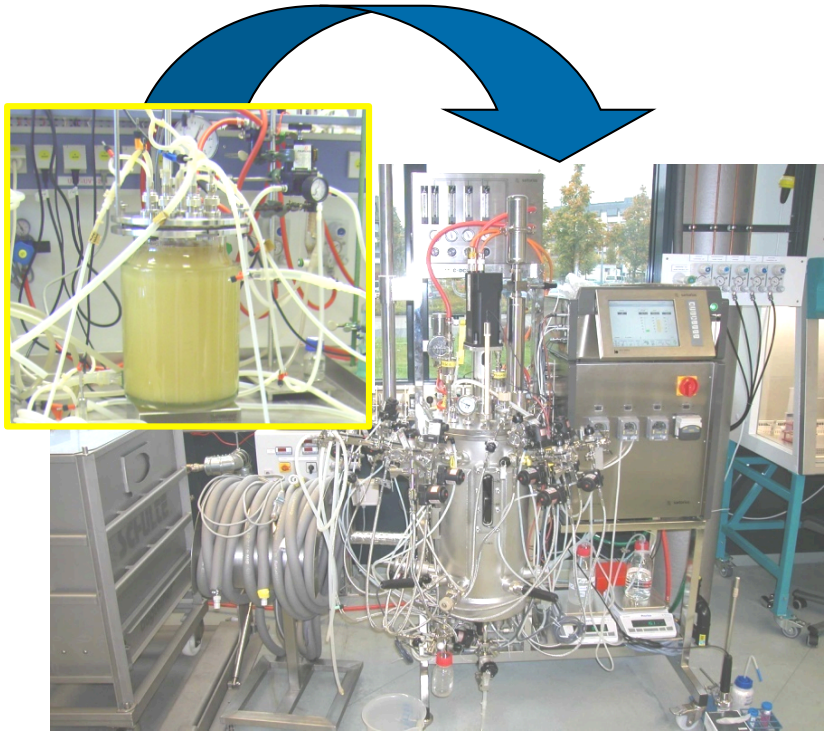
	Bacteria	Yeast	Insect	Mammalian
Cell Growth	Rapid	Rapid	Slow	Slow
Complexity of the growth medium	Minimum	Minimum	Complex	
Cost wth medium	Low	Low	H	
Expre			L gh	e
Extra expressi	Secretion to	Secretion to	S to M	n to
Prote		y	R may b ed	olding
N-link sylatio		e	S o s	
O-link sylation	No	Yes	Y	Y
Phosphorylation	No	Yes	Yes	Yes
Acetylation	No	Yes	Yes	Yes
Acylation	No	Yes	Yes	Yes



Insect cell and mammalian cell culture facility

Cell cultivation

- Shake flask and adherent serum-free cultivation
- 2.5 – 30 L perfusion bioreactor (up to 200 L)
- Primary separation
- In-process analytics
- Large scale protein purification



Technology transfer laboratory

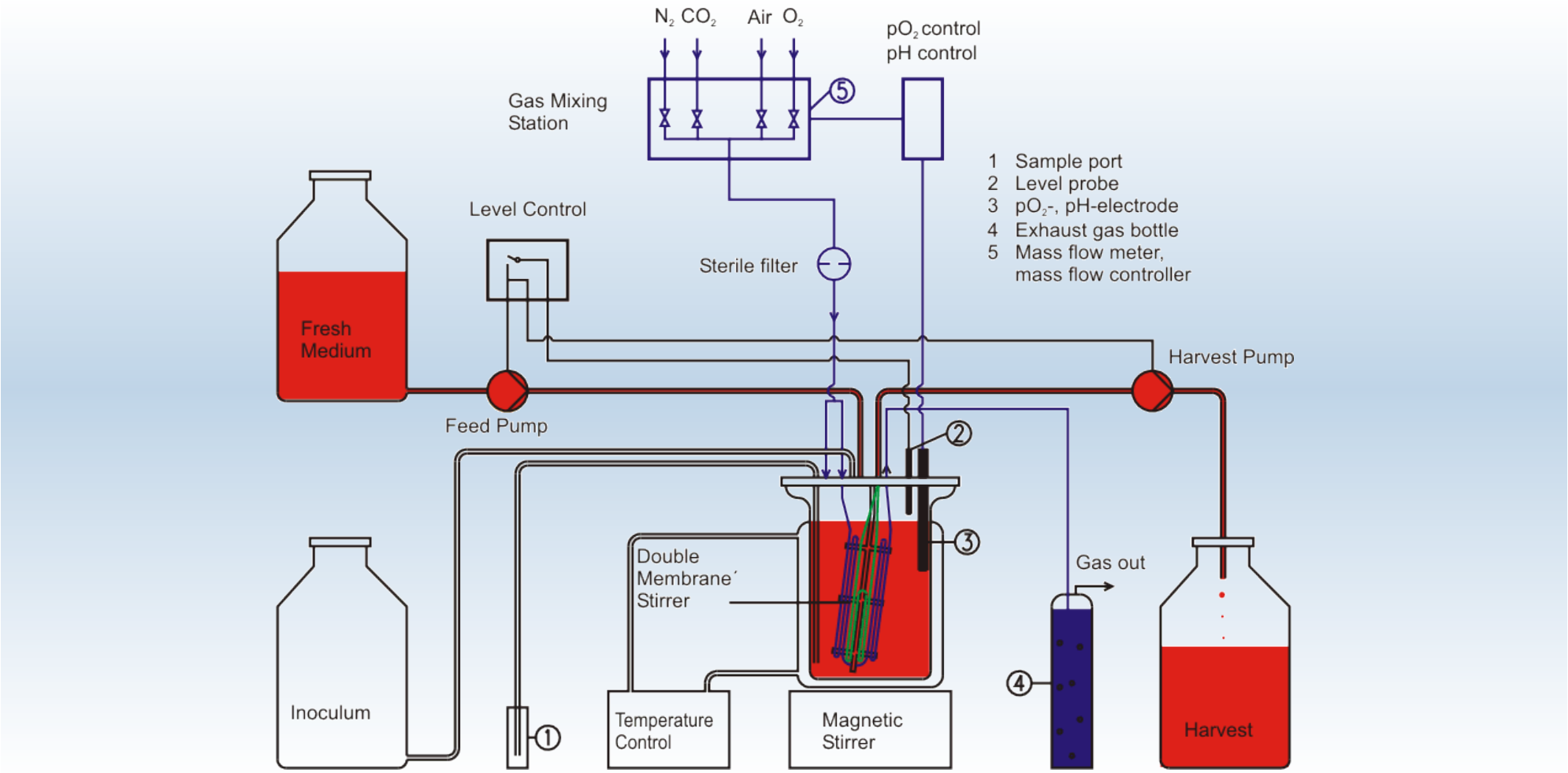
- Free laboratory space available for cooperation partner

Mammalian cell expression system

Stable transfected CHO lec3.2.8.1 or lec8 Cells

- Routine bulk production of proteins from stable transfected CHO lec cell lines with random integration
- Expression of complex mammalian proteins or protein complexes
- Serum-free cultivation
- Minimized micro heterogeneity for better crystallization
- Development of new CHO lec master cell lines with directed gene integration into *hot spots* by RMCE

Benchtop Bioreactor System with Continuous Membrane Perfusion



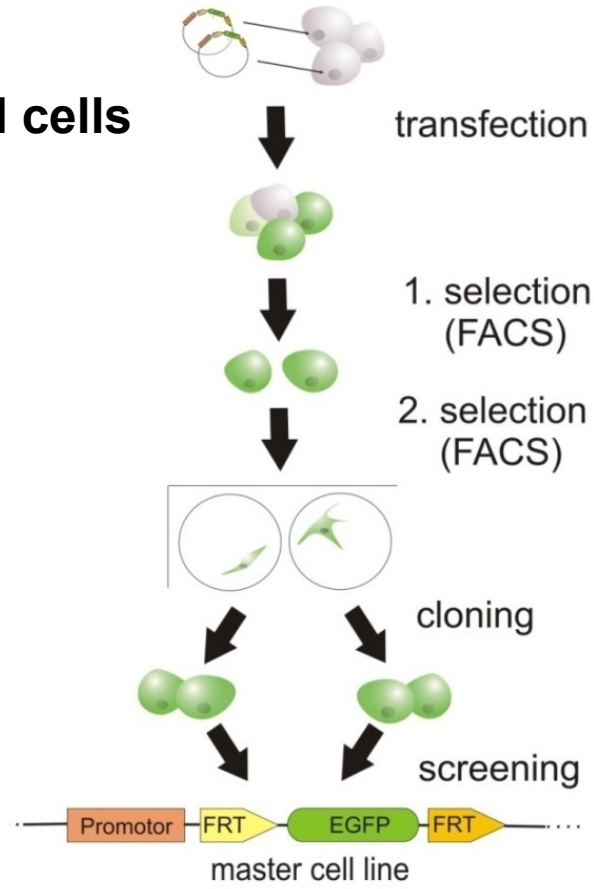
PSPF- Unique expertise

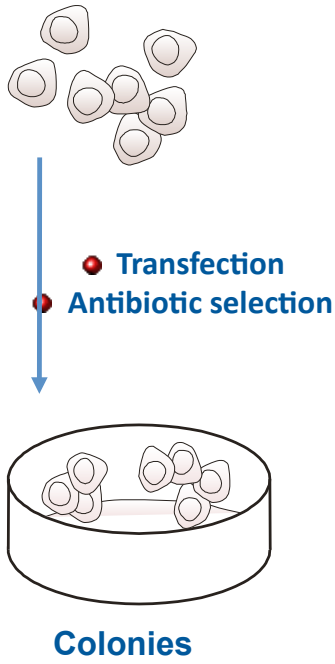
Development of a FACS-based selection method to isolate high producer mammalian cell lines for structural biology (Büssow)

FACS-sorting of GFP-transfected cells

Avoids antibiotic selection

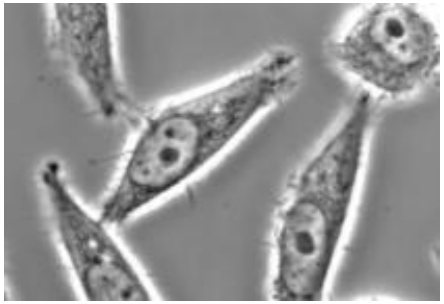
Selection for strong, stable GFP expression

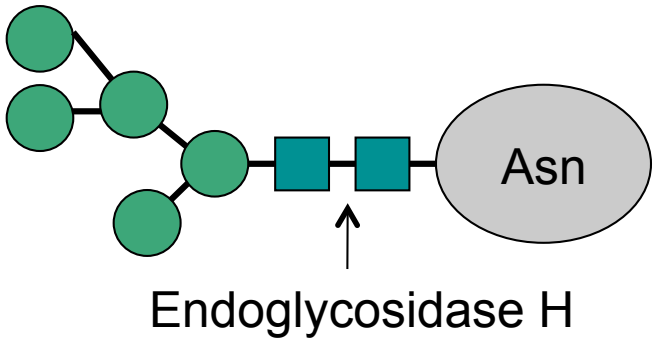


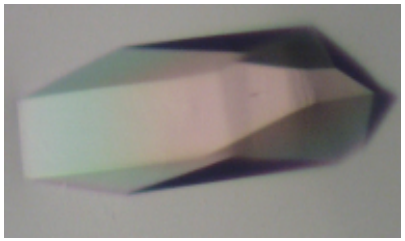


CHO Lec3.2.8.1 expression system for crystallography

- Recombinant production of mammalian glycoproteins with animal cell lines
- Glycosylation deficient CHO Lec3.2.8.1 cells (Stanley, 1989) express shorter and more homogeneous glycan chains.
  → well diffracting crystals

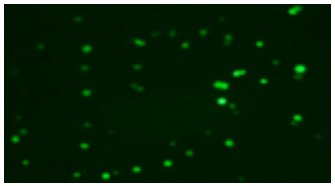






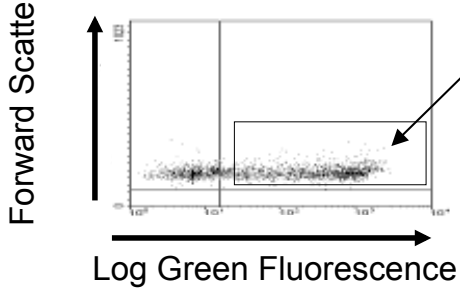
Tagging and FACS

Day 2



Transfection

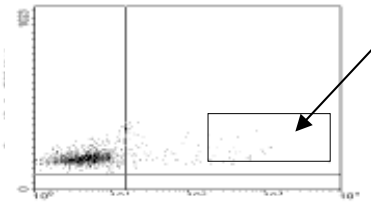
Day 7



Cells sorted in a population

First FACS

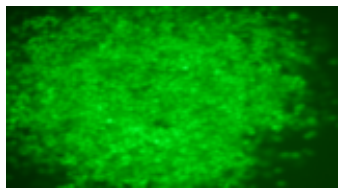
Day 14



Cells sorted as single cells

Second FACS

Day 30



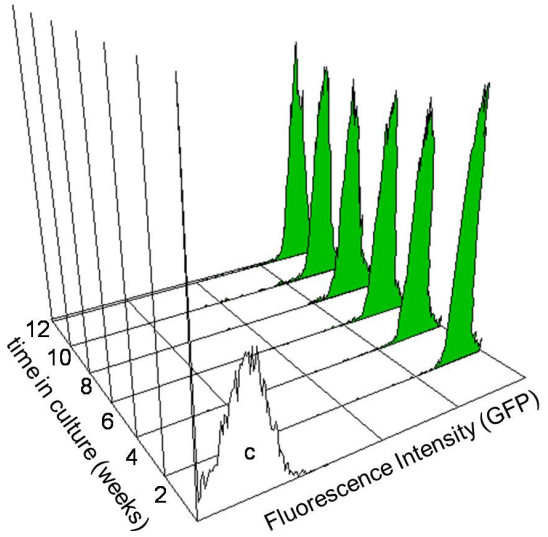
Cloning

Sonja Wilke

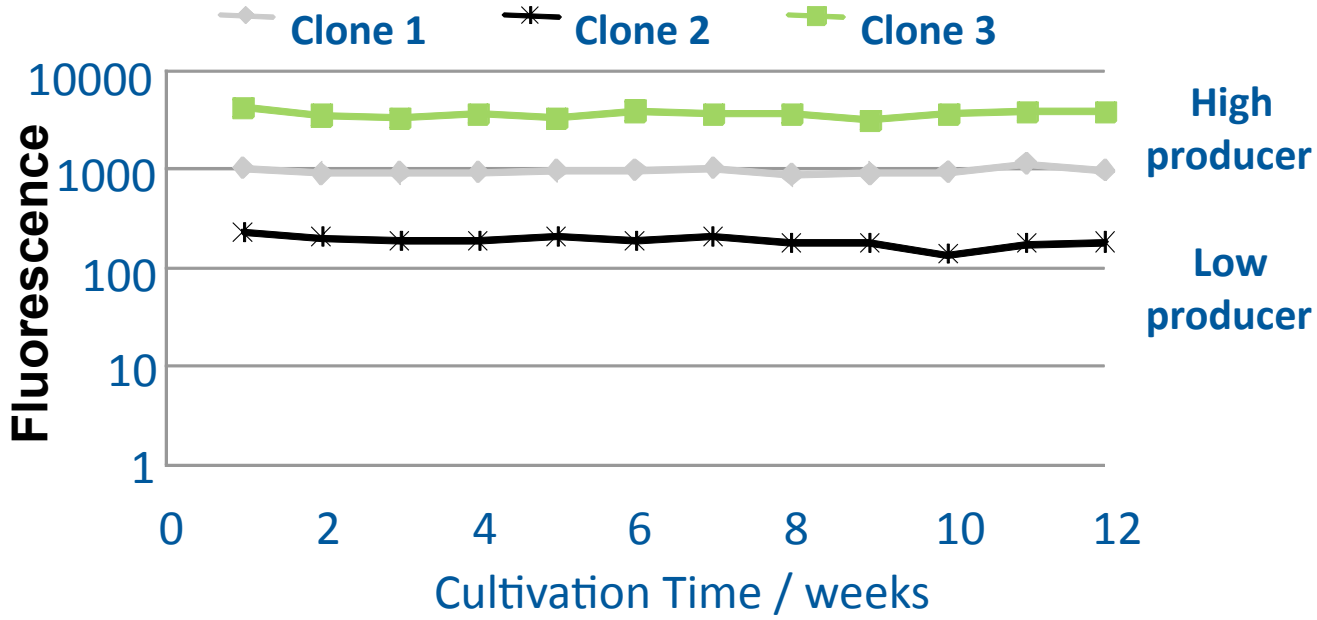
Improved stability of the selected expression cell lines

(Wilke *et al.* (2010) *Prot. Sci.*)

Stable CHO-Lec3.2.8.1 cell lines which show homogeneous GFP expression

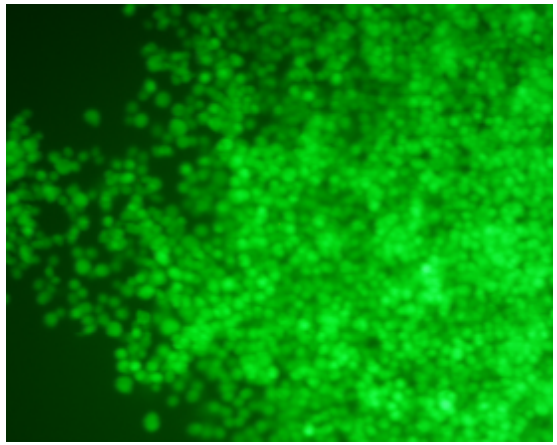


Fluorescence over time



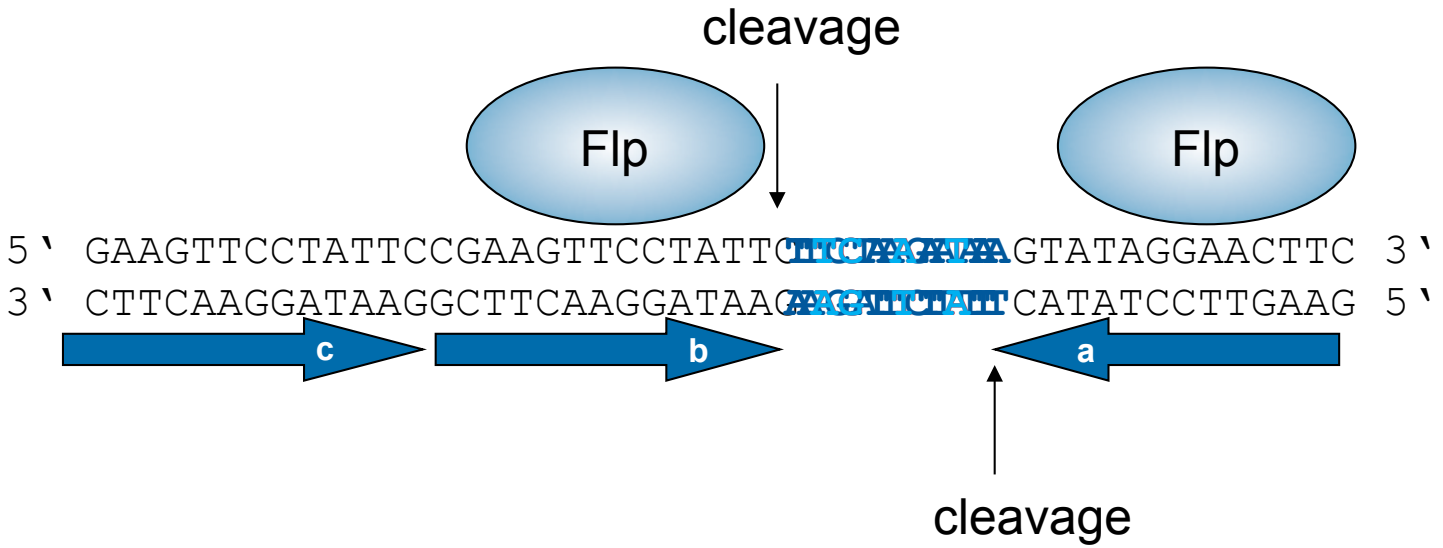
High producer

Low producer



Recombination Mediated Cassette Exchange (RMCE)

- *Flp-recombination-target site*
  - 48 bp sequence with inverted repeat sequence
  - Asymetric 8bp spacer involved in DNA-pairing during exchange
  - Its asymmetry determines the direction of site alignment in the recombination event
  - Different FRT-sites known

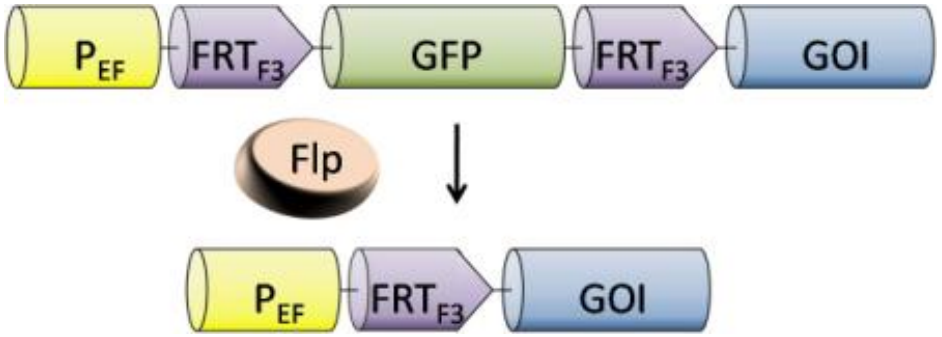


F5

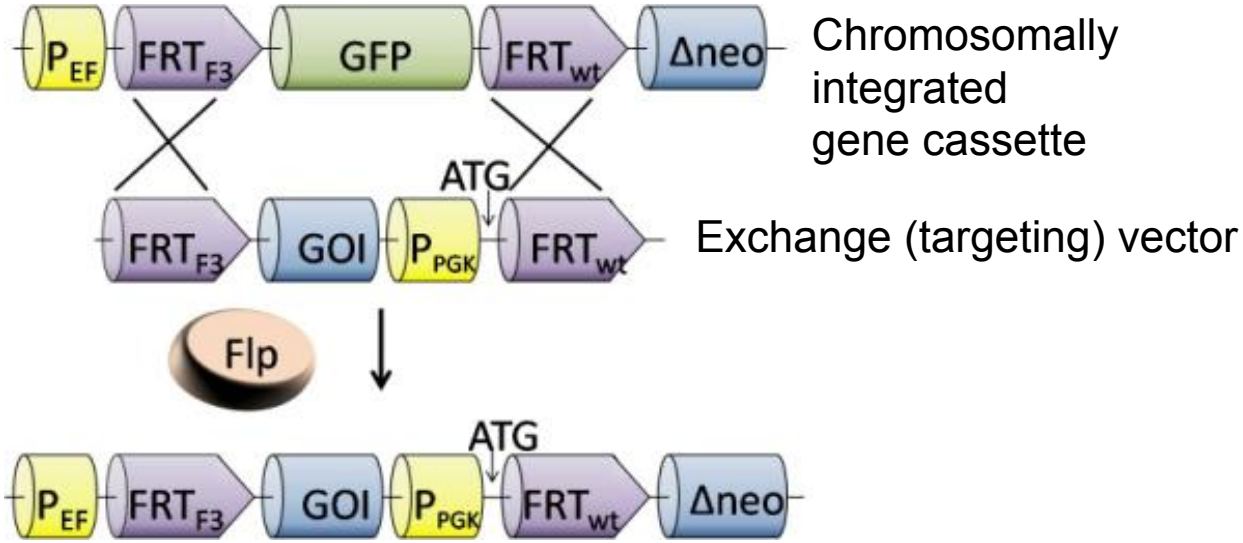
Flp/FRT site-specific recombination

- Recombinase-mediated reporter gene excision

Chromosomally integrated gene cassette



- Recombinase-mediated cassette exchange (RMCE)



Chromosomally integrated gene cassette

Exchange (targeting) vector

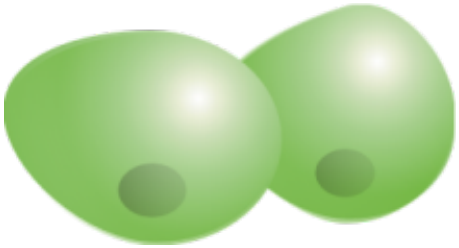
Recombinase-mediated cell line development

or

Random integration

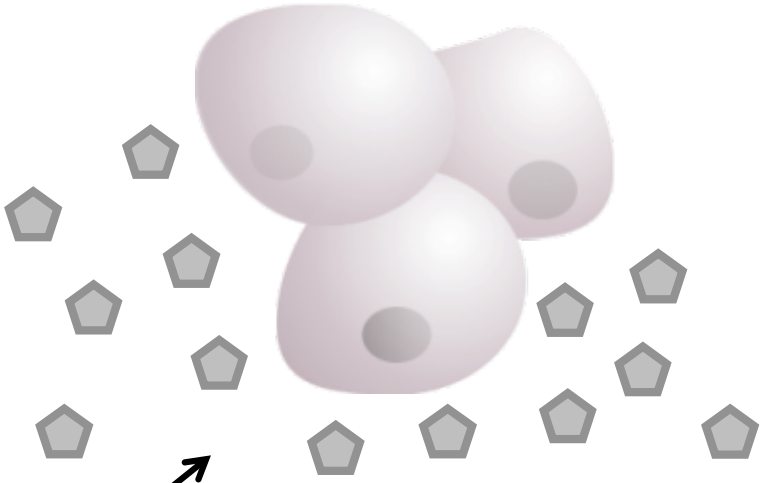
Tagging of a single site in the host cell genome

Master cell line

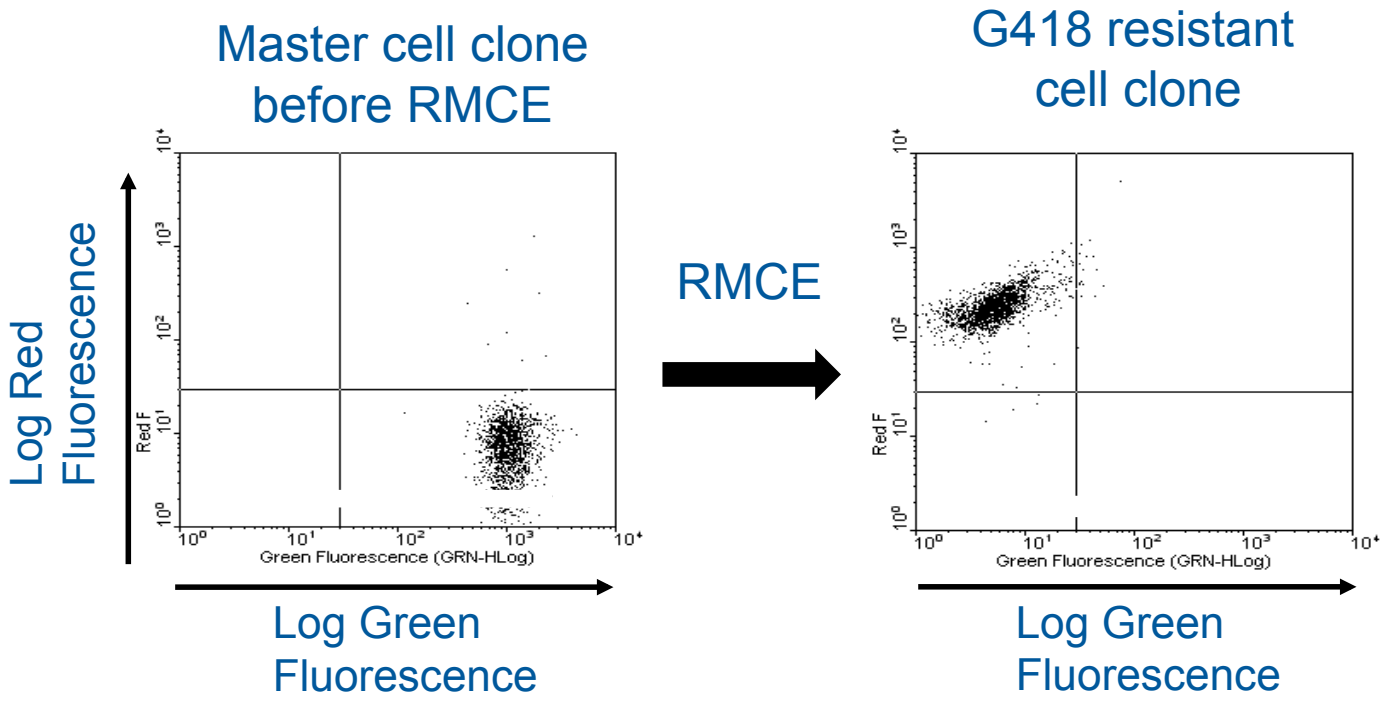


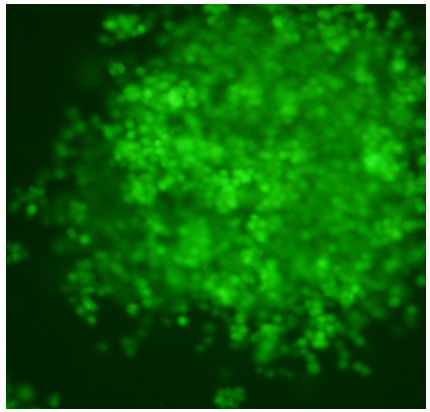
Excision or exchange of GFP reporter

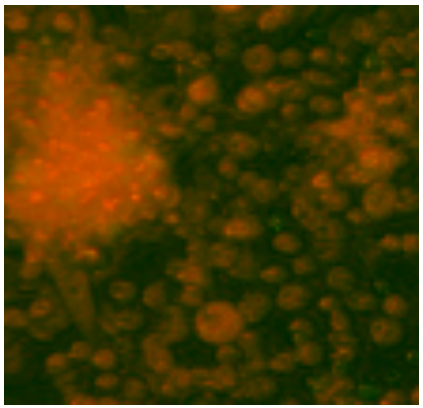
New production cell line



Fast exchange of GFP into RFP by recombination mediated cassette exchange (RMCE)

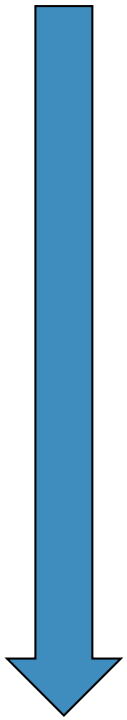






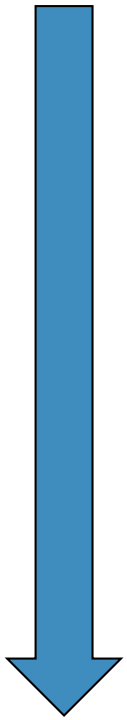
Advantage of the recombination mediated cassette exchange system

Classic method



~0.5 - 1 year

GFP – FACS sorting

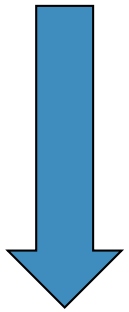


~0.5 - 1 year

Master cell line

RMCE method

Master cell line



~2-4 months

Applications:

1. Production of Met Receptor/HGF complex

   - Role of Met signalling in infection and cancer
     (cooperation with MRC Cambridge and University of Bielefeld)

2. Development of Novel Expression Systems

   - Flp recombinase mediated cassette exchange for stable insect cell lines
     (EU Grant application and cooperation with EMBL Grenoble)

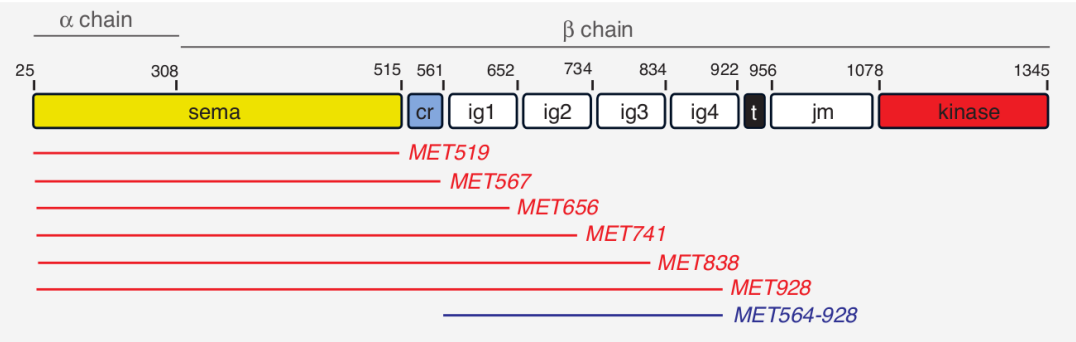
1. Production of HGF and Met in CHO lec3.2.8.1

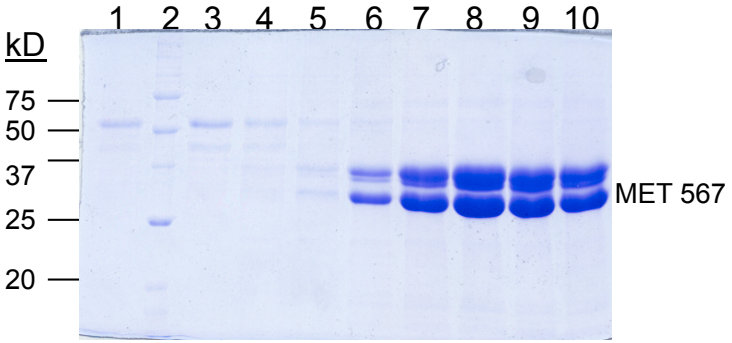
Mammalian expression

- Required for “complete authenticity”
- CHO lec3.2.8.1. glycosylation deficient cell line
- Time consuming
- Expensive
- low yields (1-3 mg/L)

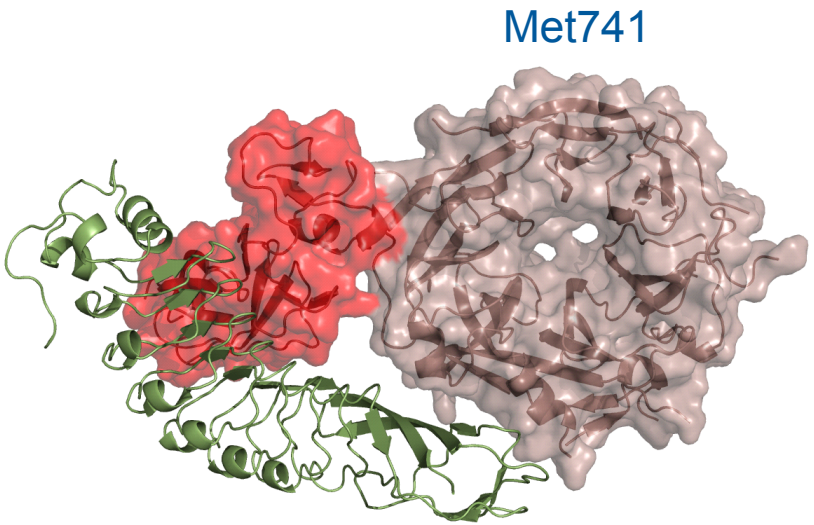
Expression of soluble extra-cellular Met receptor domains

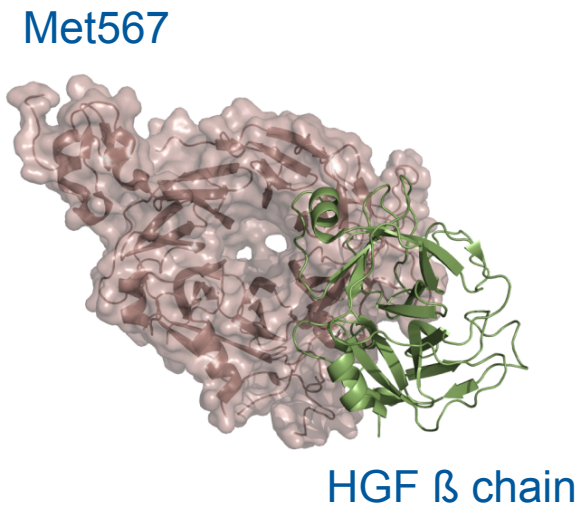
(Cooperation with MRC Cambridge and University of Bielefeld)





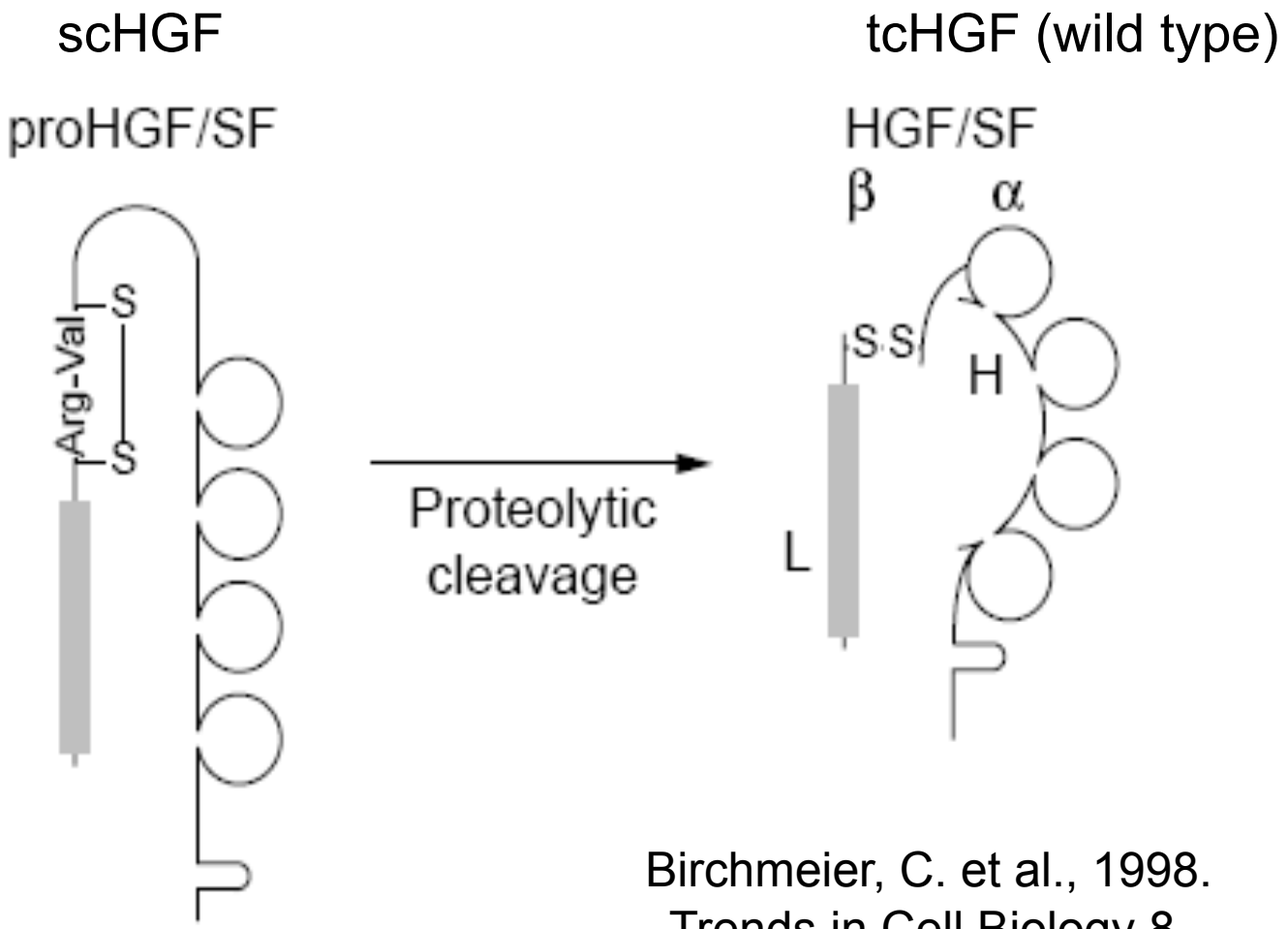
c-Met ecto-domain in complex with the Listeria InlB or human tcHGF/SF





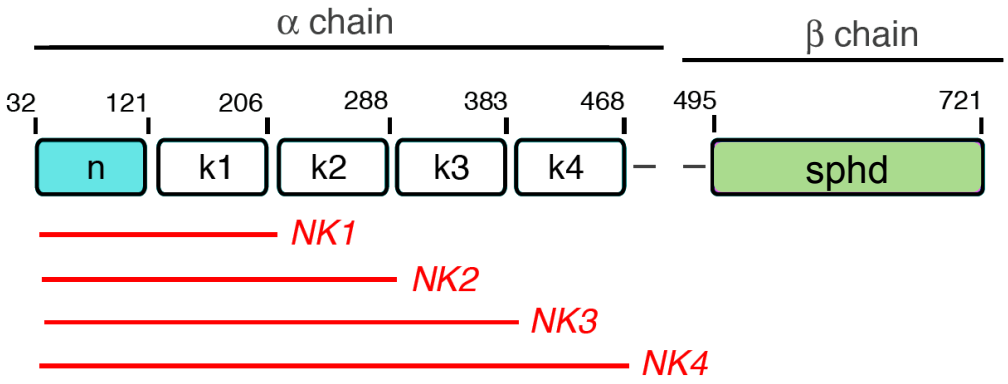
How does the c-Met receptor discriminates between the two different ligands?

Human hepatocyte growth factor (HGF/SF)



Birchmeier, C. et al., 1998.
Trends in Cell Biology 8,
404-410

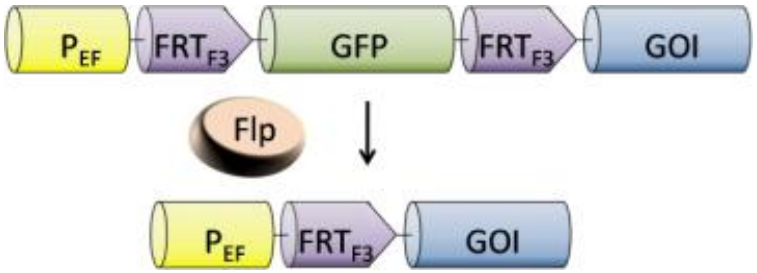
Large scale production of fl-HGF in CHO Lec3.2.8.1



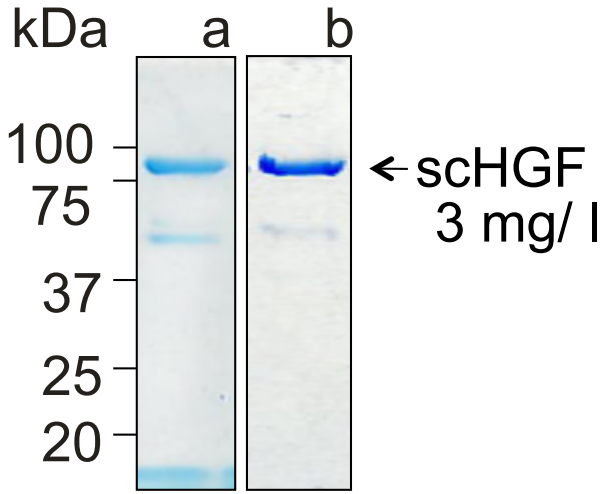
Challenges for full-length HGF/Met complex purification

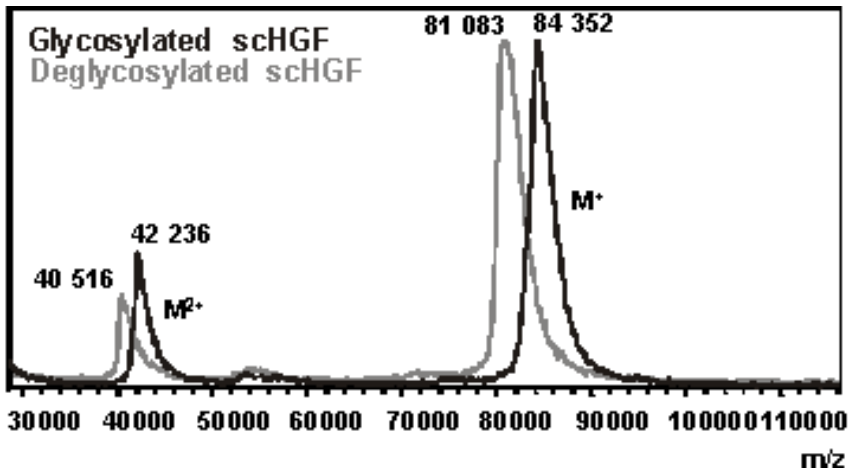
- Extensive glycosylation → CHO lec3.2.8.1
- Activation by proteolytic cleavage
- Both sc- and tc-HGF bind Met
- Activation of HGF by proteolytic cleavage with HGFA – pure tc-HGF
- Expression of sc-HGF proteolytic cleavage site mutant K491D; R494E

scHGF CHO Lec cell line



- Production of scHGF





Wilke et al. Protein Science 2010

Conclusion

- 6-9 weeks required for exchanging a GOI into the master cell line
- 10 proteins up to now have been expressed
- Expression level of soluble receptor ecto-domains in low mg/L level
- Expression homogeneous for all cells
- Stable long-term expression

Future developments

- New tools required for flexible generation of multigene baculoviral expression vectors for multi protein assembly in stable insect cells
- New RMCE-CHO cell lines required for fast, stable and efficient protein expression of multi protein complexes
- Compatibility of baculoviral and CHO stable cell culture expression systems
- Shortening of time from clone to produced protein

Acknowledgements

- PSPF
  - Sonja Wilke
  - Steffen Meyer
  - Sarah Tokarski
  - Daniela Gebauer
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