Biotinylation of recombinant proteins by co-expression with BirA in a range of different cell hosts

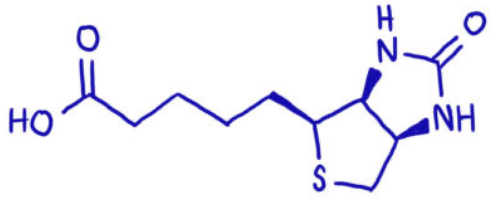
Dr. Chris Cooper

Director and Head of Protein Sciences
CHARM Therapeutics
Cambridge, UK
(ex-Peak Proteins/Sygnature)

20th P4EU Meeting HZI/ Helmholtz Centre for Infection Research
Braunschweig, Germany

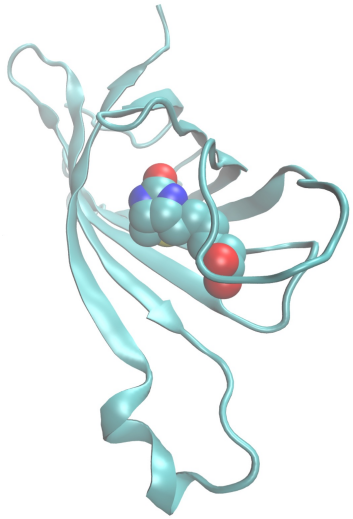
What is biotin?

- Biotin (Vitamin B7) is an essential co-enzyme, literally meaning “life-giver”
- Covalently linked near the active sites of four major classes of carboxylase enzyme
- Higher eukaryotes cannot synthesise; humans obtain most biotin from gut microflora
- The egg-white glycoprotein **avidin** was discovered as a binder of biotin
- Streptavidin is a related protein isolated from *Streptomyces avidinii* and is also **tetrameric** but not glycosylated; both thought to provide anti-bacterial protection
- Both are extremely tight non-covalent binders of biotin ($K_d \approx 10^{-15}$ M), resulting from extensive hydrogen bond and hydrophobic contacts, with a ‘lid’ closing over the biotin
- Streptavidin-biotin interactions have significant usage in molecular biology/biotechnology:
    - small and unlikely to peturb function
    - can be added to molecules enzymatically or chemically to proteins and nucleic acids
    - near covalent interaction strength *e.g.* stable immobilisation on surfaces
    - resistant to many denaturing agents, detergents and extremes of temperature and pH



Biotin

$C_{10}H_{16}N_2O_3S$

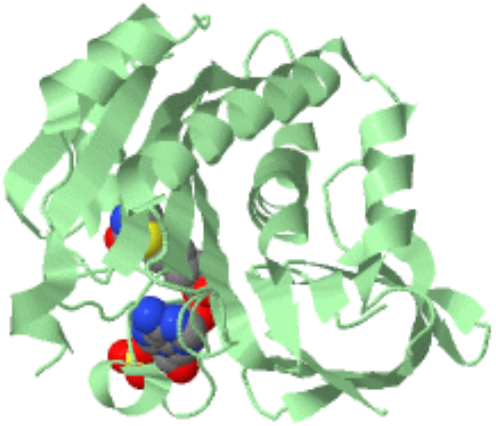


de.wikipedia.org/wiki/Avidin

What is biotinylation?

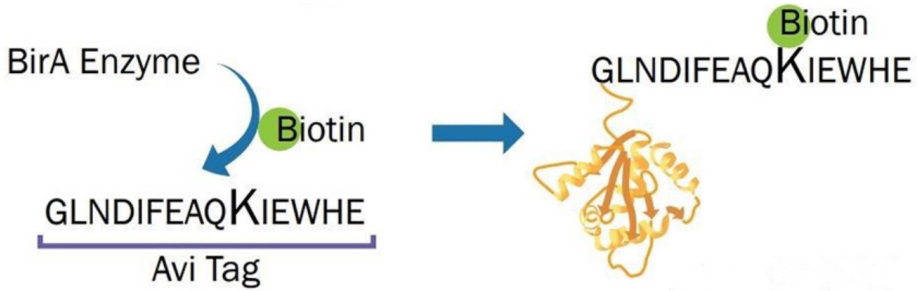
- Biotin ligases (BPLs) catalyse **covalent** addition of biotin moieties to acceptor proteins
- BirA from *E. coli* is one of the most studied BPLs and contains both BPL and transcriptional repression functionalities
- BirA substrate in *E. coli* is Biotin Carboxyl Carrier Protein (BCCP, Lys^{122})
- Cronan *et al* fused 75 residues to a target protein to site-specifically biotinylate it
- However Beckett *et al* optimised this BCCP sequence using phage display

Biotin protein ligase complex with biotinyl-5-AMP and sulphate (PDB code 4OP0)



proteopedia.org

- Resulting 5-residue peptide is commonly known as the ***Avi tag***
- Avi tag allows precise biotinylation on target proteins, and in conjunction with streptavidin and other avidins, has seen wide applicability in biotechnology

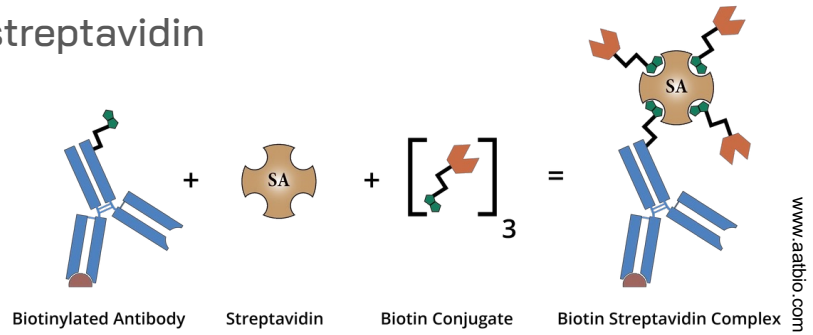


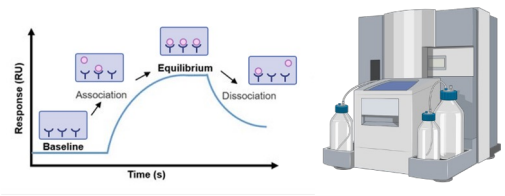
www.creativebiomart.net/avi-tag-biotinylated-proteins.htm

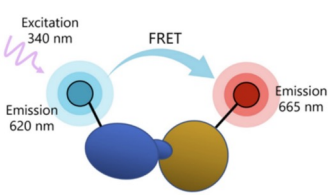
Uses of biotin in molecular biology, drug discovery.....

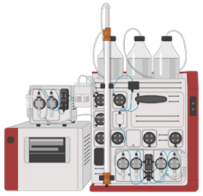
PEAK PROTEINS
A SYGNATURE DISCOVERY BUSINESS

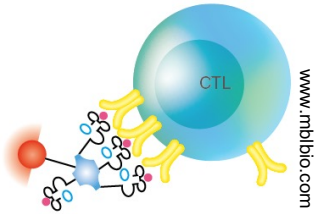
- Very stable interaction between anything conjugated to biotin and streptavidin
- Hence, biotinylated proteins can be **immobilised** at specific sites on many different surfaces with (strept)avidin
  - solid surfaces – key for surface plasmon resonance *etc*
  - agarose/magnetic beads – HTRF/TR-FRET assays
  - purification resins – including reversible Strep-Tactin resin

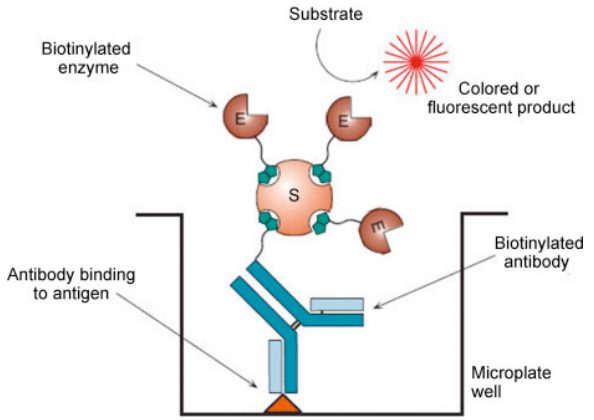












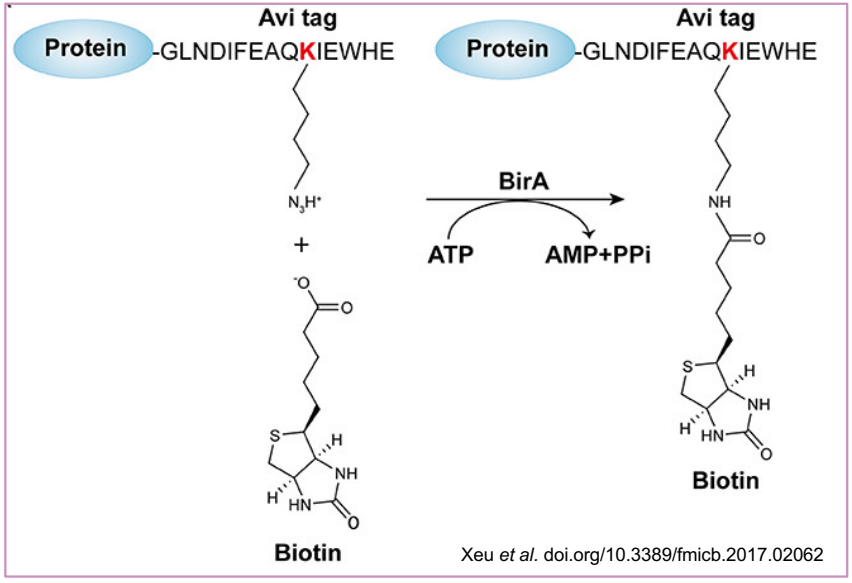
- Streptavidin **tetramerisation** conveys useful properties:
  - MHC tetramers – higher binding affinity for T-cell labelling
  - ELISA - signal amplification from 1:3 ratio of antibody: enzyme

Feng et al. doi.org/10.1016/B978-0-12-815053-5.00012-X

CHARM THERAPEUTICS

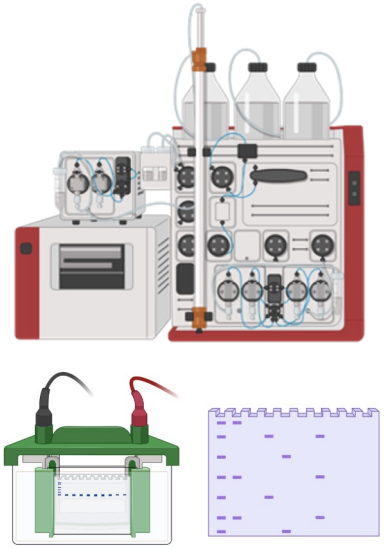
Protein biotinylation (*in vitro*) – how does it work

- Site-specific biotinylation on Avi tag is performed with recombinant BirA biotin ligase (EC 6.3.4.15)
- BirA can be readily purified from *E. coli* (and available in kit form)
- BirA activates biotin to form biotinyl 5' adenylate, transferred covalently to the ε-amino group of acceptor lysine residue
- Incubate in ATP-containing optimum BirA buffer, 30°C incubation for ~1 hour



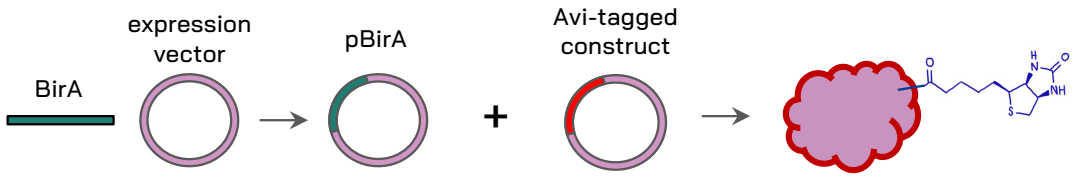
Xeu *et al.* doi.org/10.3389/fmicb.2017.02062

- % biotinylation dependent on many factors, *e.g.* Avi tag accessibility at the N/C- termini
- Buffer composition can affect BirA activity (>100 mM NaCl, > 5% glycerol)
- BirA most likely still needs to be removed
- Further purification may be required (*e.g.* SEC)



Protein biotinylation – how to biotinylate *in vivo*

- Why bother to biotinylate (Avi-tags) *in vivo*?
- *In vitro* biotinylation is has some issues, an alternative is available!
- *In vivo* biotinylation is well-established in a number of cell systems



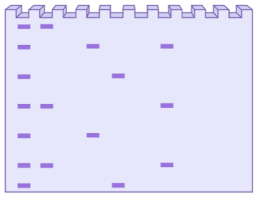
dev.to/quantumsheep/why-you-should-reinvent-the-wheel-4ha2

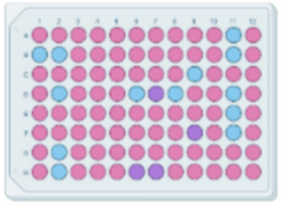
- A number of considerations are needed however:
  - Avi-tag biotinylation may happen to a degree in *E. coli* during expression
  - however exogenous BirA may be needed for full Avi-tag biotinylation
  - codon optimisation is likely needed for BirA in non-bacterial hosts
  - biotin is water soluble but sparsely – however 100 mM solutions can be prepared by neutralising with NaOH to ~pH 7
  - maybe biotin powder can be added directly to cultures (anyone want to try? 😃)
  - does the end user want ALL the protein to be biotinylated?

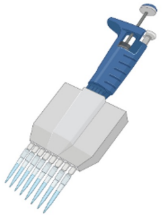
In vivo protein biotinylation – detection methods

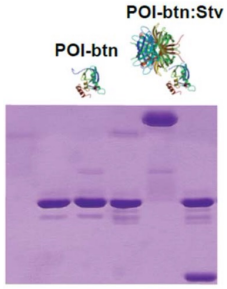
- Why do we need to confirm biotinylation?
  - biotinylation is not always efficient; Avi-tag accessibility can factor
  - homogeneity may not be important for some downstream functions (*e.g.* SPR)
  - however homogeneity may be crucial for some applications (process development, HTRF *etc*)

- Different modalities of biotinylation detection
  - Western blot – reasonably sensitive and quantitative, but does not detect unbiotinylated protein
  - ELISA – similar to western blot but more sensitive, quantitative and HTP
  - streptavidin gel shift –detects both un/biotinylated species and can be partially quantitative with GFP
  - mass spectrometry (*e.g.* LC/ESI-TOF MS) – super sensitive and (semi)-quantitative *BUT* super £$€¥

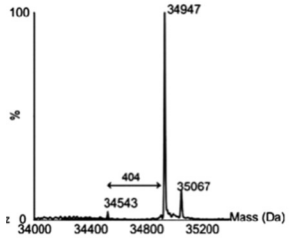




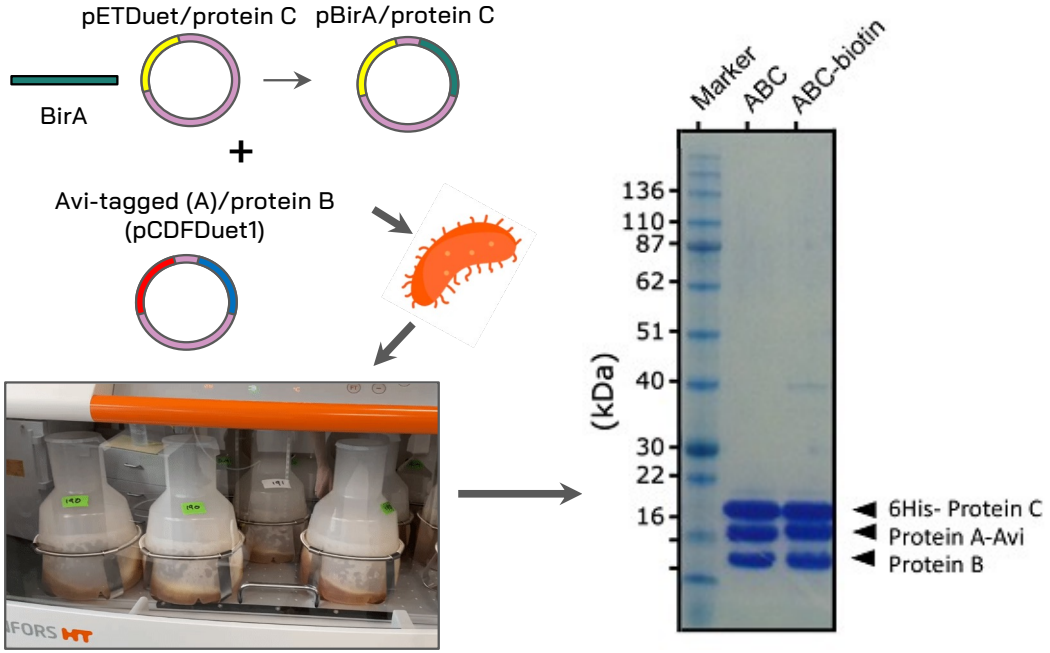


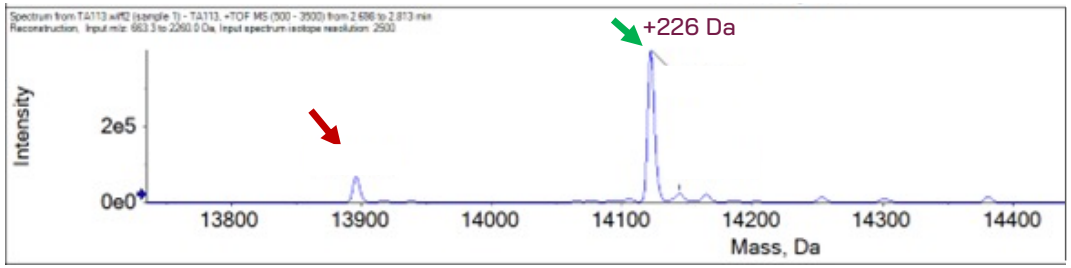


Sorenson et al. DOI: 10.1039/C4AY02666G



Bacterial *in vivo* biotinylation



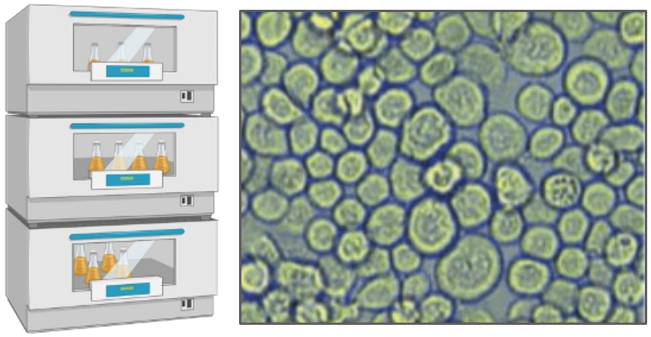


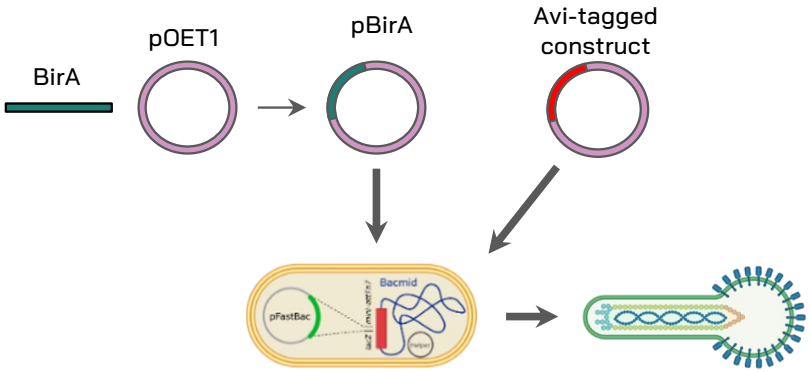
- Heterotrimeric human complex (A-B-C) expressed in *E. coli*
- BirA & protein A (C-terminal Avi) – pETDuet vector
- Proteins A & B (N-terminal 6xHis/TEV in pCDFDuet1
- Expression was induced by IPTG and 100 μM biotin added

- Mass shift of +226 Da indicates single biotinylation
- ~80% of the complex has been biotinylated (peak heights)

- Modified system with BirA in pCDFDuet background as pET28/Kan background is more commonly used
  - Free MCS2 in pCDF for cloning (BirA in MCS2)
  - And/or cotransform with pET28/pCDF-BirA competent cells
  - Biotin addition at point of cooling cells ~1 hour prior to induction may work better…?

Insect cell *in vivo* biotinylation



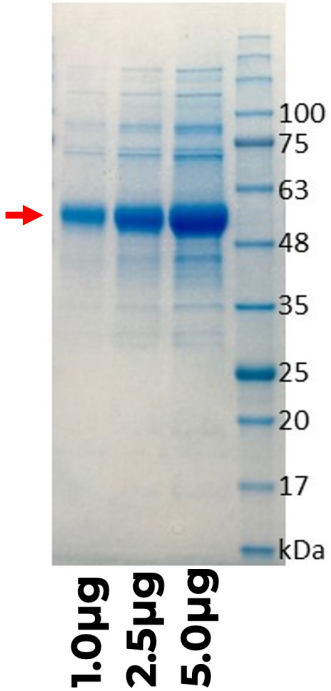


- A biotinylated version of a highly disordered protein was required for a high-throughput screening assay
- Insect BEVS system chosen due to nature of IDP
- Codon-optimised BirA cloned into pOET1
- Avi-tagged target protein cloned separately into pOET1
- Viruses made and Sf21 cells were co-infected with both
- Cells were supplemented with 4 μM biotin at infection.

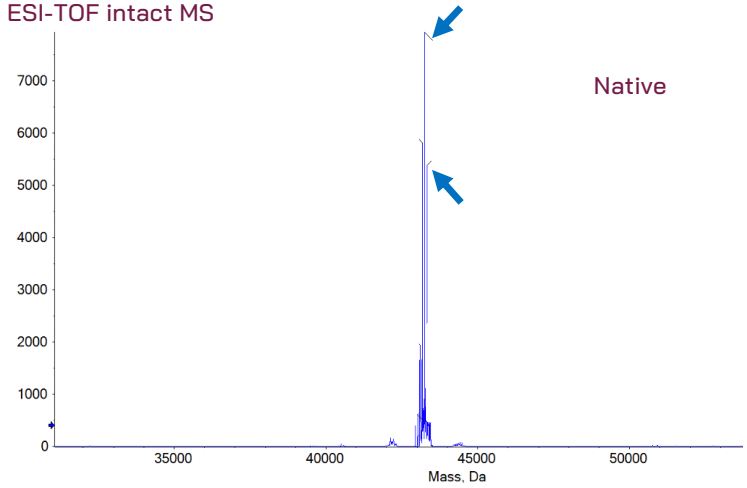
- Some useful considerations:
  - no BirA in insect cells so Avi-tagged protein can be made +/- biotinylation
  - exogenous BirA otherwise needed
  - BirA found to associate with biotinylated proteins
  - BirA: target virus to 1:10 reduced this issue AND increased target yield

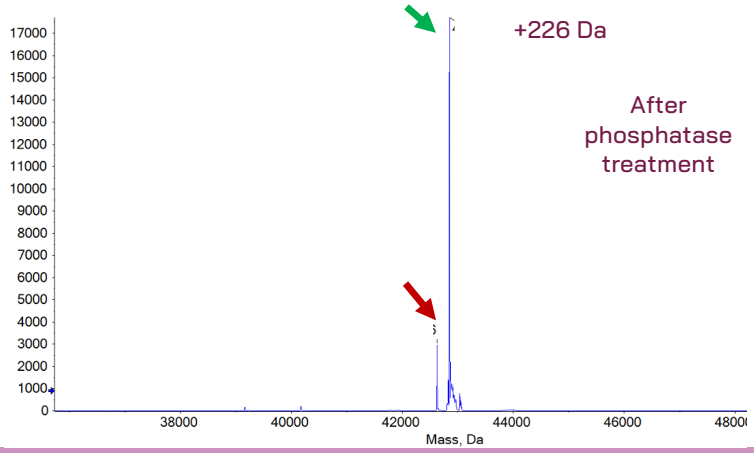
Insect cell *in vivo* biotinylation

Ni-IMAC purification



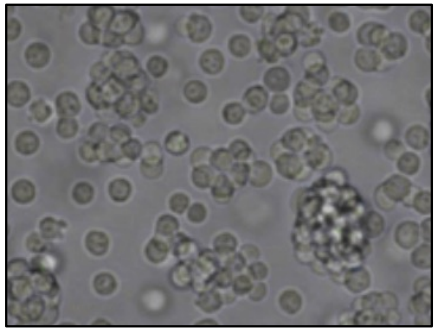
ESI-TOF intact MS

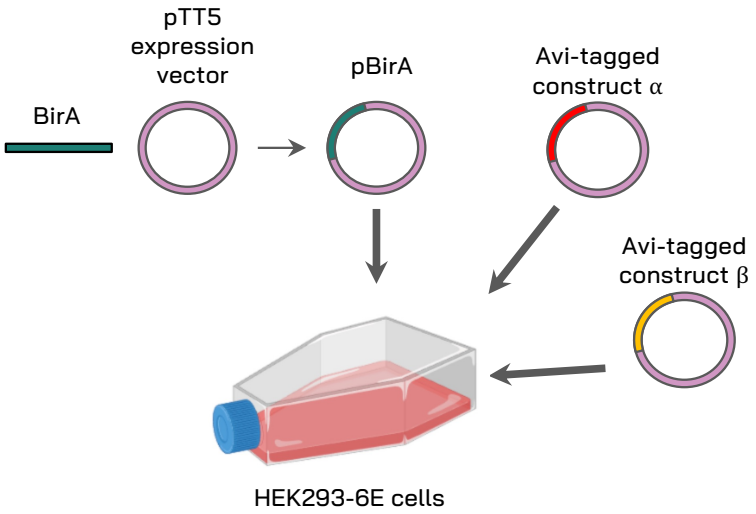




- Expression was good and ~pure after IMAC
- Multiple PTMs complicated intact MS analysis:
  - multiple phosphorylations (blue arrows)
  - acetylation
  - single biotinylation
- Dephosphorylation with λ phosphatase allowed intact MS deconvolution
- Almost complete biotinylation of single site
- Demonstrates power of intact MS combined with PTM removal

In vivo biotinylation for mammalian secretion

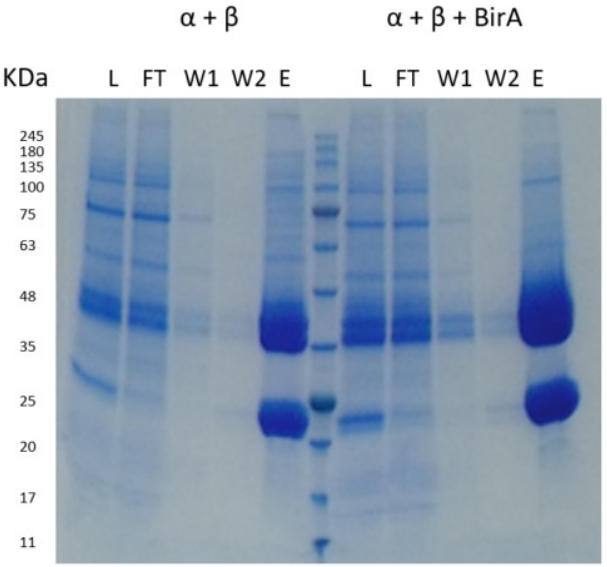




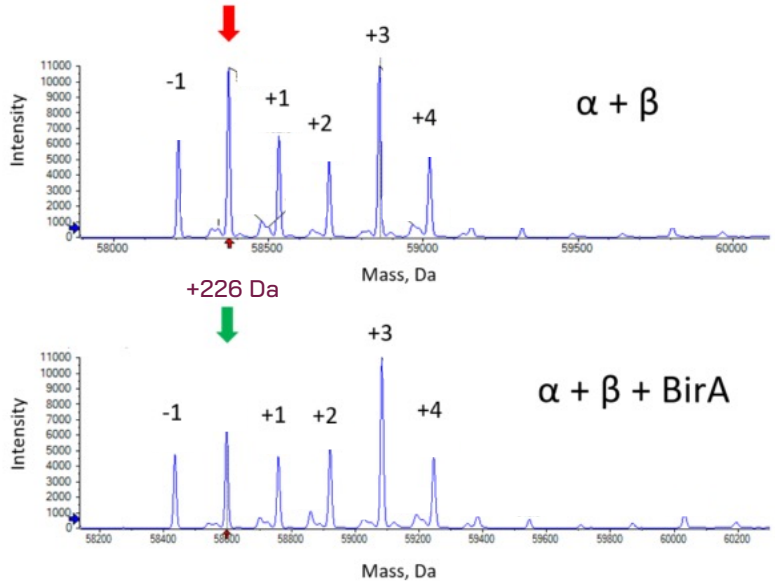
- Licenced HEK293-6E and pTT5 expression system chosen for expression of a heterodimeric cytokine (α + β subunits)
- Secretion required due to disulphide linkages (IgK leader peptide)
- Biotinylation required too – BirA required but it is intracellular…
- Codon-optimised BirA cloned into pTT5 with IgK leader
- BirA plasmid transfected at 1/10th of amount of other two plasmids
- Cells were supplemented with 4 μM biotin at transfection
- Experiment was also performed in absence of BirA (+biotin)
- Some useful considerations:
  - no BirA in mammalian cells so Avi-tagged protein can be made +/- biotinylation
  - Some literature suggestions that intracellular BirA may not be able to biotinylate secreted protein fully – co-secrete so both pass through the ER secretory system

In vivo biotinylation for mammalian secretion

Ni-IMAC purification from supernatant



ESI-TOF intact MS



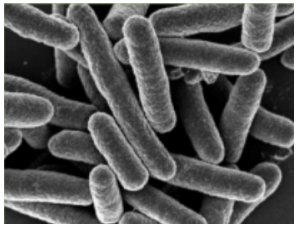
- The main species in both conditions is highlighted above with an arrow
- Multiple glycan species are shown all increasing by a single hexose unit (162 Da).
- Each glycan species was +226 Da following co-expression with BirA indicating single biotin addition
- The shift in size was 100%, indicating complete biotinylation of all the secreted protein.

- This approach has also been successful in CHO cells
- Potential disadvantage – cannot capture straight onto streptavidin
  - biotin in media/supplemented likely to block interaction/sequester streptavidin
  - initial purification capture step would be required
  - also possible to remove biotin *e.g.* BioLock (£$€¥)

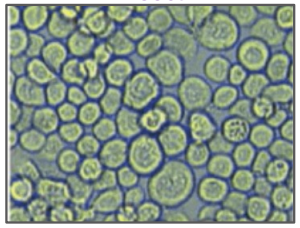
In vivo protein biotinylation – future directions?

- Lots of advantages to *in vivo* biotinylation:
  - Reduced processing times & cost
  - No requirement to remove BirA (slight caveat….)
  - No BirA buffer compatibility issues/issues with temperature incubation
  - Still lots to improve on however!
- More studies on how much biotin is needed in exogenous media (especially eukaryotic systems)
- Limitations of biotinylation - accessibility of Avi-tag (problem for *in vitro* too)
- Genome engineering – tagged BirA for subtractive removal/genomically encoded? Knock into Sf21/HEK293?
- Lower copy plasmids/weaker or inducible promoters to drive BirA expression in *E. coli*
- *In vivo* biotinylation in other less common expression systems – already exists in some!

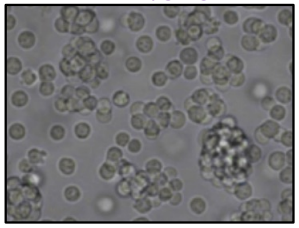
Bacteria



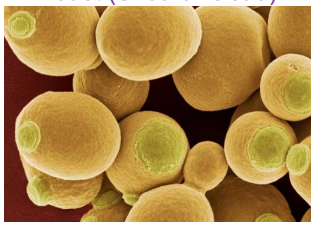
Insect



HEK/CHO

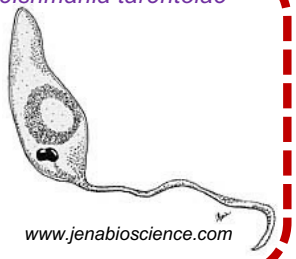


Yeast (*S. cerevisisae*)



creative-biolabs.com/drug-discovery/

Leishmania tarentolae



www.jenabioscience.com

Acknowledgements

Sophie Huber

Dr Gurdeep Minhas

Ailsa Townley

Emma Cains

Ros Brant

Catherine Geh

Dr Mark Elvin (Head of Expression)

Entire Protein Sciences and Mass Spectrometry team