Small-scale expression and purification of the cytokine receptor, MPL, using the ALiCE® cell-free system

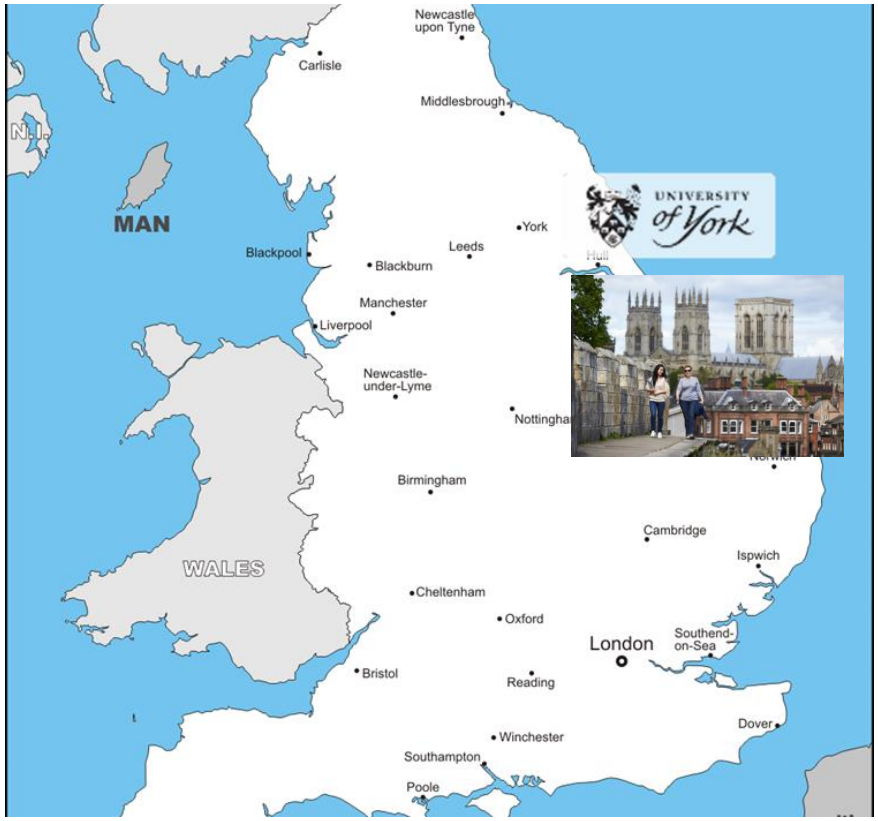
Julie Tucker

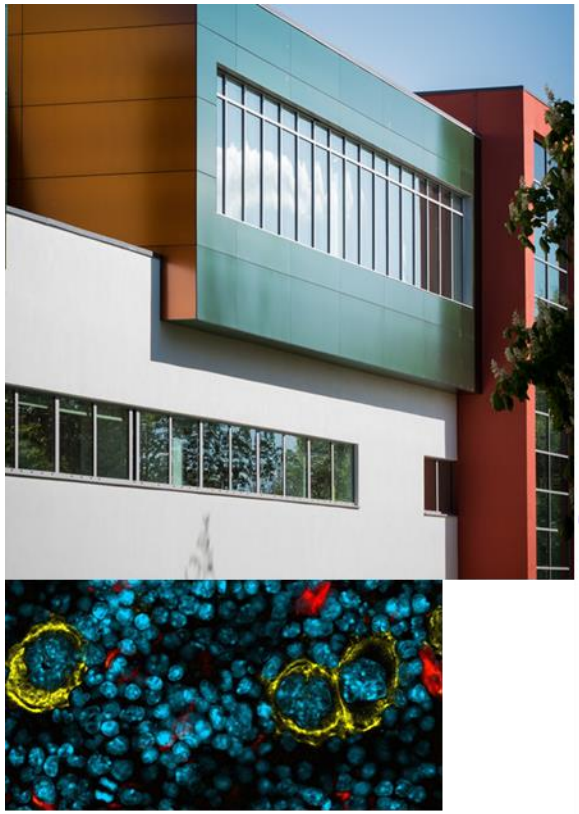
LINXS membrane protein workshop

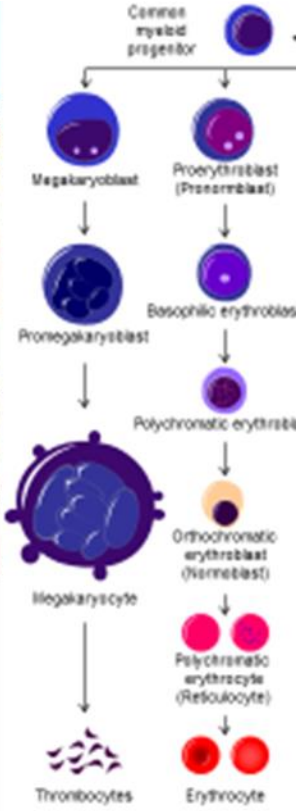
26th May 2021

“ALiCE® adventures in membrane-protein-land…”

The Hitchcock Lab at the University of York







Department of Biology, T block / Megakaryocytes and macrophages in healthy bone marrow / Extract from 'Haematopoiesis in Humans'

Image credits: University of York / John Houlihan / Alex Holland / Joanna Greenman / Motofolio

TPO and MPL, the 'master regulators' of platelet production

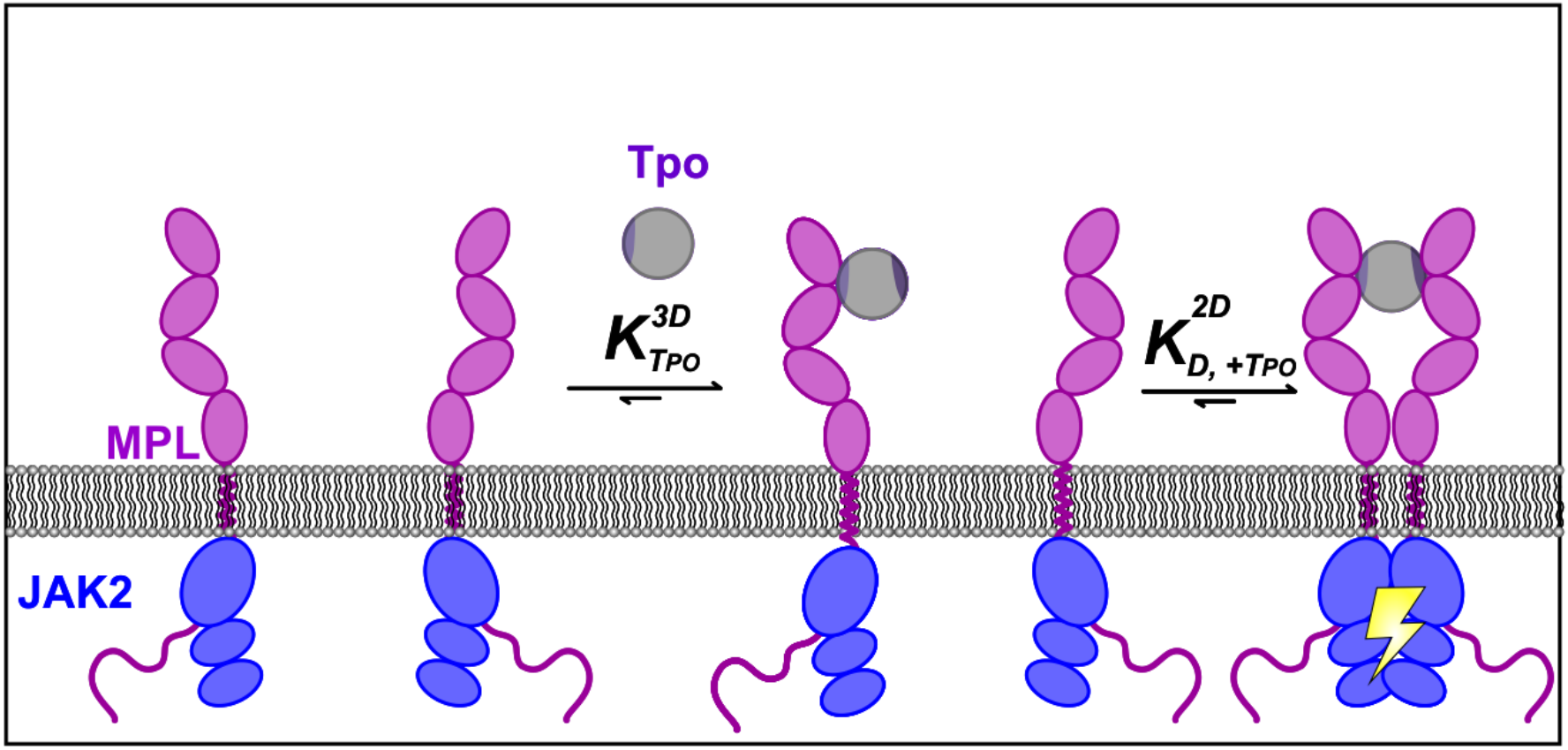
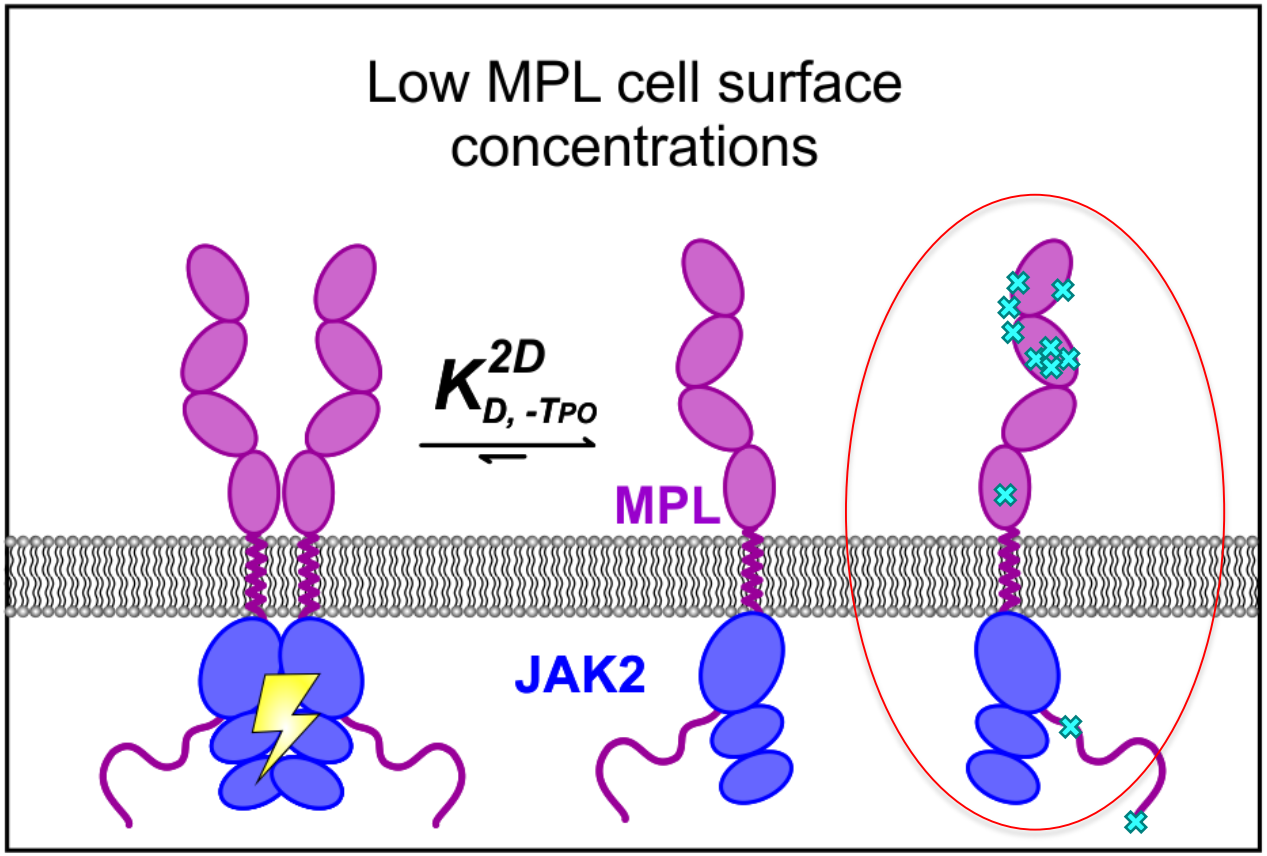


Figure adapted from Hitchcock *et al.*, Platelets, *in press*

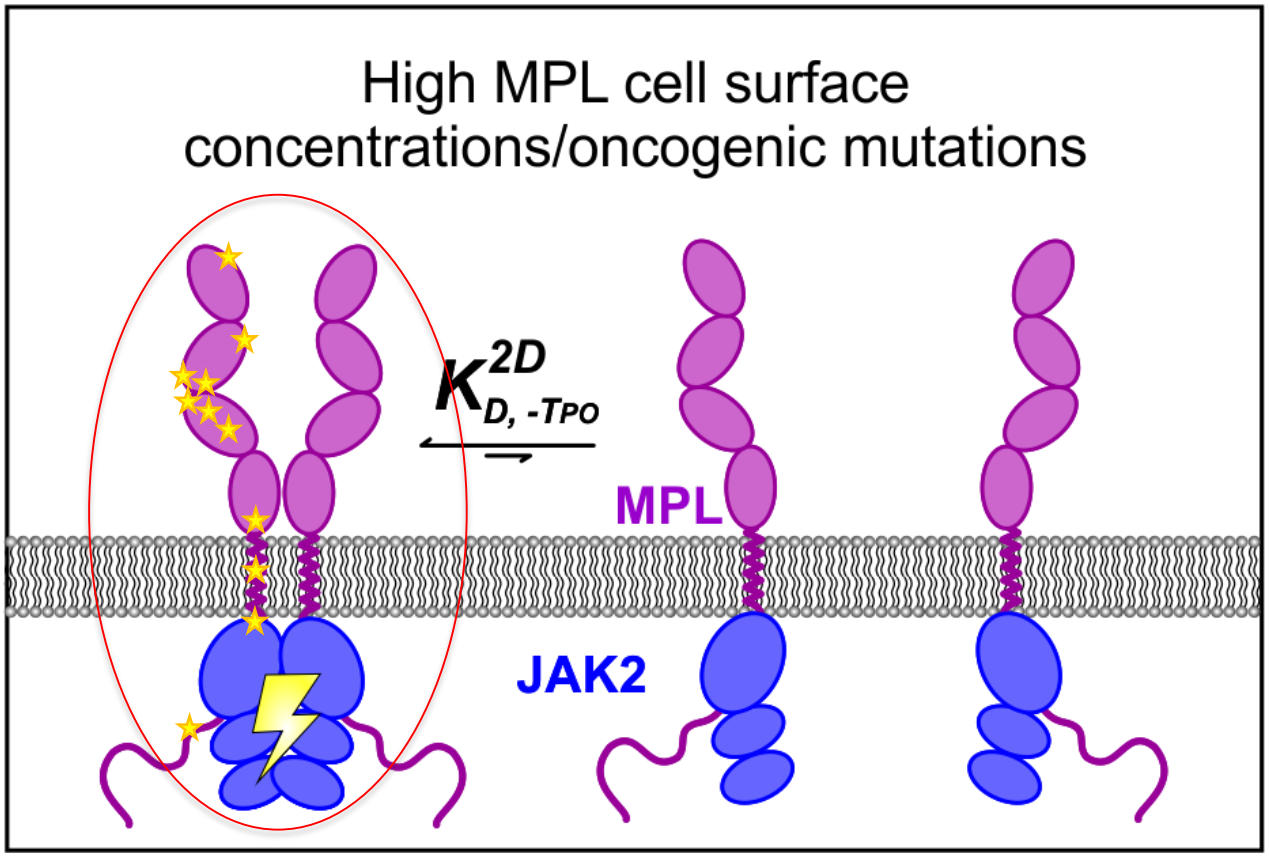
Mutations in MPL underlie haematological diseases: loss of function



- Reduced platelet count (thrombocytopenia)

Figure adapted from Hitchcock *et al.*, Platelets, *in press*

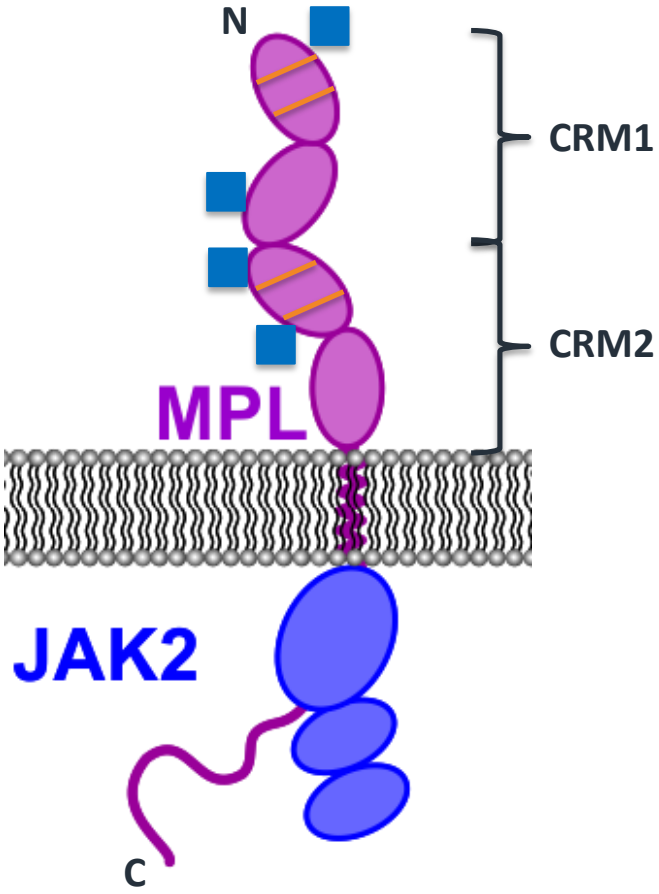
Mutations in MPL underlie haematological diseases: gain of function



- Elevated platelet count (essential thrombocythaemia)
- Bone marrow hyperplasia (primary myelofibrosis)

Figure adapted from Hitchcock *et al.*, Platelets, *in press*

Let's look at MPL in a bit more detail…



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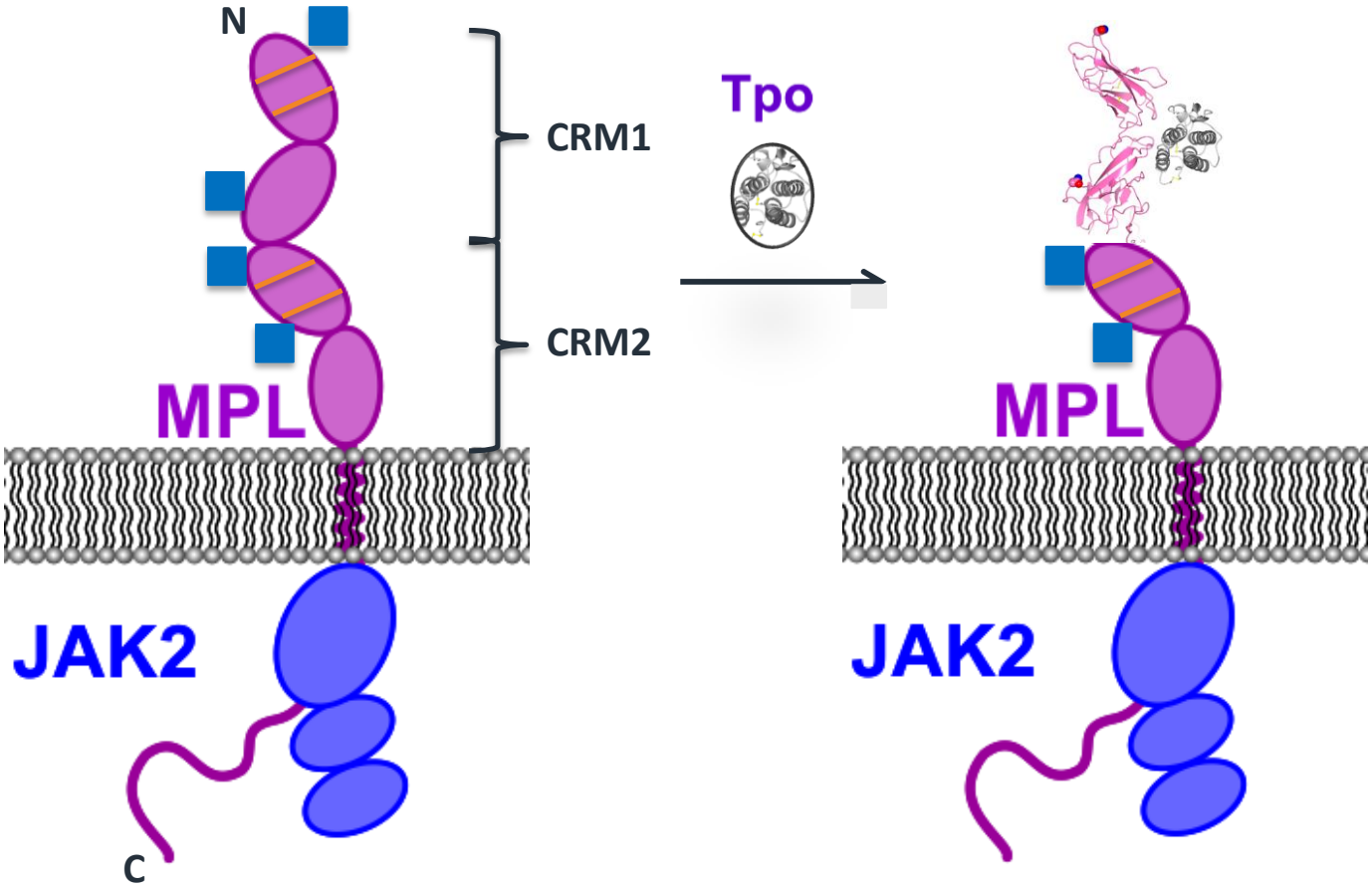

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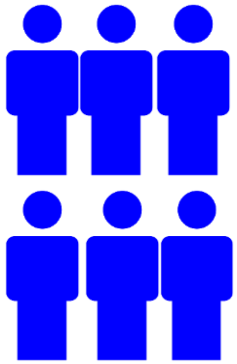
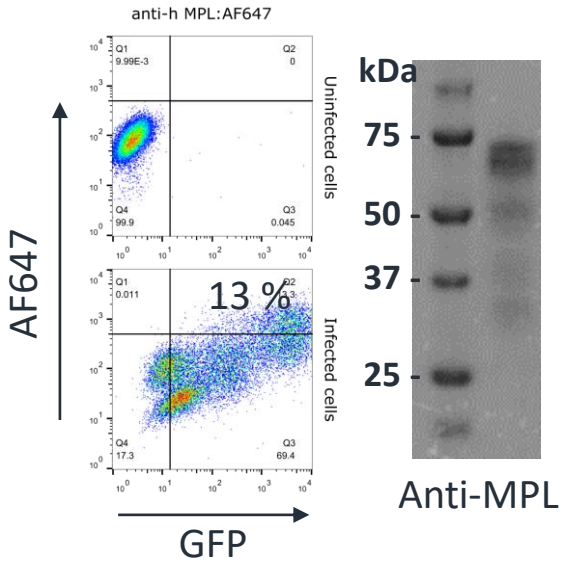
How can we make MPL for structural studies?

Physiological expression level ≤ 2 receptors/µm^2



Platelets from
6 people
(or 1 cow)

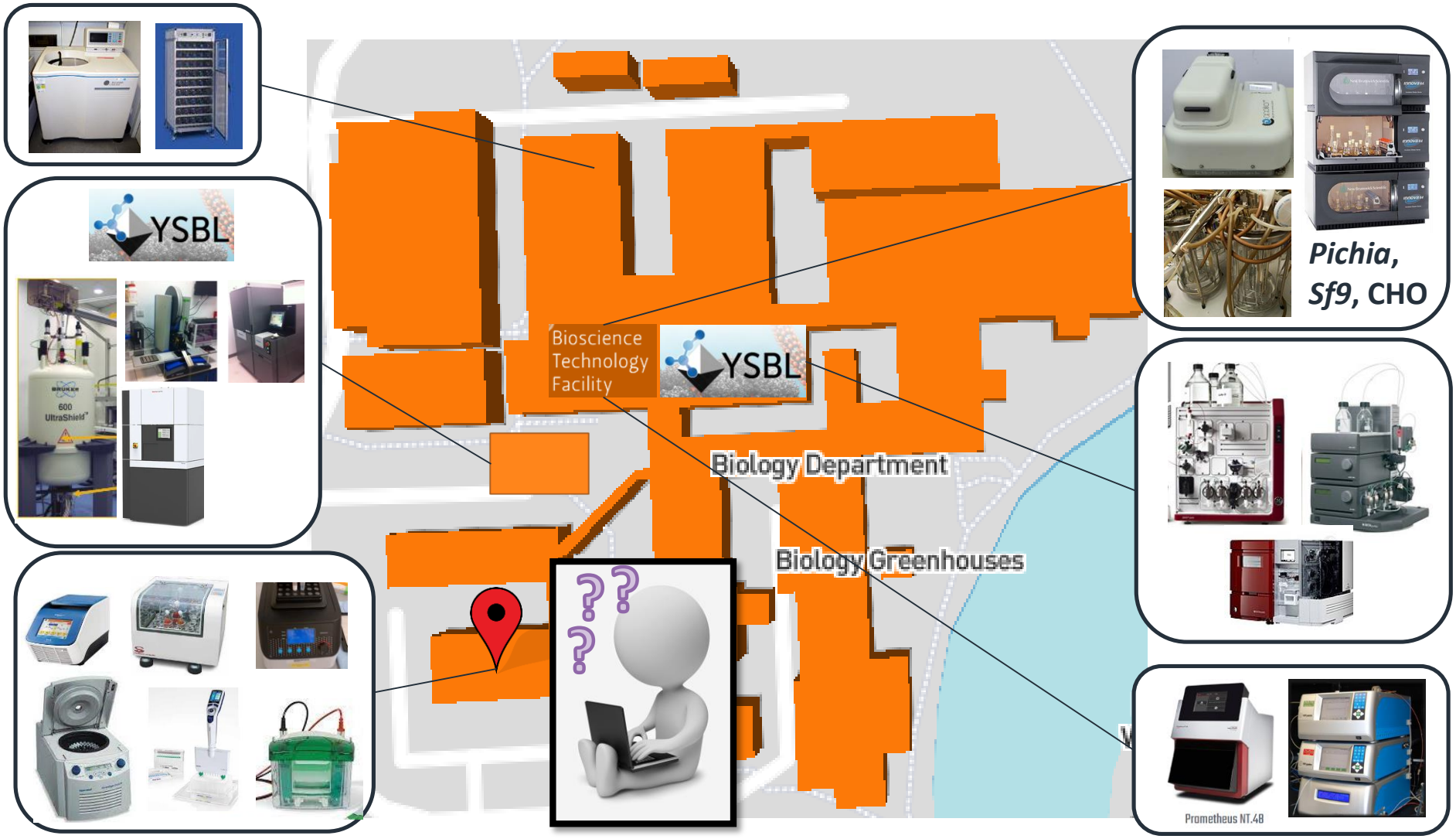
10 L Ba/F3 cell
culture



Baculovirus-infected
Sf9 insect cells

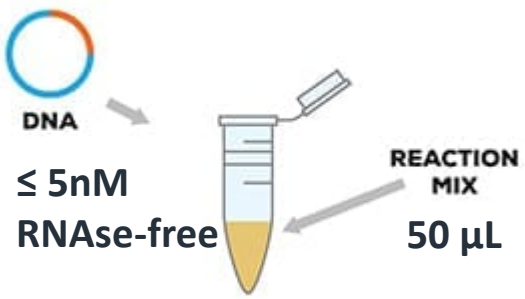
Yield?

Why use a cell-free protein synthesis system?



Who (or what) is ALiCE?

A tobacco BY-2 cell-based cell-free protein synthesis system



BY-2 lysate - intact organelles provide continuous energy supply and promote folding and glycosylation

Image credit: LenioBio GmbH

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A plate-based set up is also possible

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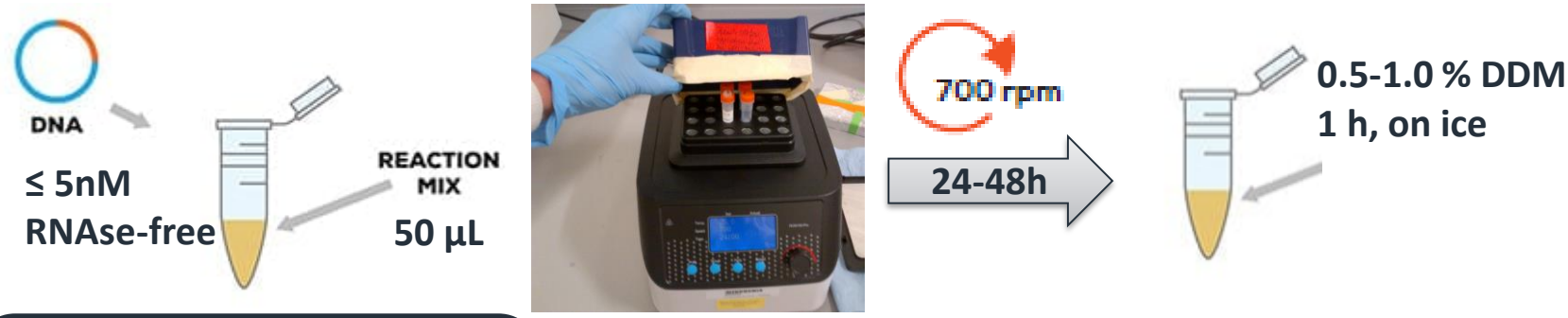

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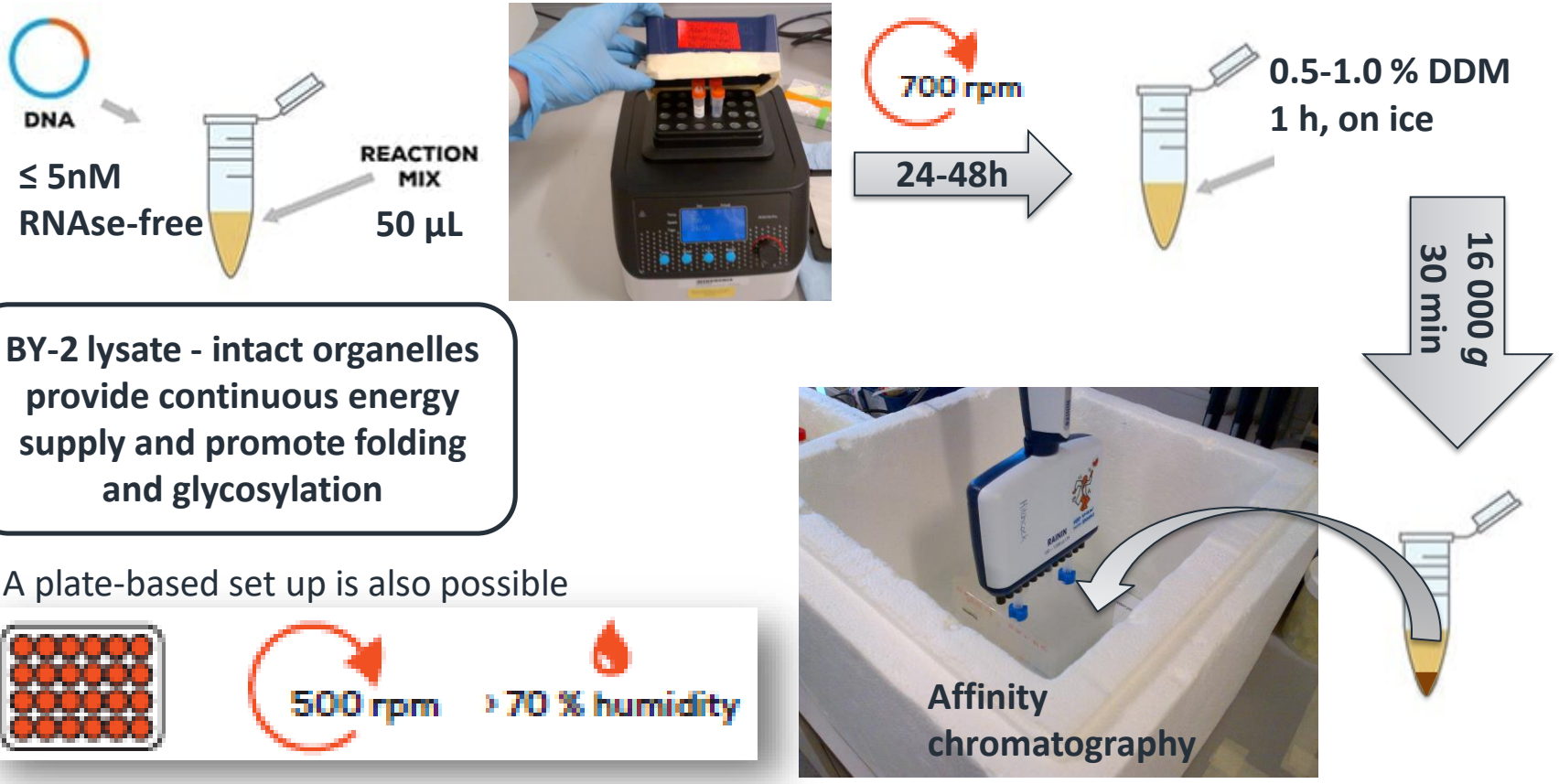

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A plate-based set up is also possible

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Step 1: sub-clone into pALiCE2

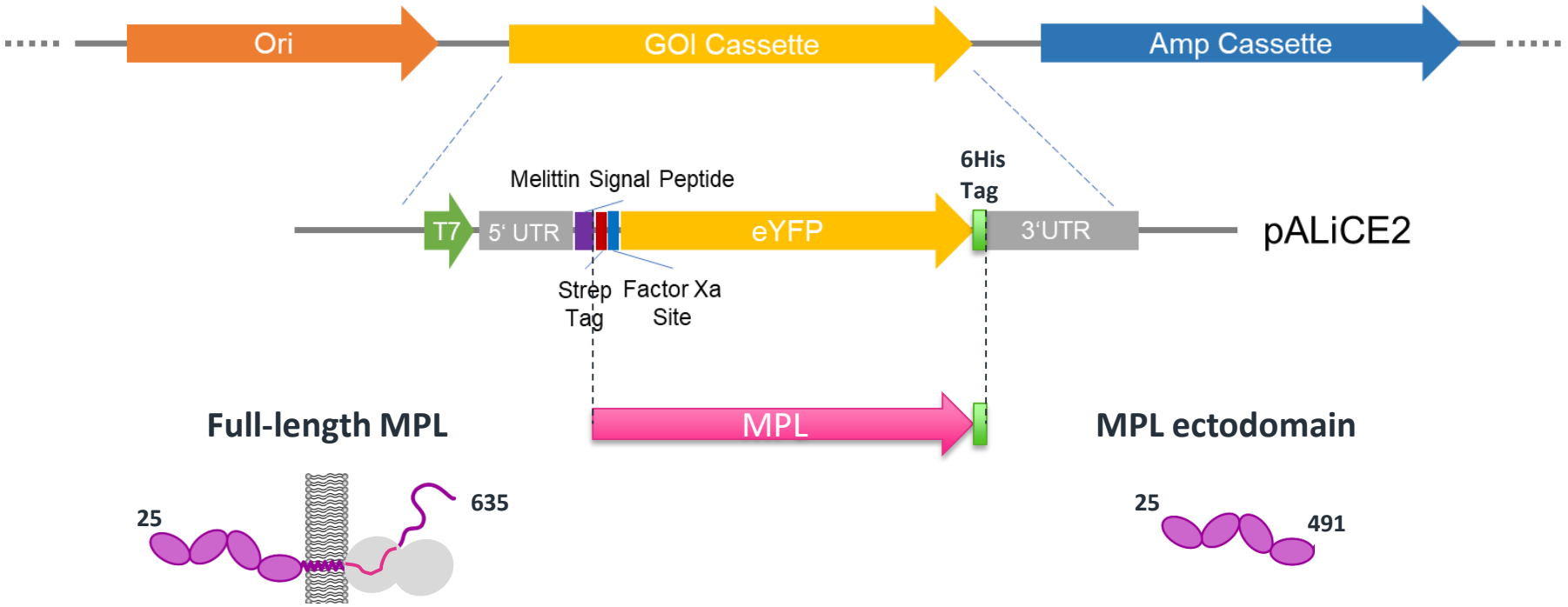
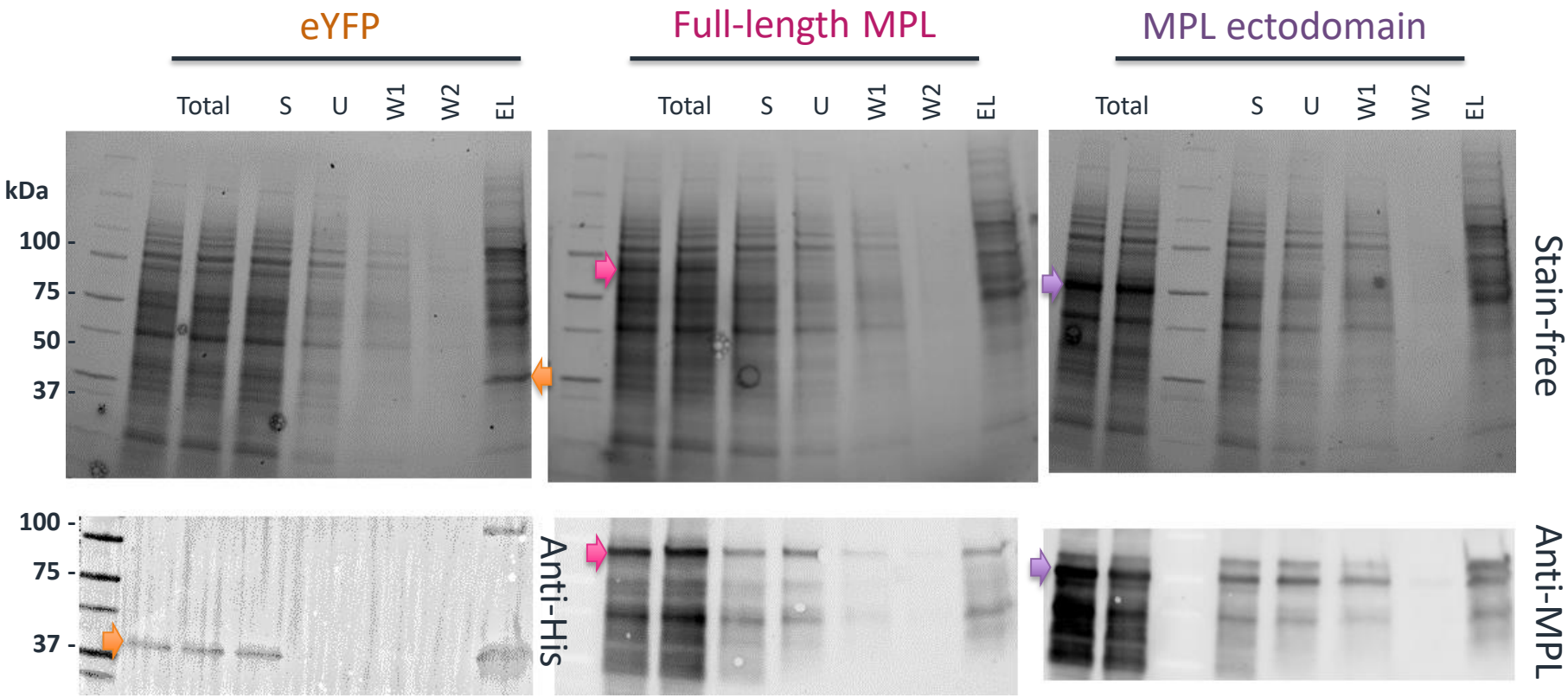


Figure adapted from DasGupta *et al.*, *in preparation*

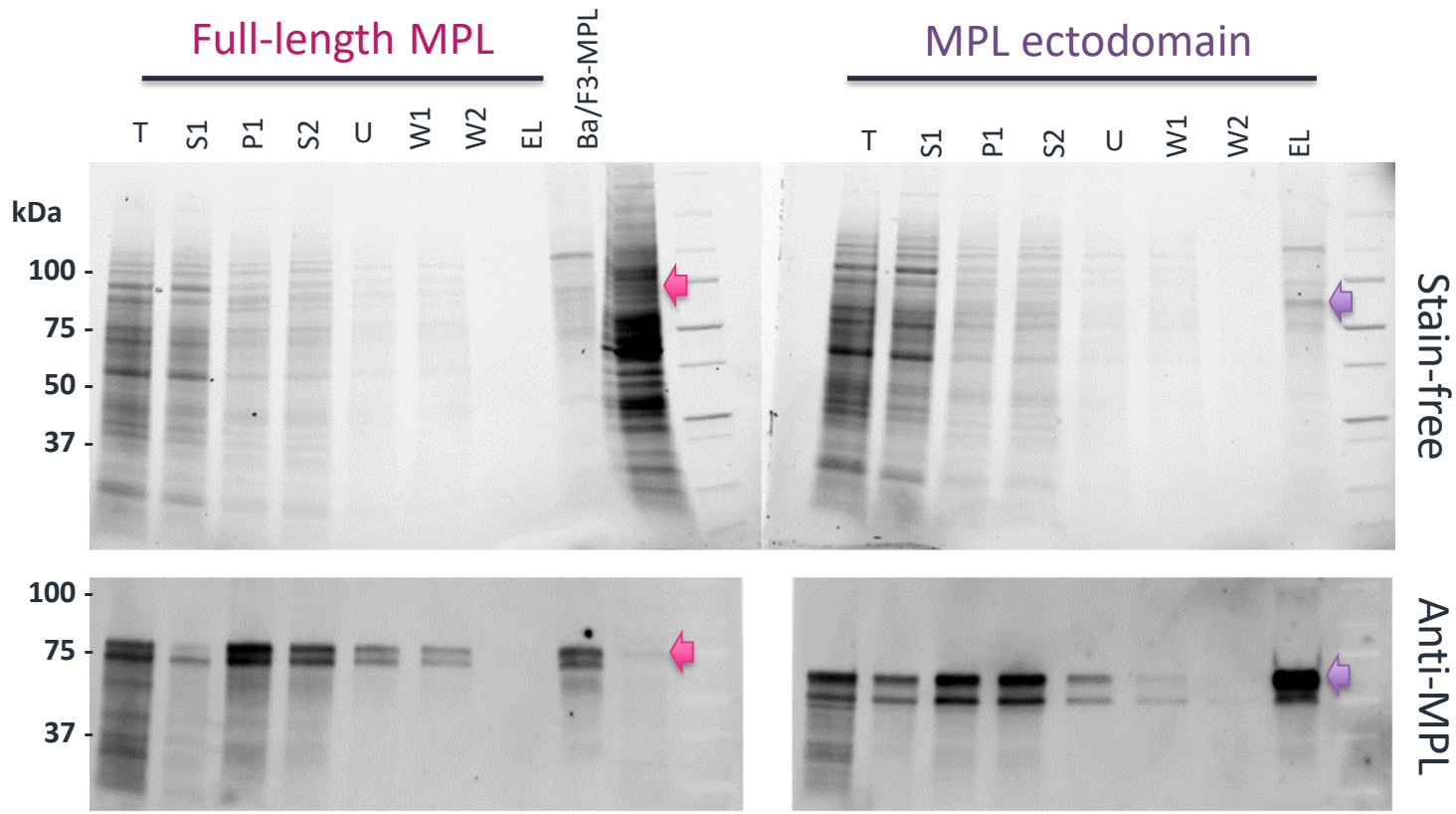
Step 2: test ‘expression’



Initial conditions = 48 h + 5 nM plasmid; detergent solubilisation of total reaction

- Full-length MPL and ectodomain visible in total sample!
- BUT largely insoluble and many truncated products

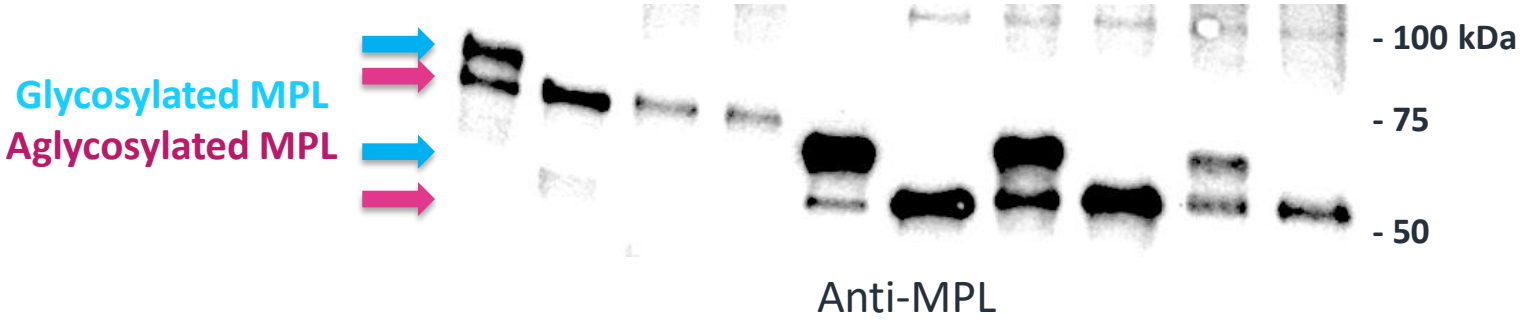
Step 3: optimise reaction conditions: 'less is more'



- Shorter reaction time (24 h) and lower [plasmid] (2.5 nM) increases yield of intact protein in the detergent solubilised fraction.
- Additional microsome isolation step improves purity.

ALiCE MPL is *N*-glycosylated

Construct	Full-length				Ectodomain					
Reaction time, h	24h		48h		24h				48h	
[plasmid], nM	2.5		5		2.5		5		5	
PNGaseF	-	+	+	-	-	+	-	+	-	+

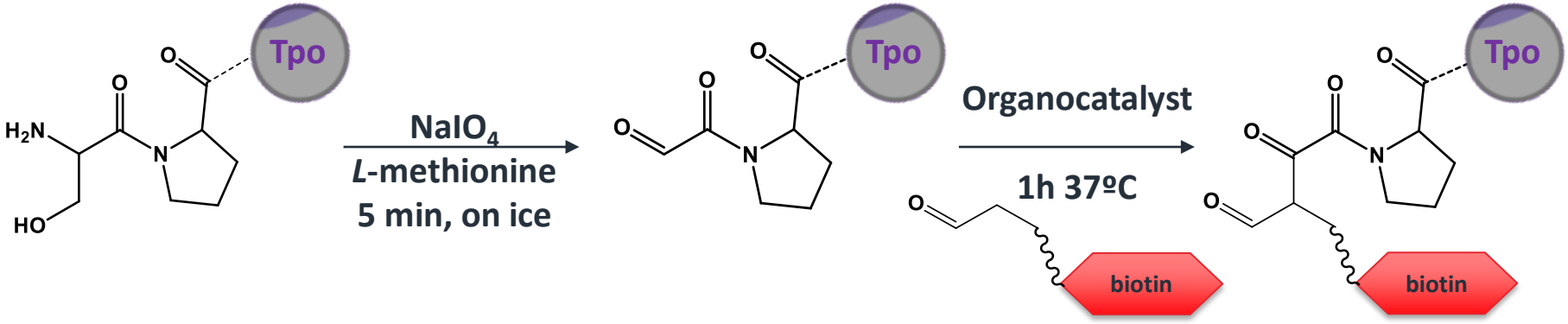


'Less is more'!

- Glycosylation inversely proportional to reaction time and [plasmid]
- Maximal levels > 50 % for full-length and > 90 % for ectodomain

Is ALiCE MPL functional?

1. Streptavidin pull-downs with biotinylated TPO



For more details of the OPAL conjugation method, see Spears *et al.*, Chem. Sci., 2018

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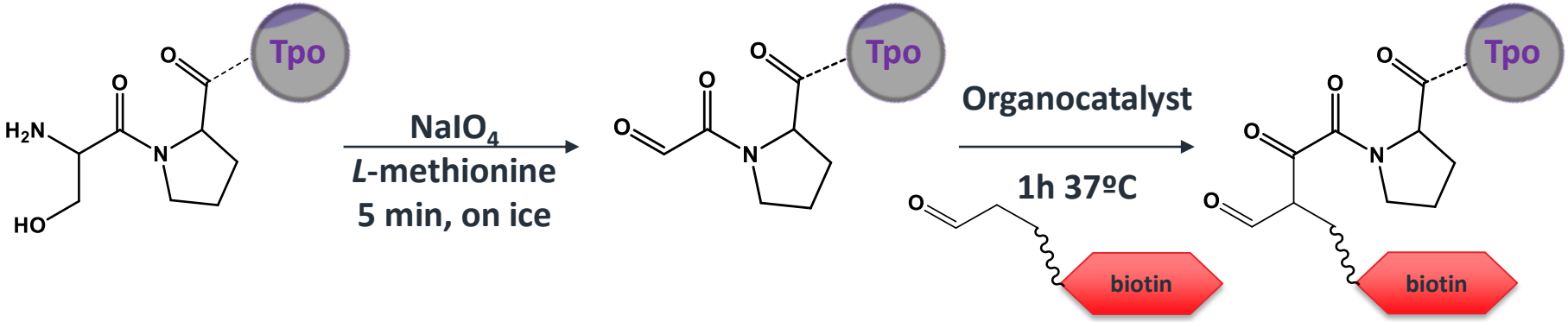
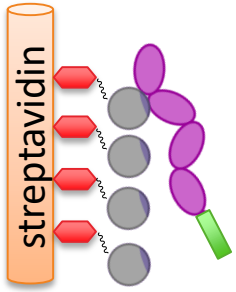
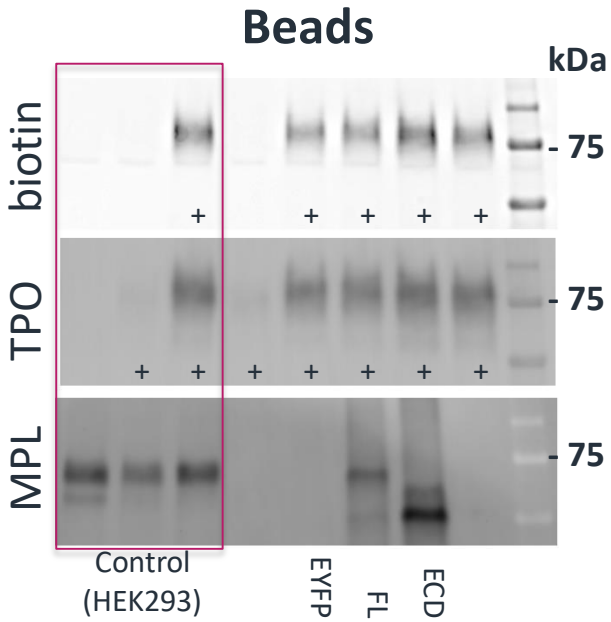
1. Streptavidin pull-downs with biotinylated TPO





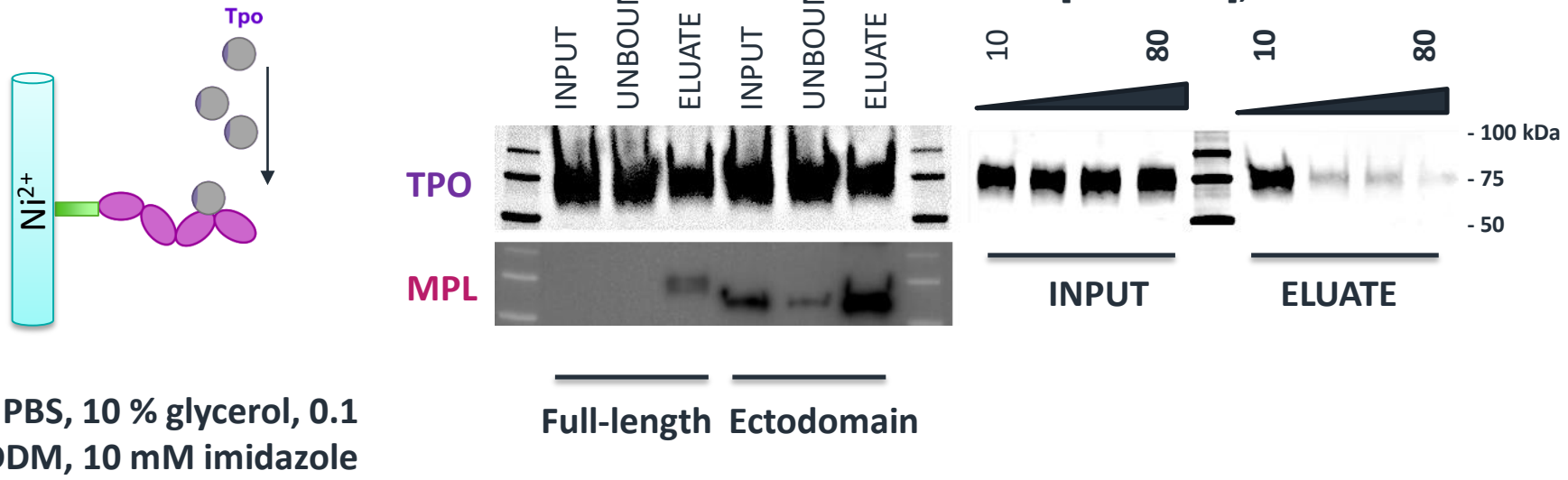
1h pre-incubation (4ºC)
Batch binding (1h, 4ºC)
1 X PBS, 10 % glycerol, 0.1 % DDM, ≤ 25 mM imidazole



MPL binds to streptavidin beads in absence of TPO!

Is ALiCE MPL functional?

2. IMAC pulldowns of untagged TPO with His-tagged MPL

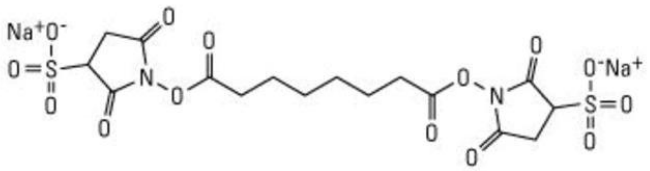


1 X PBS, 10 % glycerol, 0.1 % DDM, 10 mM imidazole

- TPO binds to IMAC resin in the absence of MPL.
- Non-specific binding is reduced in the presence of ≥20 mM imidazole.

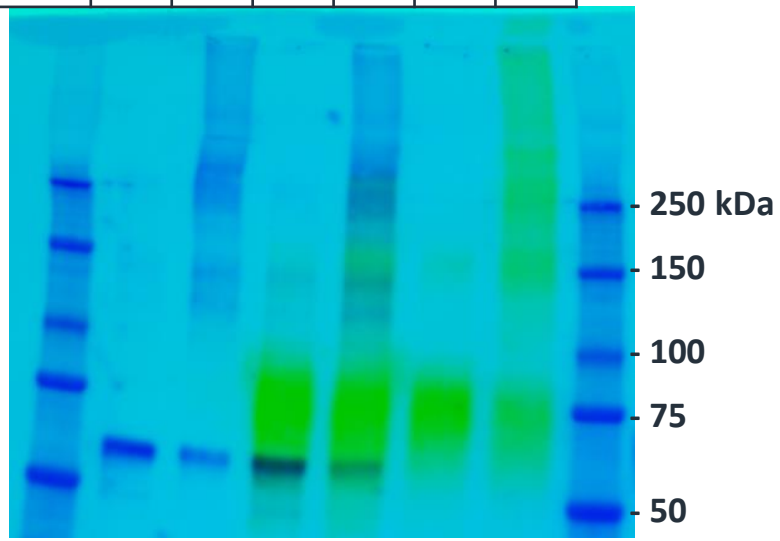
Is ALiCE MPL functional?

3. Chemical cross-linking of MPL and TPO



BS3
Bis(sulfosuccinimidyl) suberate
MW 572.43
Spacer Arm 11.4 Å

MPL	+	+	+	+	-	-
TPO	-	-	+	+	+	+
BS3	-	+	-	+	-	+



- **0.25 mM BS3 + 1.5 µM TPO + 3 µL MPL ectodomain in 1 X PBS**
- **60 min, RT**
- **Ammonium bicarbonate quench**

- Higher molecular weight species apparent even in absence of binding partner

Next steps

- Is ALiCE® MPL monodisperse (and monomeric)?
- Optimise pull-down assays and/or…
- Label TPO with OPAL-fluorescein for functional assay development.
- Further purify glycosylated ALiCE® MPL using concanavalinA Sepharose.
- Co-expression of MPL with TPO and JAK2 to make holo-signalling complex…

+ Lots more ideas from this workshop!
  - (fSEC, detergent/SMA/lipid screening, alternative tags…)

Summary

- Using the ALiCE® cell free system, full-length MPL and its ectodomain are:
  - ✓ Expressed into microsomal compartment
  - ✓ Solubilised with DDM
  - ✓ Glycosylated
  - ✓ Partially purified via 6His tag
  - ✓ Yield 5-30 µg / mL (ectodomain)*
  - ? Functional?

Watch this space!

* reassessed post workshop based on a 0.2 mL synthesis reaction

Acknowledgements

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Cristina Viola

University of York Department of Chemistry
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Martin Fascione
Natasha Hatton
Jenny Hayes
Amanda Noble
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University of Reading
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Joanne Walter

We are recruiting!

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