Camelid single domains antibodies: small but mighty

Assunta Senatore

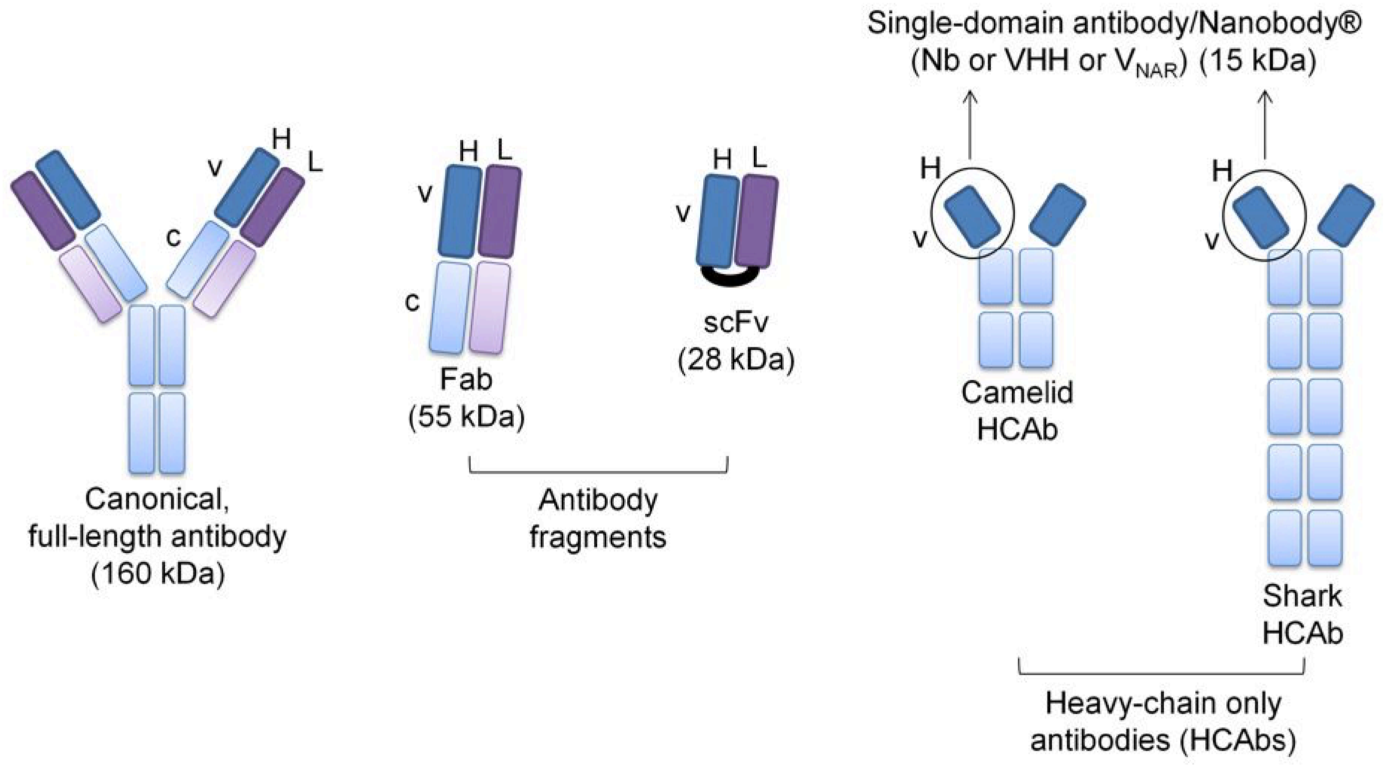
July 2nd 2019

Outline

- Features and brief historical overview
- Nanobodies as therapeutic tools
- Novel platform for nanobody generation
- Use of nanobodies as research tools

Credit: Hennadii/iStock/Getty Images

Traditional antibodies and their fragments vs nanobodies (Nbs).



Compared to regular antibodies (160 kDa) and their fragments (Fab – 55 kDa; scFv – 28 kDa), the molecular size of single-domain Nbs is the smallest (15 kDa).

Nbs can be derived from the heavy-chain only antibodies (HCAbs) that occur naturally in camelids (VHH) and sharks (V_{NAR}) (v-variable, c-constant, H-heavy chain, L- light chain).

Doshi R., Scientific reports, 2014

Characteristics

- HCAbs are produced from the same *igh* locus as conventional Abs but with distinct sets of genes. A point mutation disrupts the splicing site and causes omission of CH1 exon.
- no CH1 domain > a direct connection of the rearranged V_HH exon to the hinge region;
- different amino acid composition and a broader length distribution for CDR3 .
- HCAbs repertoire diversification involves:
  - a large number of VHH gene segments recombining with DH and JH minigenes, with additional non-templated nucleotide insertions leading to longer CDR3 loops;
  - somatic hypermutation of extended CDR1 regions
- HCAbs antigen binding:
  - involvement of FR2 residues in antigen binding and in structuring the CDR3 loop
  - due to the loss of VL domains, VHH paratopes display a higher structural complexity by involving more residues in antigen binding compared to classical VHs.

Pros

- VHHs Small size: 12-30 kDa, 2.5 nm in diameter and about 4 nm in length
- High affinity and specificity
- better stability and solubility
- higher penetration rate into tissues
- Binding to targets that were out of reach to full-length Ab: intracellular targets, GPCR etc
- suited for cytosolic expression as “intrabodies,” due to their ability to fold in the reducing intracellular environment (single disulfide linkage present in the VHH domain)
- low immunogenicity
- Lego-like modularity>> bi and multi-specific Ab
- technical advantages of the camelid VHH platform over conventional antibody platforms:
    a) direct cloning of VHH repertoires from immunized camelids
    b) smaller library sizes required to capture the immune VHH repertoire
    c) feasibility of displaying VHHs on display formats
    d) ease of sub-cloning and expression of antigen-specific VHHs

Cons

- ❖ **Short half-life** of VHHs in blood circulation
  well suited to applications such as tumor imaging or delivery of toxin/radioisotopes to diseased tissues where rapid clearance is required.
  The pharmacokinetic of VHHs can be improved by different formatting options (i.e. PEGylation or fusion to serum albumin).

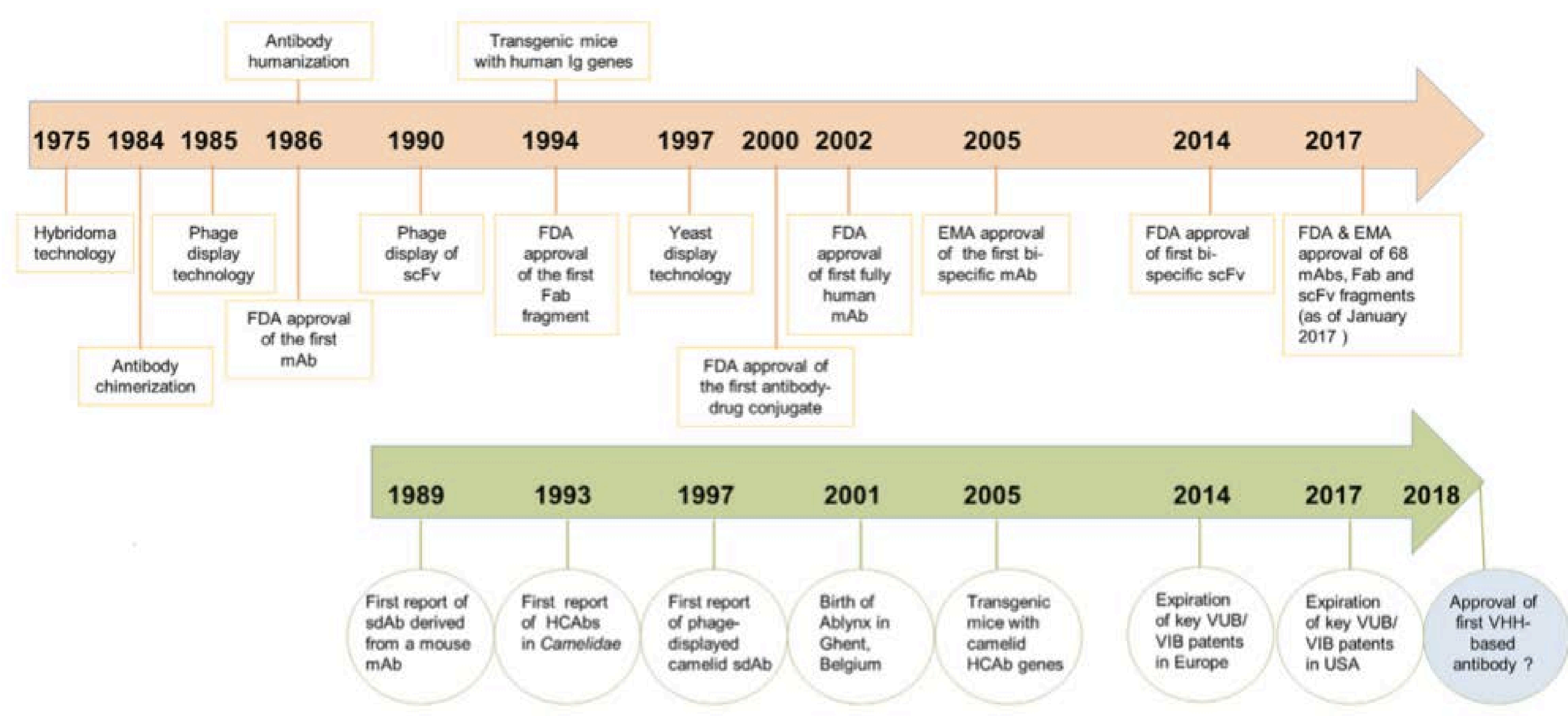
- ❖ **Limited engineering** that can be tolerated by antigen-specific VHHs.
  Complete humanization of camelid VHHs involving the mutation of residues outside the antigen-binding loops often compromises antigen-binding affinity, VHH stability, the expression yield and specificity.

- ❖ **Low propensity to bind small molecules**, likely due to their dominant convex surface topology as compared to the flat or concave topologies of conventional Ab fragments.

- ❖ **Immunization of large animals** and heterogeneity in immune responses among individual outbred animals is another consideration which is important when alternative immunization techniques such as DNA immunization are applied.

  To overcome this limitation, transgenic mice bearing either a rearranged dromedary γ2a chain or hybrid llama/human antibody loci have been generated that produce a form of dromedary or human heavy chain antibodies.

Chronological timeline in the field of antibody engineering: Developments of mAbs and VHHs/heavy chain-only antibodies (HCAbs)



The serendipitous discovery of HCAbs occurred as part of a student-run project aimed at developing a serodiagnostic test for trypanosome infection in camels.
Besides conventional IgG1 (150 kDa), two other immunoglobulin fractions (IgG2 and IgG3; ~90 kDa) were present which contributed up to 75% of all serum IgGs.

Outline

TABLE 1 | SELECT LIST OF DOMAIN ANTIBODIES IN DEVELOPMENT

Drug	Sponsor	Domain properties	Target	Indication	Status
Caplacizumab	Sanofi (Ablynx)	V_{HH}	vWF	aTTP	Approved
Ozoralizumab	Taisho (Ablynx)	V_{HH}	TNF	Rheumatoid arthritis	Phase III
M1095	Merck KGaA (Ablynx)	V_{HH}, bispecific	IL-17A, IL-17F	Psoriasis	Phase IIb
Vobarilizumab	Sanofi (Ablynx)	V_{HH}	IL-6R	Rheumatoid arthritis	Phase II
LCAR-B38M	Legend/Janssen	V_{HH}, incorporated into a CAR-T	BCMA	Multiple myeloma	Phase II
V565	VHsquared	V_{HH}	TNF	Inflammatory bowel disease	Phase II
M6495	Merck KGaA (Ablynx)	V_{HH}	ADAMTS5	Osteoarthritis	Phase I
BI 836880	Boehringer Ingelheim (Ablynx)	V_{HH}, bispecific	VEGF, Ang2	Solid tumours	Phase I
BI 655088	Boehringer Ingelheim (Ablynx)	V_{HH}	CX_3CR1	Renal disease	Phase I
AD-214	AdAlta	i-body	CXCR4	Idiopathic pulmonary fibrosis	Preclinical
TXB4	Ossianix	VNAR, with mAb payload	TfR1	Primary CNS lymphoma	Preclinical

Source: BioMedTracker

- In February 2019 the FDA approved Sanofi's caplacizumab for acquired thrombotic thrombocytopenic purpura (aTTP), a rare disease characterized by excessive blood clotting in small blood vessels.
  Caplacizumab is the first domain antibody to be approved by the FDA.
  It is the first drug approved for this disease and the first to target von Willebrand factor (vWF).

Universal protection against influenza infection by a multidomain antibody to influenza hemagglutinin

Nick S. Laursen[1]*, Robert H. E. Friesen[2]†, Xueyong Zhu[1], Mandy Jongeneelen[3], Sven Blokland[3], Jan Vermond[4], Alida van Eijgen[4], Chan Tang[3], Harry van Diepen[4], Galina Obmolova[2], Marijn van der Neut Kolfschoten[3], David Zuijdgeest[3], Roel Straetemans[5], Ryan M. B. Hoffman[1], Travis Nieusma[1], Jesper Pallesen[1], Hannah L. Turner[1], Steffen M. Bernard[1], Andrew B. Ward[1], Jinquan Luo[2], Leo L. M. Poon[6], Anna P. Tretiakova[7]‡, James M. Wilson[7], Maria P. Limberis[7], Ronald Vogels[3], Boerries Brandenburg[3], Joost A. Kolkman[8]§, Ian A. Wilson[1,9]§

use of diverse camelid single-domain antibodies to influenza virus hemagglutinin to generate multidomain antibodies with impressive breadth and potency

Laursen et al., Science 362, 598–602 (2018) 2 November 2018

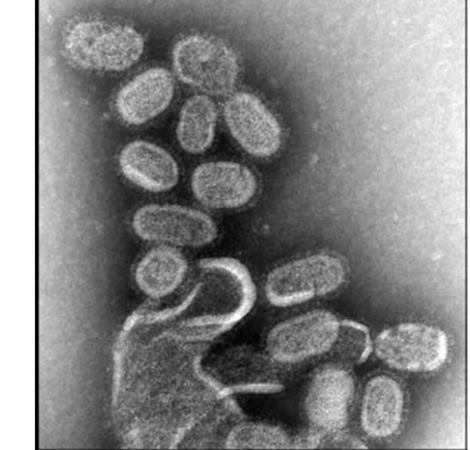
Challenges in influenza prophilaxis

Seasonal influenza epidemics cause world-wide morbidity and mortality, whereas the vast reservoir of influenza A viruses in aquatic birds represents continual pandemic threats.

Vaccines efficacy is substantially reduced in the elderly, who are at increased risk of influenza-related complications. Annual selection of vaccine strains presents many challenges, and a poor match with circulating viruses can result in suboptimal effectiveness.

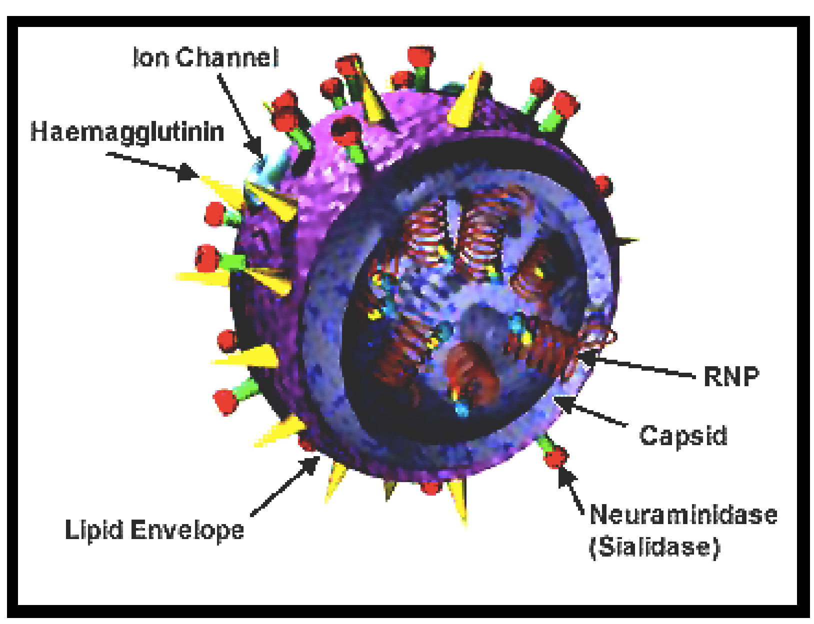
Most vaccine-induced antibodies are directed against the highly variable head region of hemagglutinin (HA) and are strain specific.

Broadly neutralizing antibodies (bnAbs) targeting influenza HA have been isolated.
However, they are limited by incomplete coverage against circulating human influenza A and B viruses, which necessitates administration of a bnAb cocktail, and the need for multiple, high-dose injections (poor distribution higher airways).



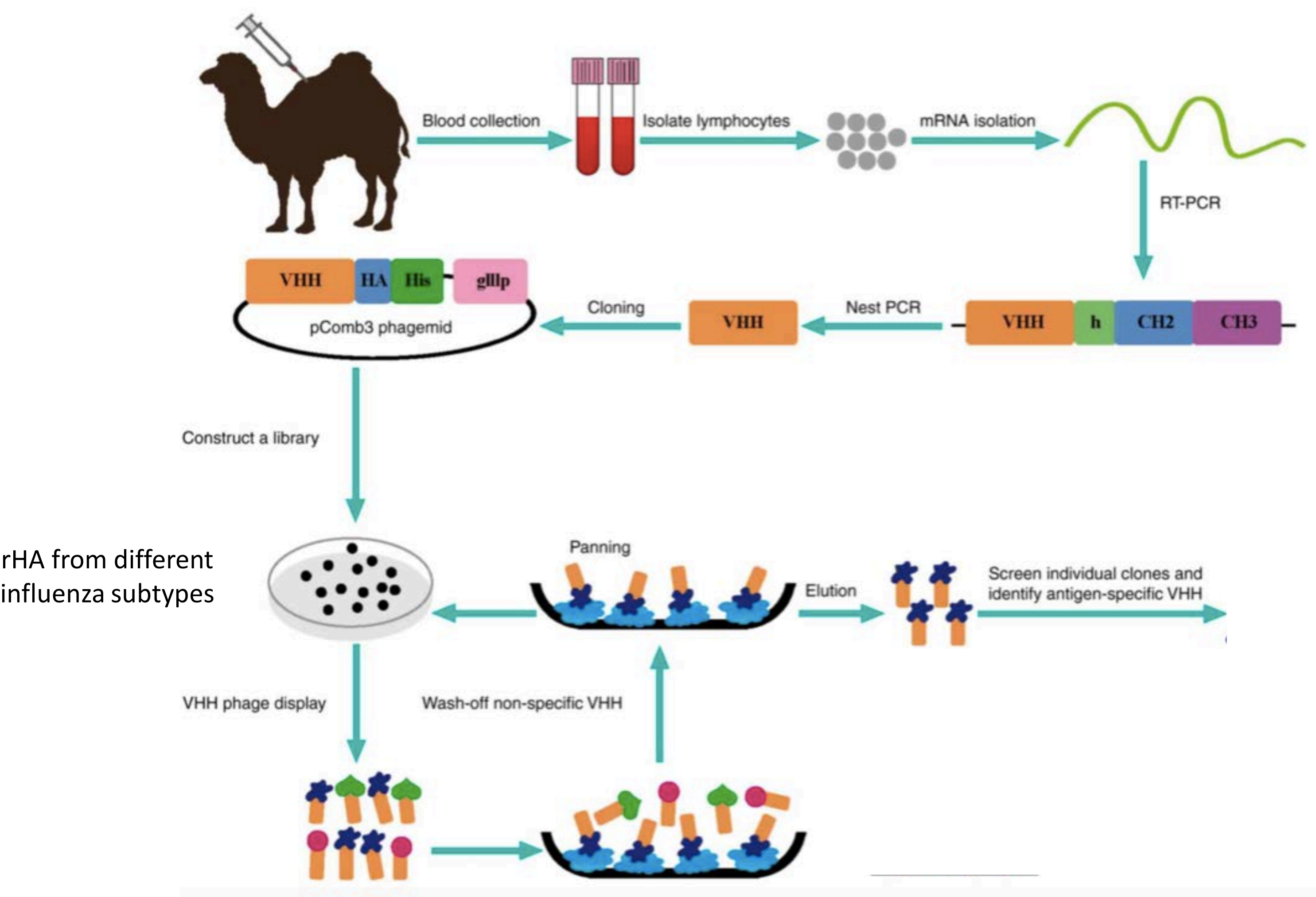
Linking Nanobodies into Multidomain antibodies For influenza prophilaxis

An alternative strategy for long-lasting protection in which single-domain antibodies with influenza A or B reactivity are linked together into a multidomain antibody (MDAb) and expressed at the nasopharyngeal mucosa through the intranasal administration of a recombinant adeno-associated virus (AAV) vector encoding the MDAb transgene.



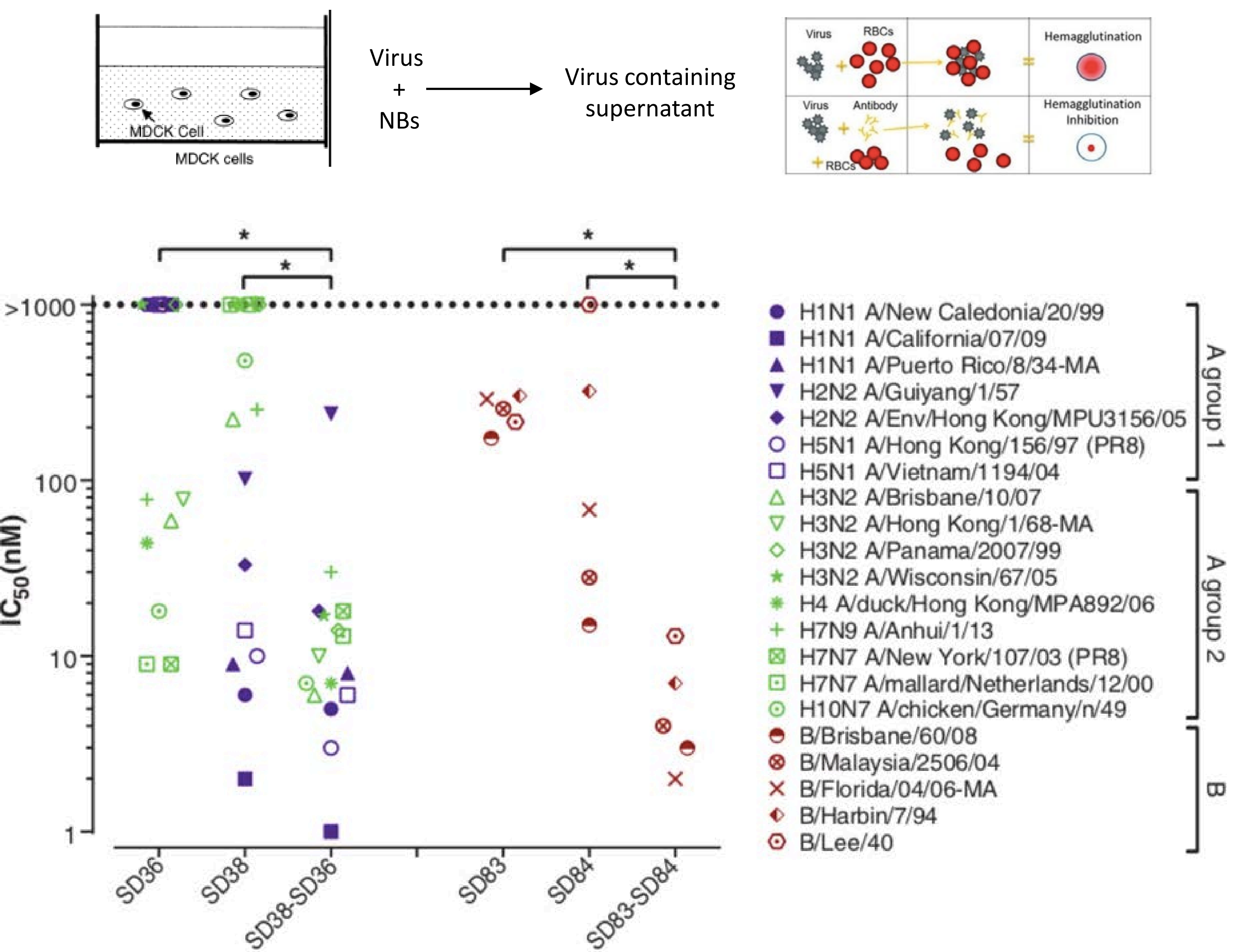
Animal immunization and phage display

rHA from different influenza subtypes

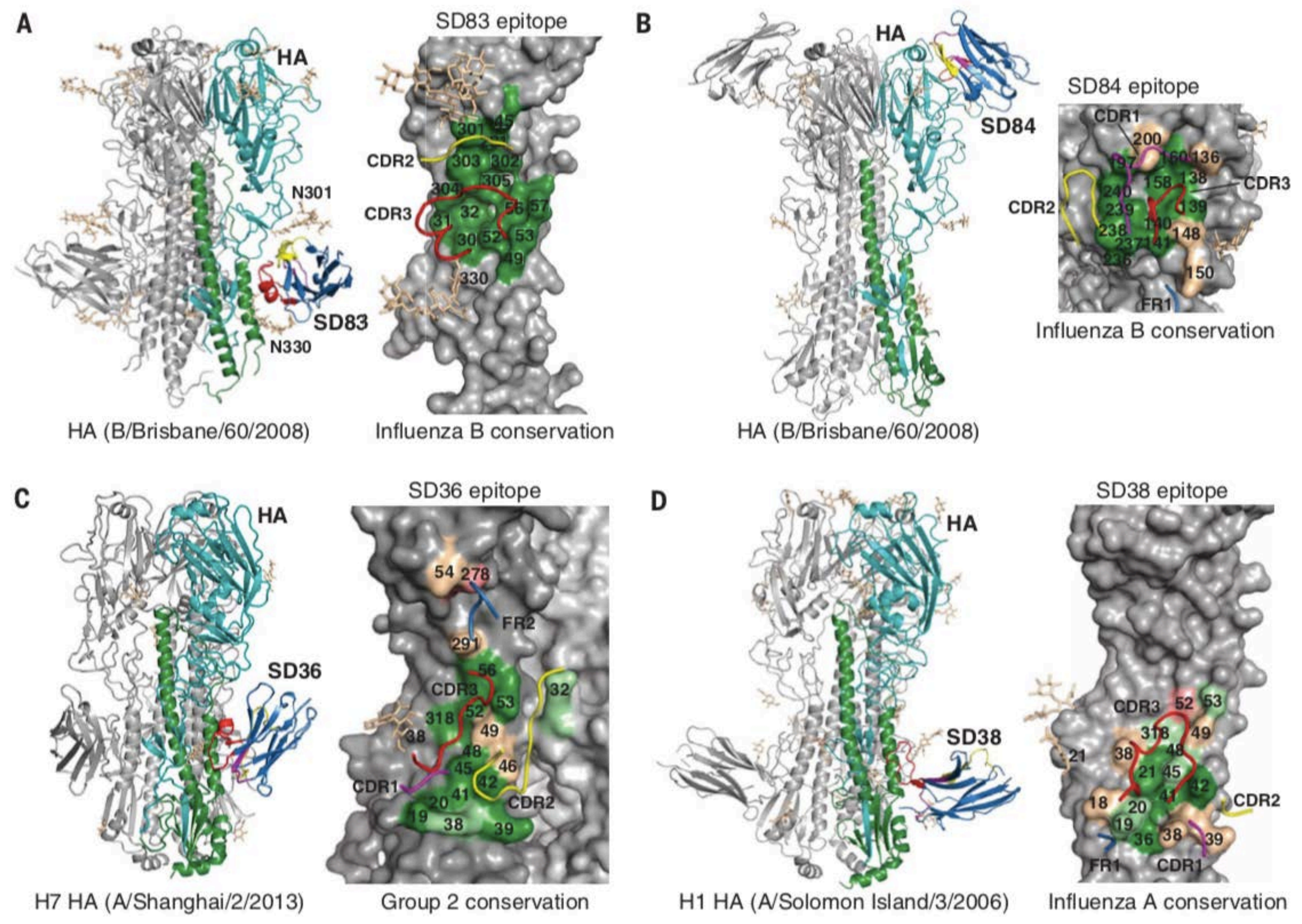


Adapted from Ruirong Wan 2017

In vitro neutralization of influenza A and B viruses by individual and genetically fused sdAbs

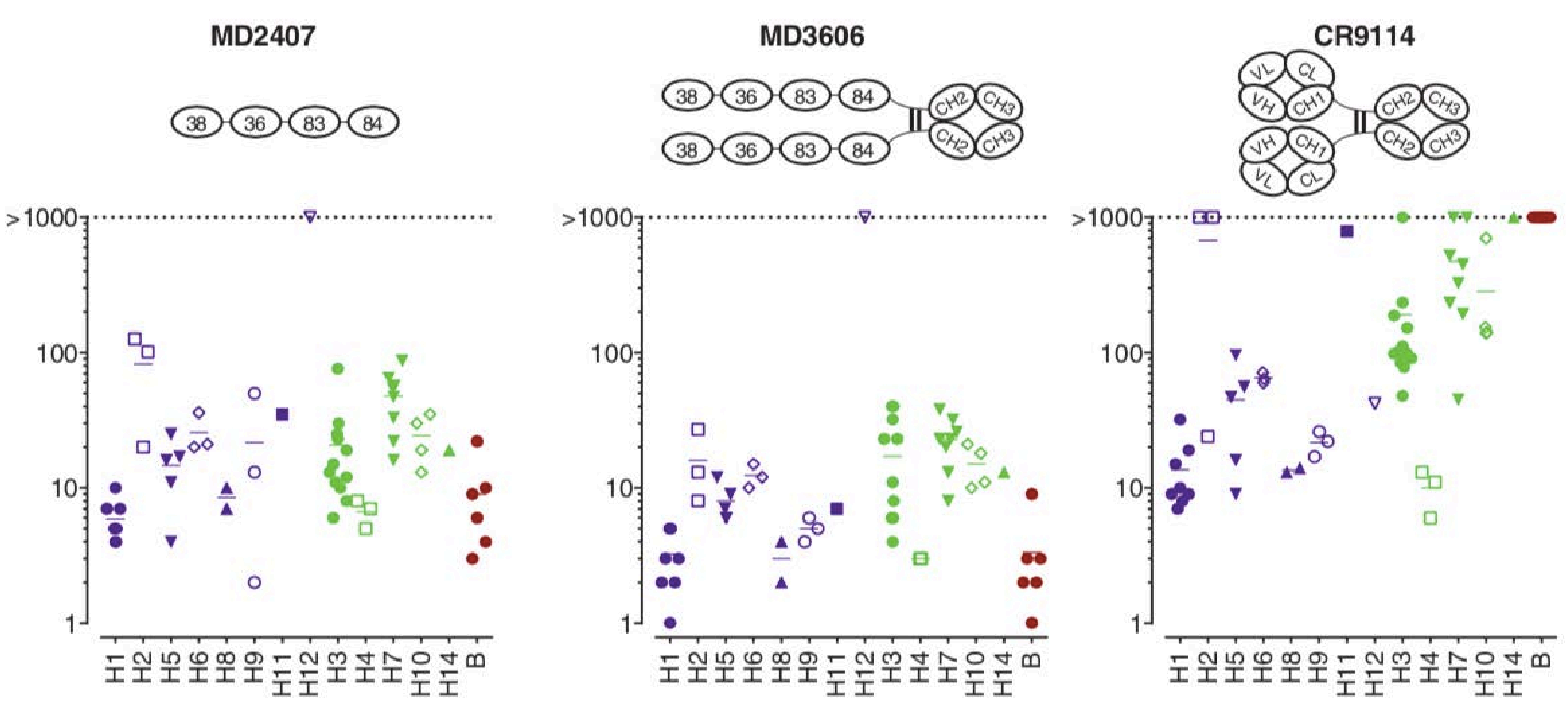


Crystal structures of sdAbs in complex with HAs and conservation of their epitopes

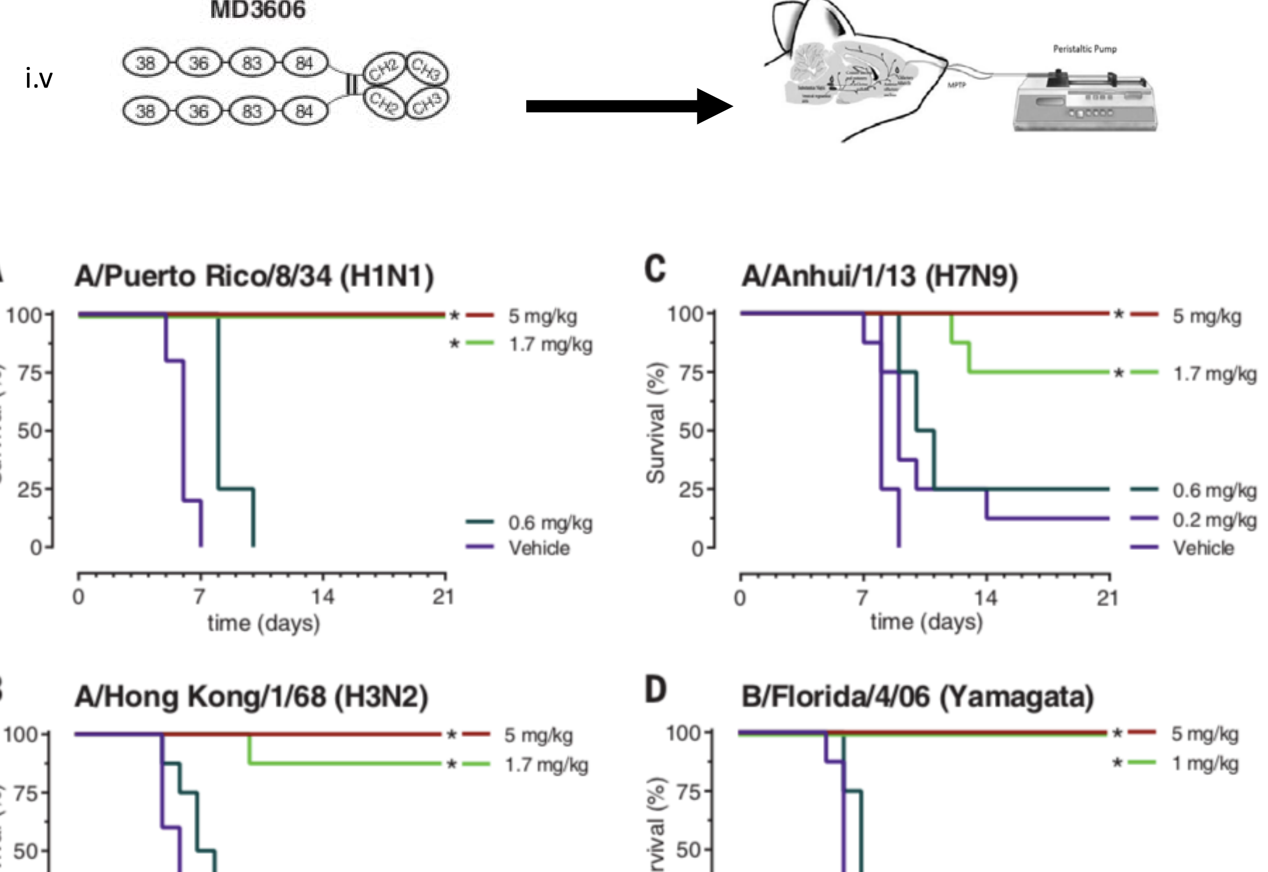


The selected Nbs recognized conserved epitopes in different regions of HA

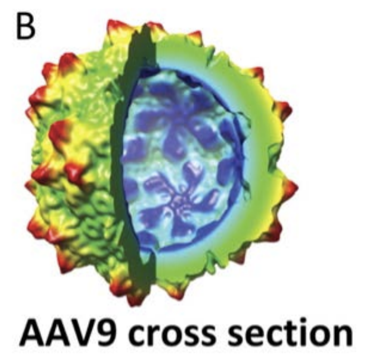
In vitro neutralization of influenza A and B viruses by pan-influenza MDAbs MD2407 and MD3606 versus CR9114



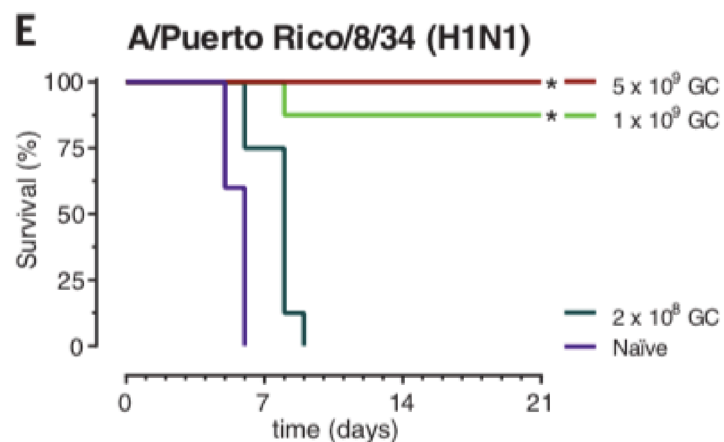
Prophylactic efficacy of recombinant MD3606 in mice challenged with influenza virus

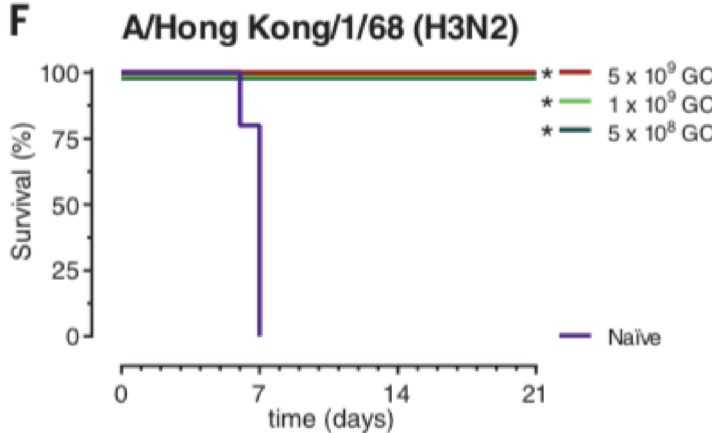


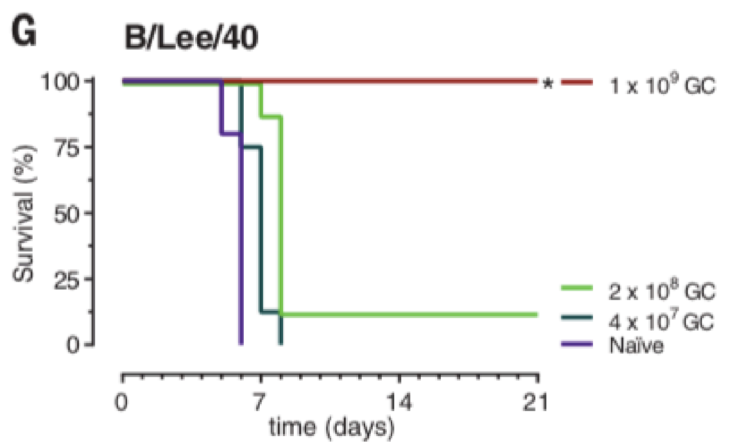
Prophylactic efficacy of AAV-expressed humanized MD3606 in mice challenged with influenza virus



AAV9 cross section







Highly potent MDAbs, having near-universal activity against influenza A and B viruses can both be expressed from a single AAV vector.

Intranasal delivery of AAV9.MD3606h provided full protection in mice at doses as low as 5×10^{8} GC

nature structural & molecular biology

TECHNICAL REPORT

https://doi.org/10.1038/s41594-018-0028-6

Yeast surface display platform for rapid discovery of conformationally selective nanobodies

Conor McMahon[1], Alexander S. Baier[1], Roberta Pascolutti[1], Marcin Wegrecki[2], Sanduo Zheng[1], Janice X. Ong[1], Sarah C. Erlandson[1], Daniel Hilger[3], Søren G. F. Rasmussen[2], Aaron M. Ring[4], Aashish Manglik[5,6*] and Andrew C. Kruse[1*]

Challenges in nanobody generation

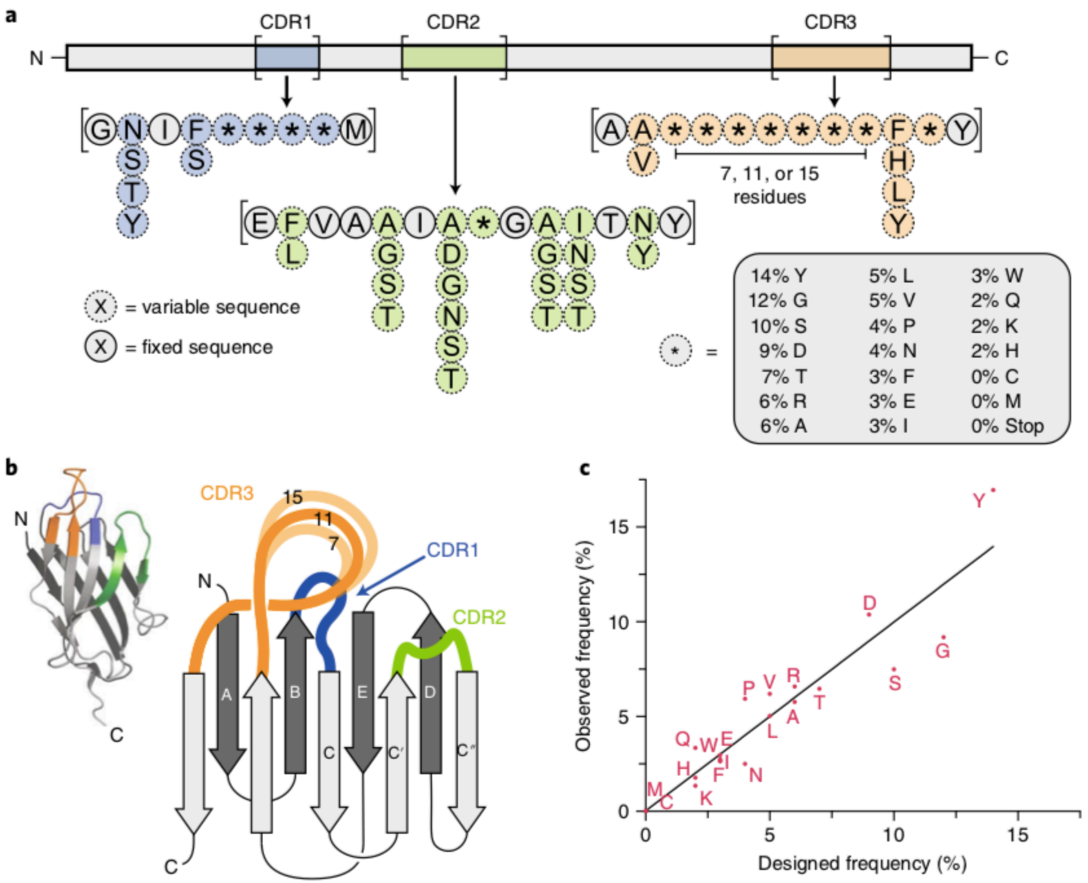
- Current methods derived from the immunization of camelids for creating nanobodies are slow, costly, and often unreliable (a significant barrier to entry for most laboratories).

- Animal-derived antibodies are frequently restricted from binding conserved epitopes because of the immunological tolerance of self-antigens.

- Nanobody identification by combining phage display with a synthetic library is reported. However, these libraries remain available only through contract work with an expensive commercial provider.

- With synthetic libraries, it is particularly challenging to identify nanobodies that not only bind to their target, but also specifically recognize a defined conformation, which represents one of the most important applications of animal-derived nanobodies. cc

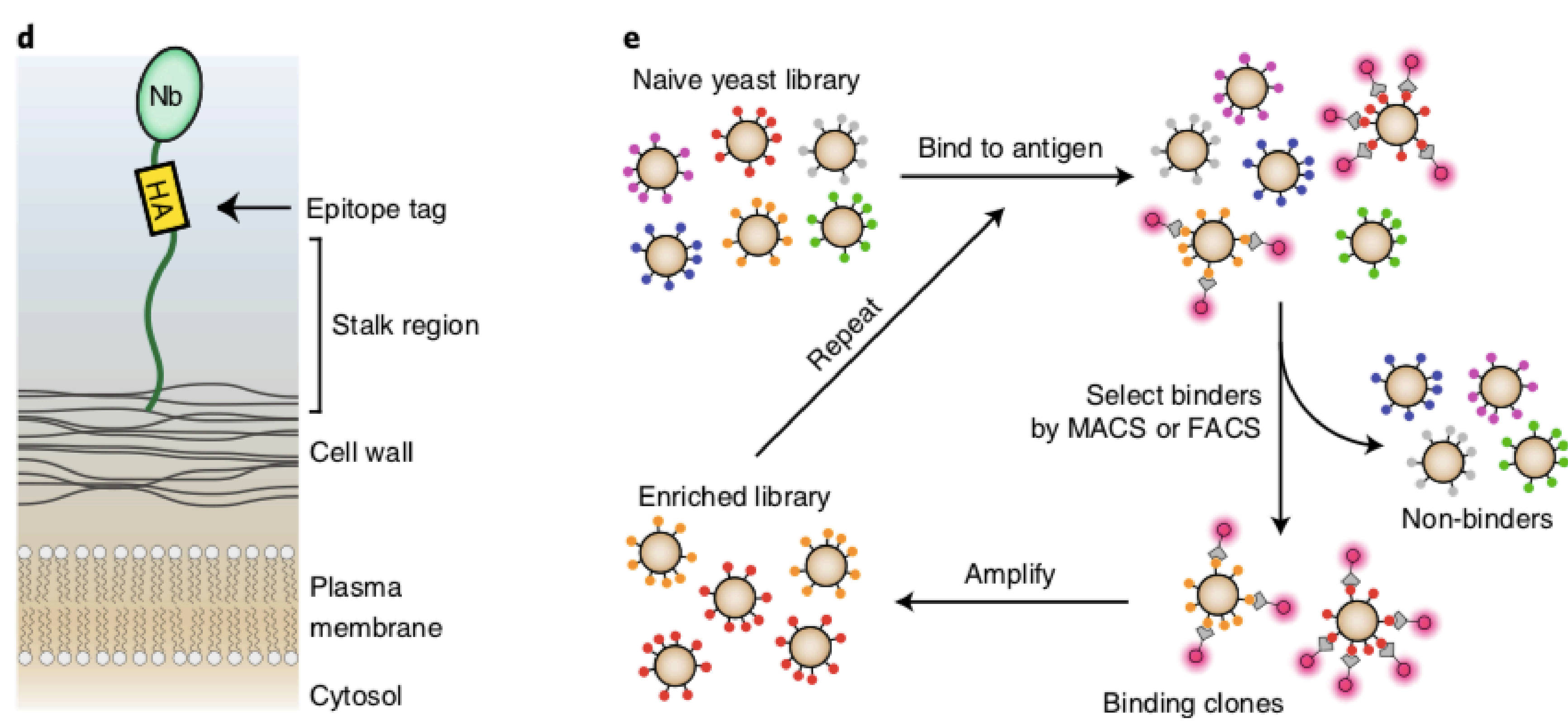
Design and construction of synthetic nanobody library



❖ Library devised using an alignment of structurally characterized nanobodies from the Protein Data Bank (PDB).

❖ Framework regions were fixed in sequence, while portions of the CDR loops were varied.
Partial randomization by mixed nucleotides to allow up to six possible residues
Highly variable regions were synthesized using a trimer phosphoramidite mixture.

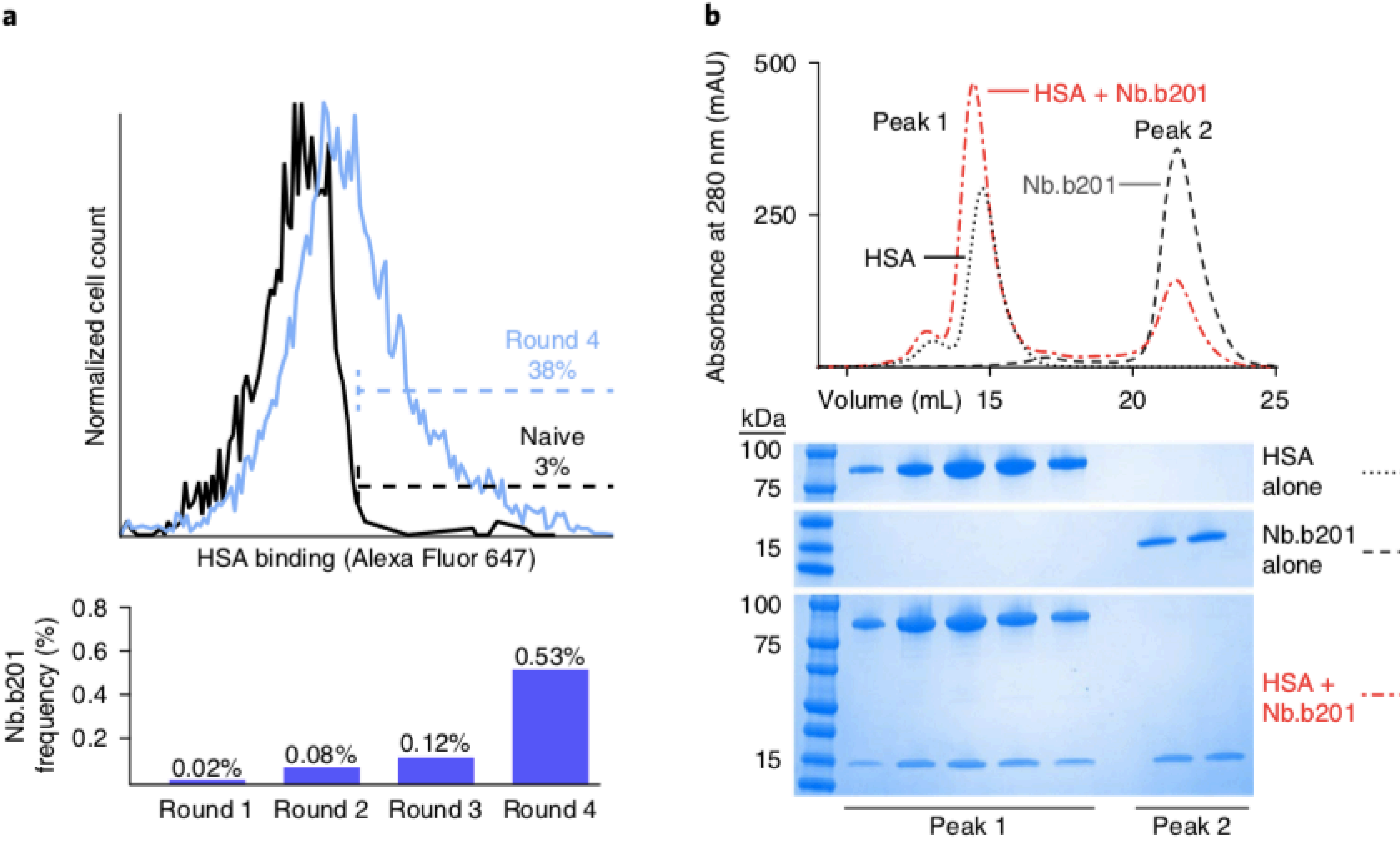
Schematic of nanobody (Nb) display on yeast and selection process



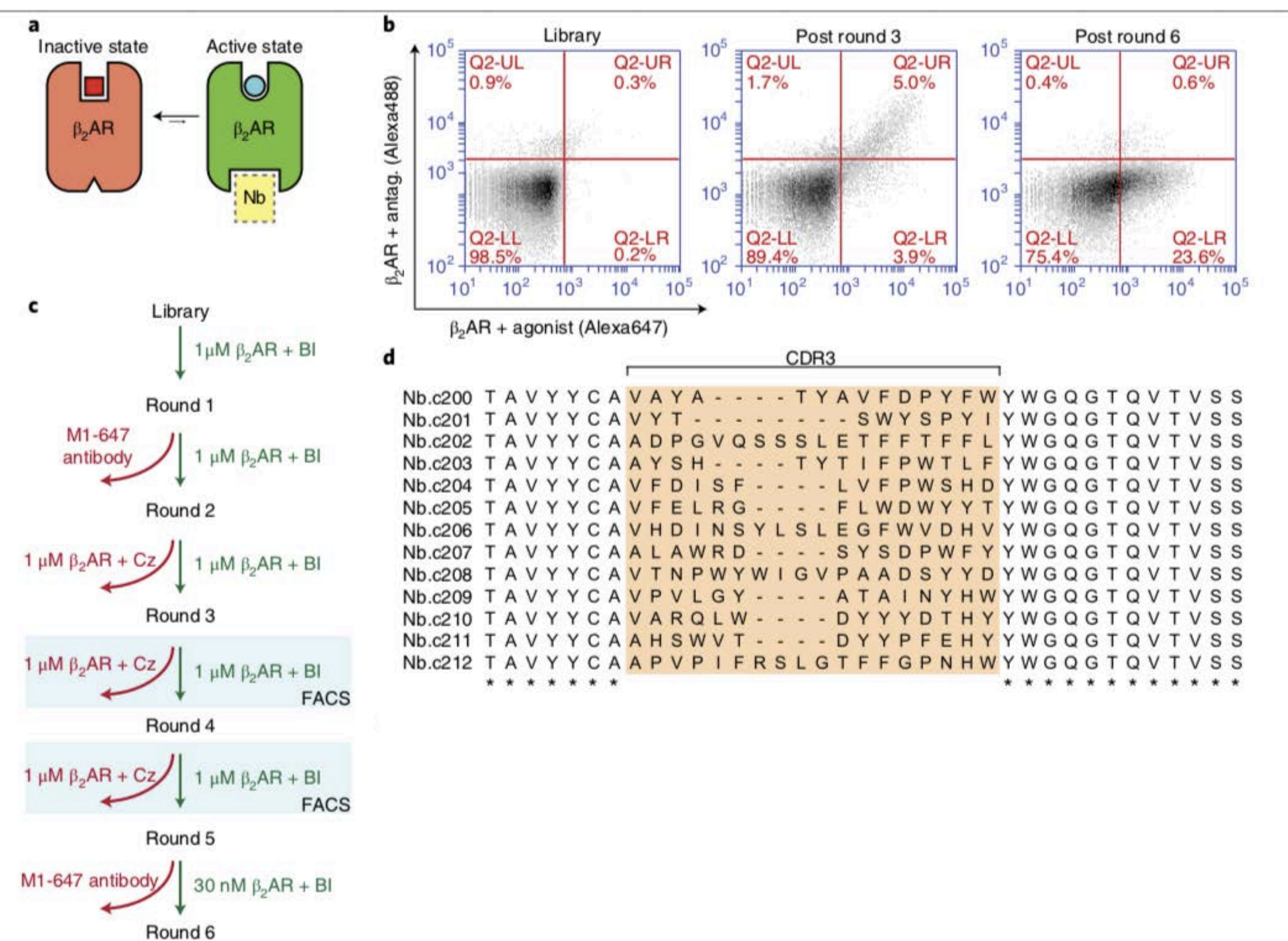
The nanobody has an HA tag at the C-term followed by a long flexible stalk which covalently tethers the nanobody to the yeast cell wall.

The antigen harbours a fluorescent tag. Yeast displaying nanobodies with affinity to antigen are isolated via MACS or FACS, amplified, and undergo iterative rounds of selection

Validation of nanobody platform using HSA as the target antigen

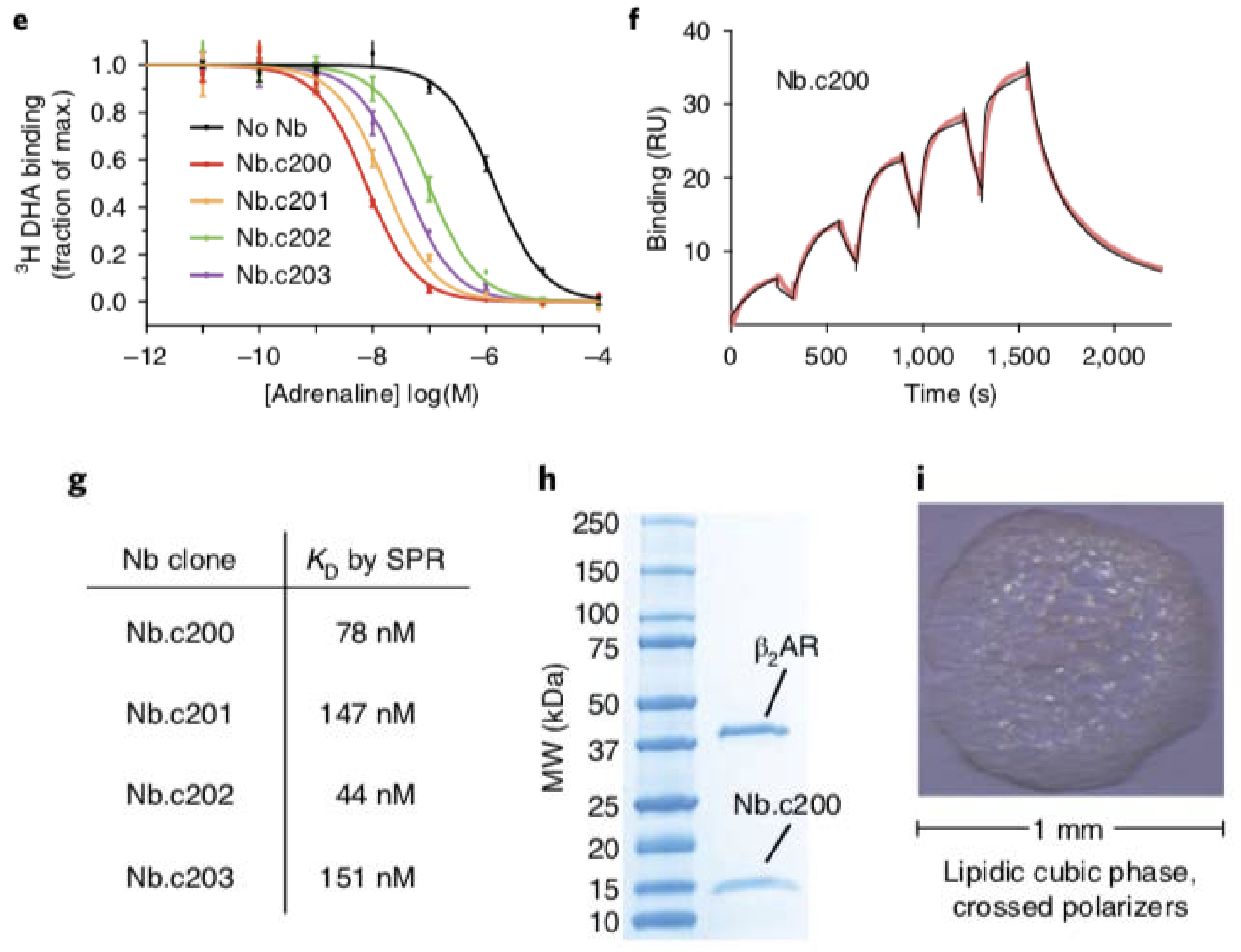


Structural and functional modulator nanobodies targeting a GPCR



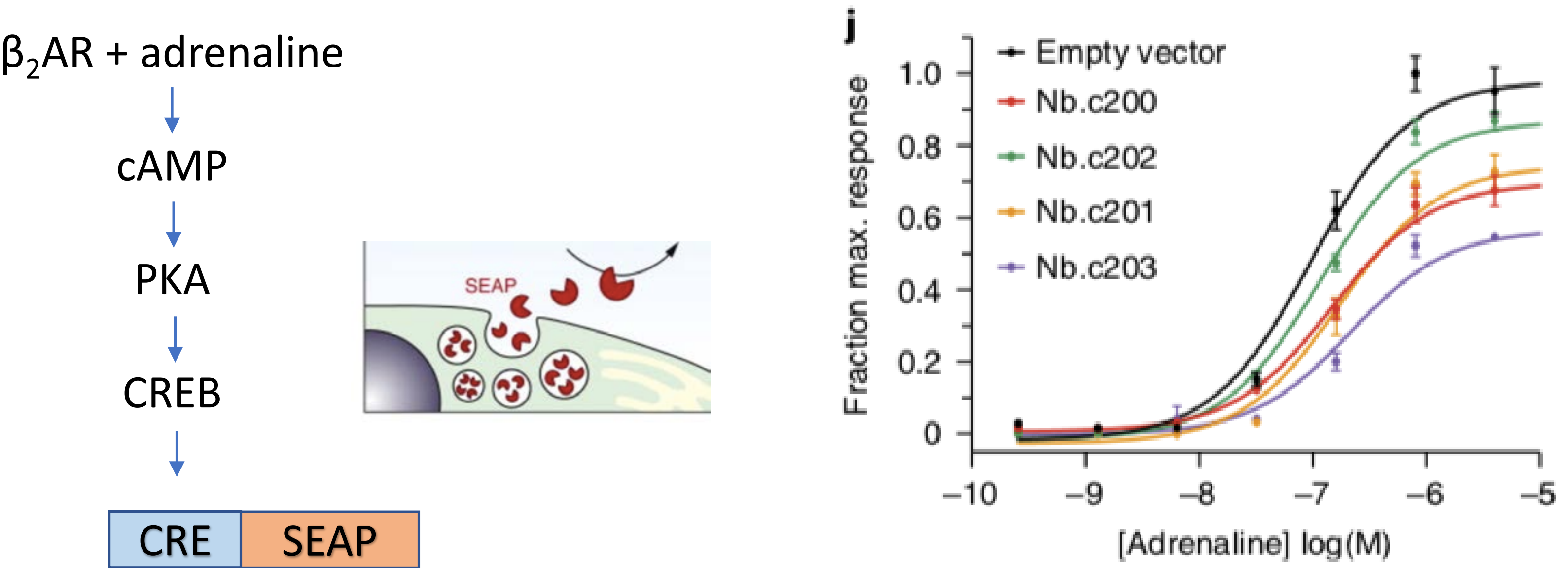
Selection schematic for isolation of active-state stabilizing Nbs. In MACS, rounds depletion and enrichment steps were performed sequentially. In FACS rounds, two-color sorting was used to enrich agonist-specific clones and deplete antagonist-specific and nonselective clones simultaneously. Cz, carazolol (antagonist); BI, BI167107 (agonist)

Binding features of selected nanobodies targeting a GPCR



Nb clone	K_D by SPR
Nb.c200	78 nM
Nb.c201	147 nM
Nb.c202	44 nM
Nb.c203	151 nM

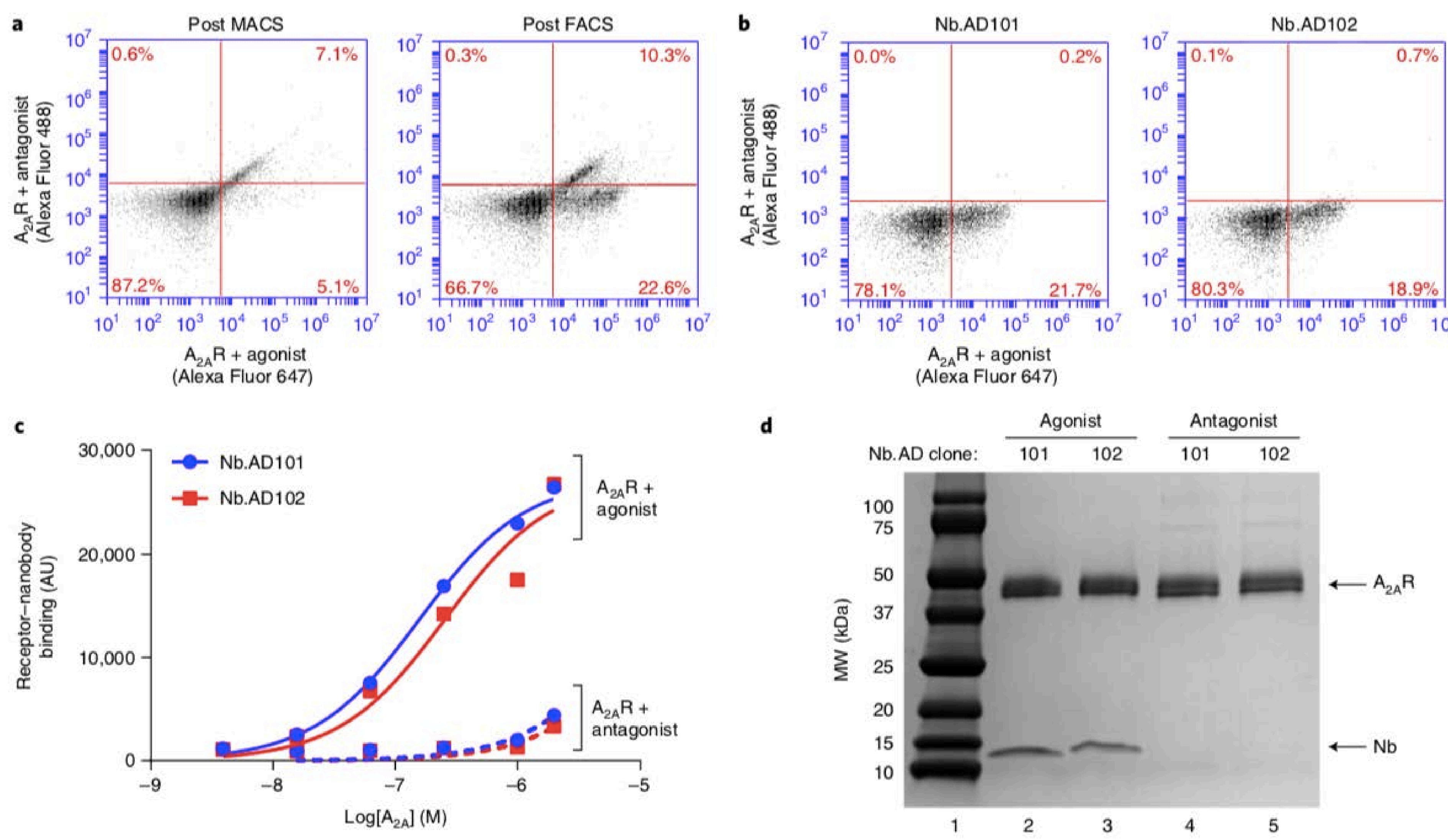
Nanobodies targeting conformationally active GPCR can modulate GPCR signaling in live cells



cAMP signaling assay to measure β_2AR signaling in the presence or absence of synthetic nanobodies.

β2AR signaling was measured using a transcriptional CRE-SEAP (secreted embryonic alkaline phosphatase) reporter to indirectly measure cAMP production.

Isolation and characterization of agonist-bound $A_{2A}R$-specific nanobodies



Relevance

- Fully in vitro platform for nanobody discovery: rapid and low cost
- Ability of generating nanobodies to a different arrays of antigens
- Nanobodies with affinity comparable or higher than Nbs derived from camelid immunization
- Ability to generate conformation specific Nbs
- Library is publicly available and free of charge for nonprofit use

LlamaTags: A Versatile Tool to Image Transcription Factor Dynamics in Live Embryos

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https://doi.org/10.1016/j.cell.2018.03.069

Bothma et al., 2018, Cell *173*, 1810–1822
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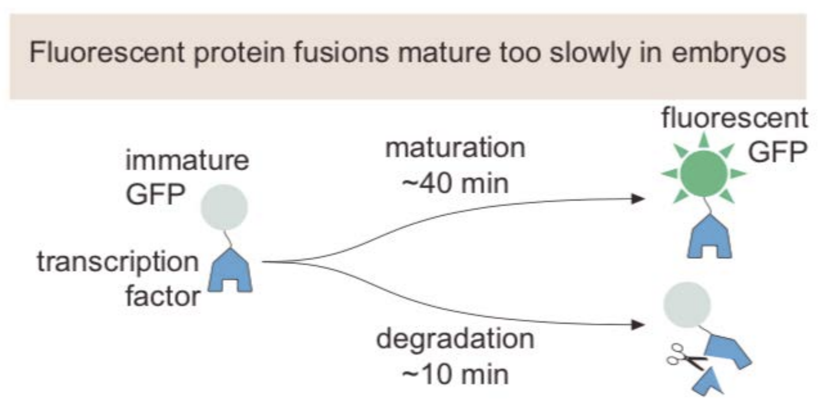
Fluorescent protein maturation kinetics remains a major hindrance to the live imaging of cell-fate decisions during embryonic development



The cells of a developing embryo often make rapid decisions about their fate.

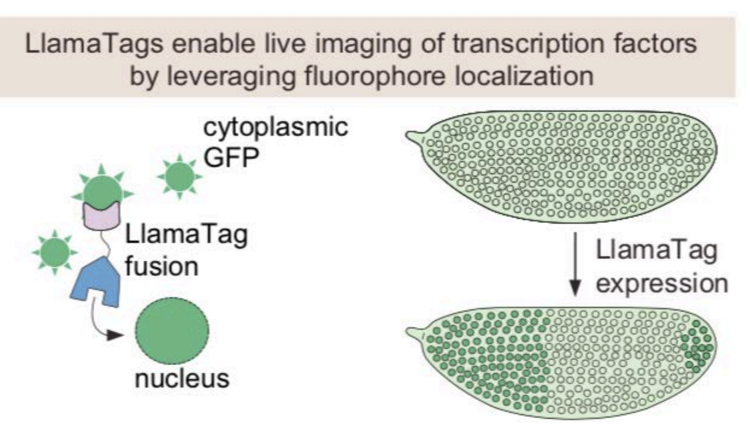
Cell-fate is driven by the concentration dynamics of transcription factors (TFs).

The use of fluorescent protein fusions to transcription factors has been limited by the slow maturation step that must occur before these fusions become fluorescent.

This rapid protein turnover makes visualizing the expression of these transcription factors in real time nearly impossible; the fusion protein of interest has already degraded by the time the reporter begins to fluoresce.

As a result, attempts to measure transcription factor patterns in live embryos with fluorescent protein fusions have yielded undetectable or significantly delayed patterns.

A novel genetically encoded tagging technique for visualizing transcription factor spatiotemporal dynamics in development



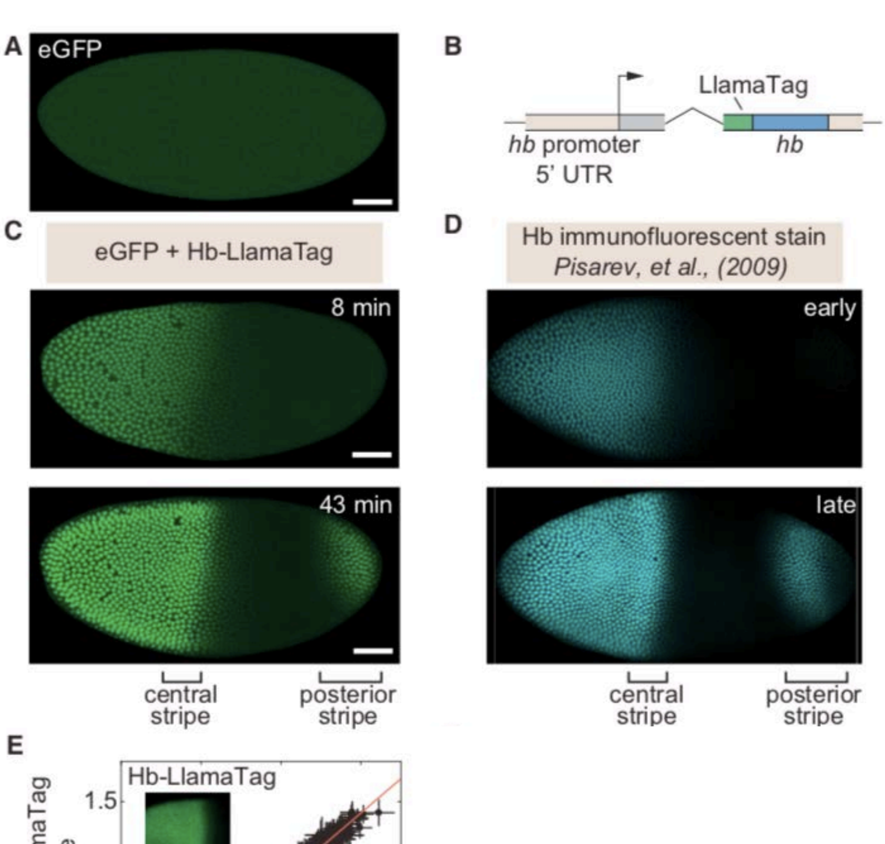
TF tag is based on nanobodies: their small size is ideal for limiting perturbations of the tagged protein's endogenous function, and the binding is fast and of high affinity.

The strategy employs the spatial localization of already mature fluorescent proteins as a reporter of protein concentration dynamic.

Strategy:
The **TF is fused to a nanobody against eGFP** and expressed under the TF 's endogenous regulatory sequence in conjunction with **maternally deposited eGFP**, ensuring ample time for eGFP maturation before the gene is expressed.

When the transcription factor-nanobody fusion is translated, it binds cytoplasmic eGFP on the timescale of seconds and increases the fluorescence of bound eGFP by 1.5-fold.
Expression of the LlamaTagged protein leads to an **increase in nuclear fluorescence** that constitutes a **direct readout** of the instantaneous transcription factor concentration in each nucleus.

LlamaTags Capture Endogenous Protein Dynamics in the Early Embryo



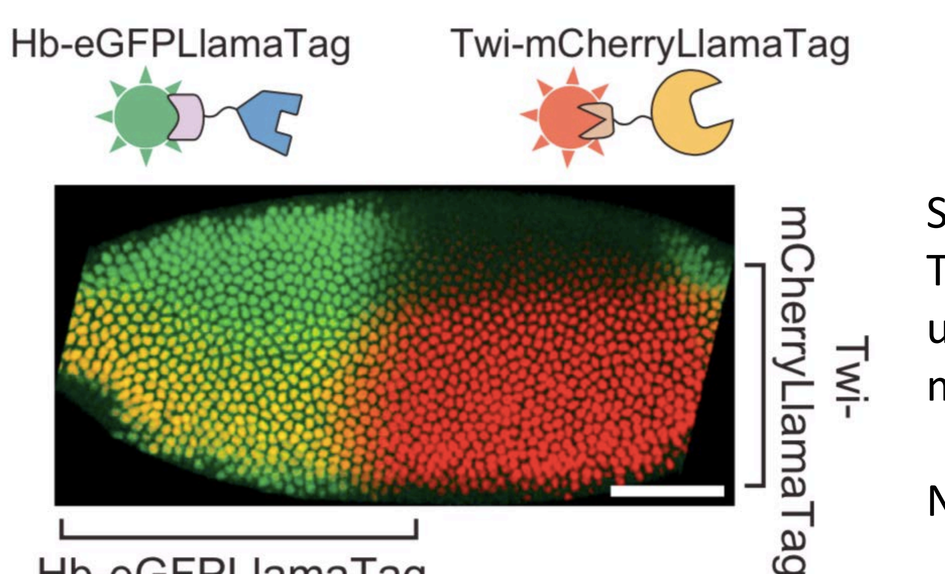
Tg flies that expresses eGFP in developing oocytes; upon fertilization, *eGFP* mRNA is translated into protein.

In the absence of a LlamaTagged transcription factor, eGFP is uniformly distributed throughout the embryo.

CRISPR-mediated fusion of *hb* to a LlamaTag. Hb-LlamaTag expression patterns recapitulate previous measurements obtained using fluorescent antibody staining.

Live embryo imaging by laser scanning confocal: Hb-LlamaTag eGFP signal per nucleus scales linearly with Hb fluorescence obtained by immunostaining.

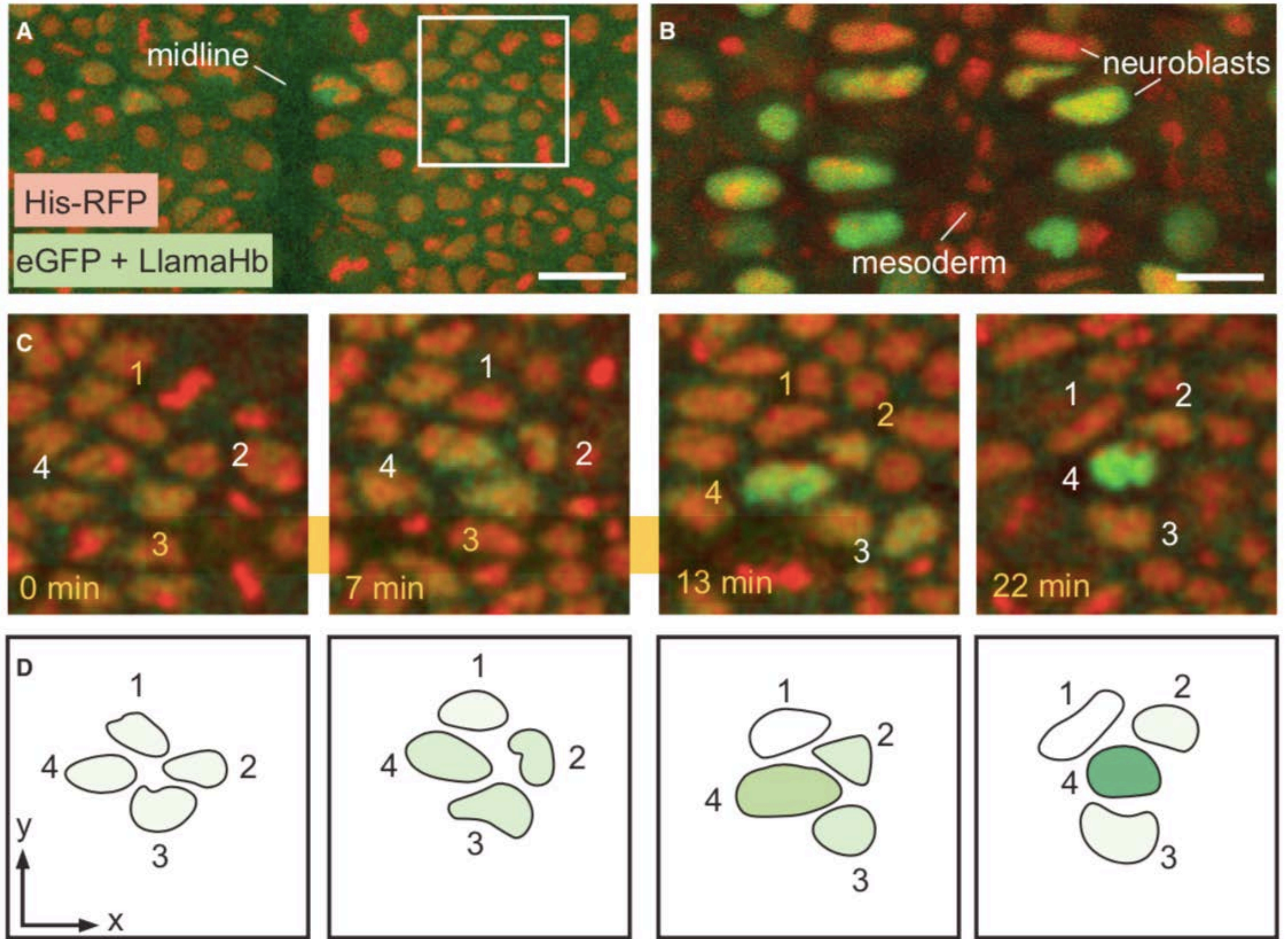
Multicolor imaging for the simultaneous quantification of multiple transcription factors



Simultaneous visualization of Hb and Twi TF in embryo initiating gastrulation using LlamaTags specific to eGFP and mCherry.

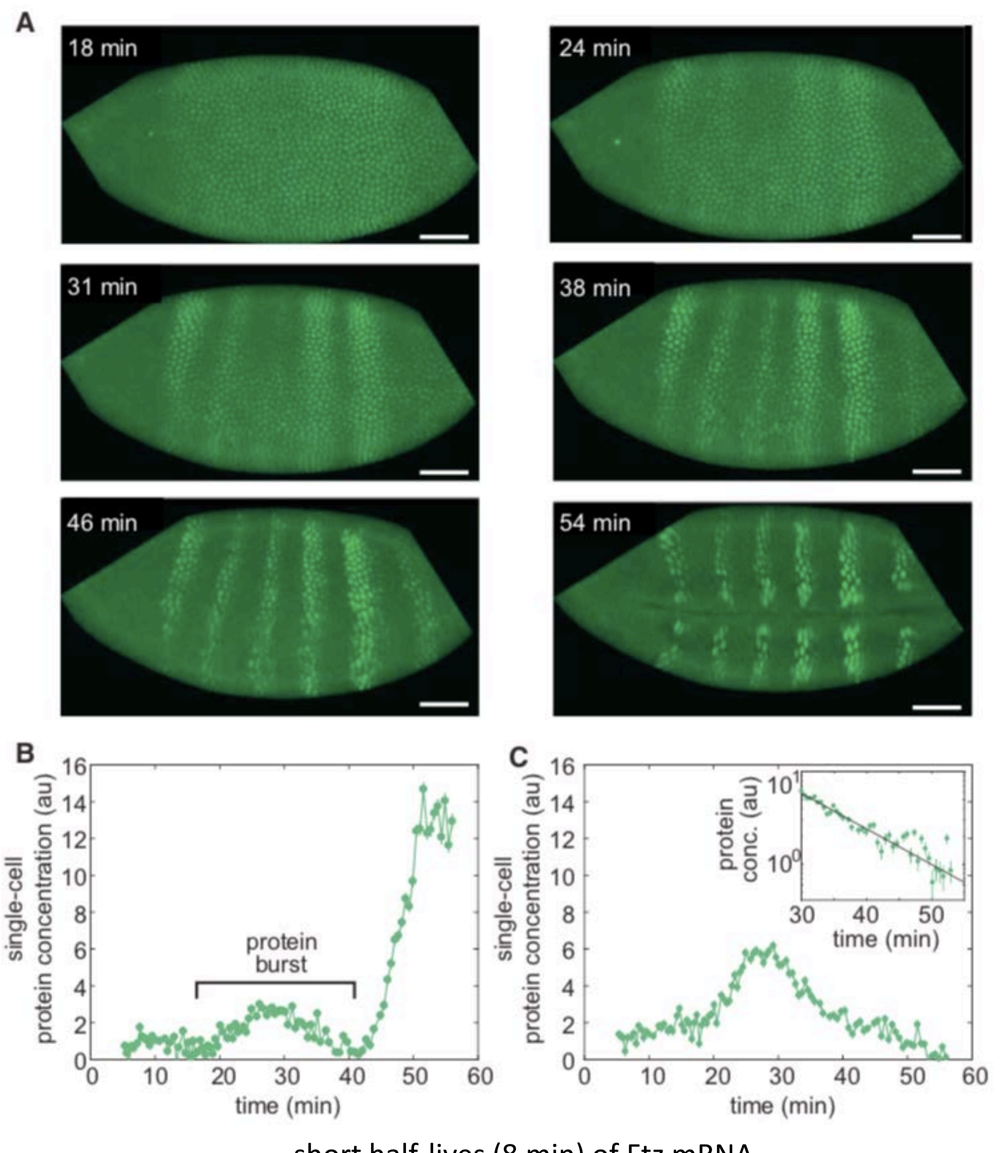
No cross reactivity

Imaging transcription factors at later development stages



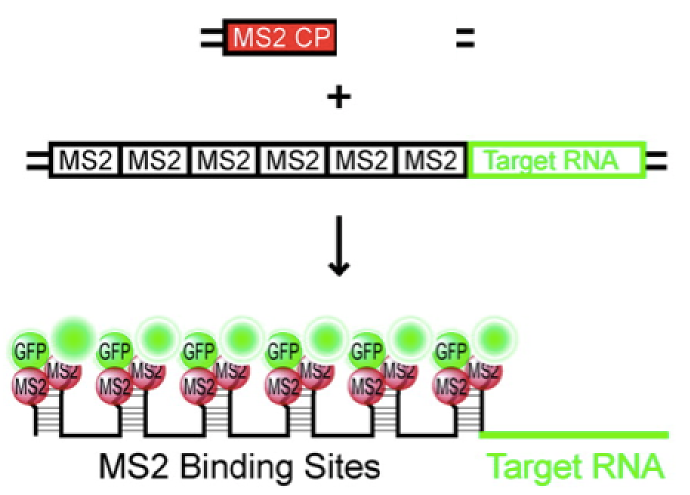
Imaging Hb's role in specifying the fate of neural progenitor cells (neuroblasts) once cellularization has occurred and cells move below the embryo surface

Capturing Fast Protein Production and Degradation Dynamics using LlamaTags



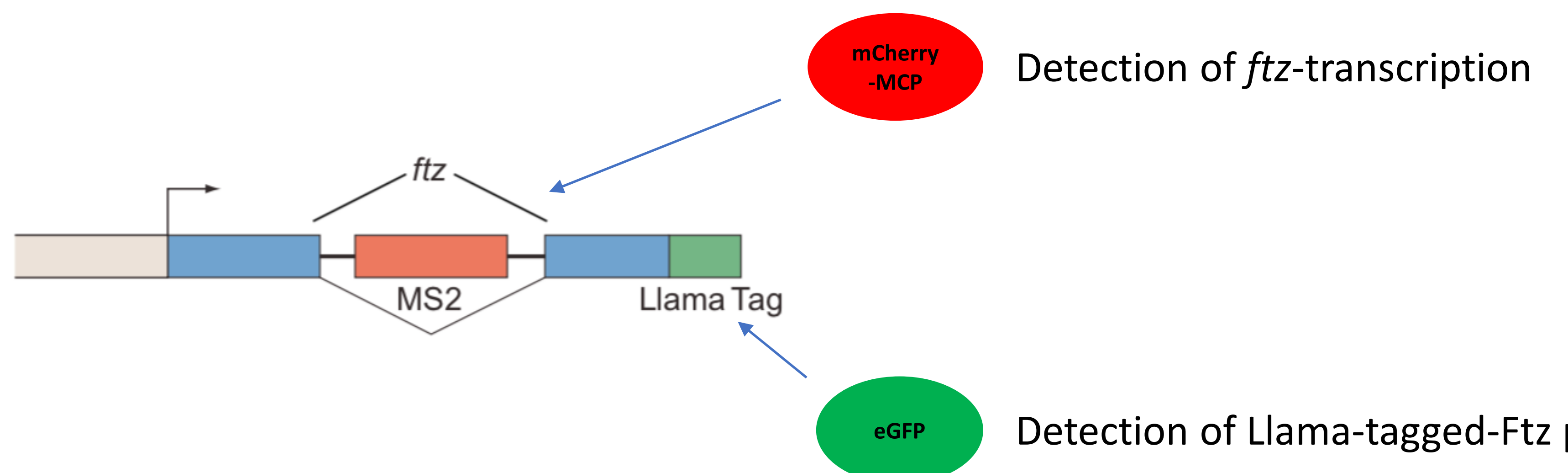
short half-lives (8 min) of Ftz mRNA

Combining LlamaTags with the MS2 aptamer system for *in vivo* tagging of nascent mRNA to simultaneously visualize transcription factor concentration and transcription

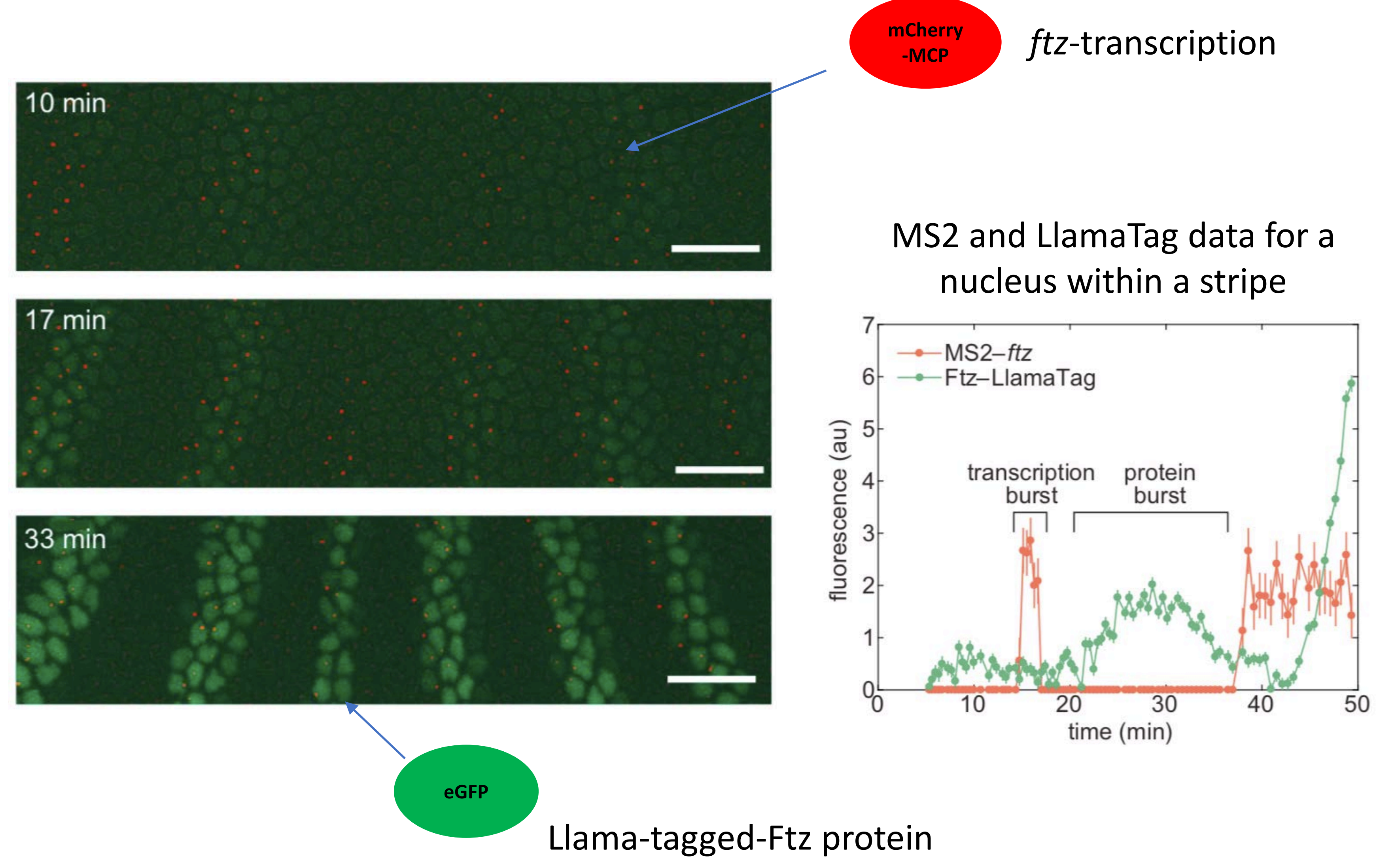


We introduced RNA MS2 loops into the intron of our LlamaTagged *ftz* mini-gene

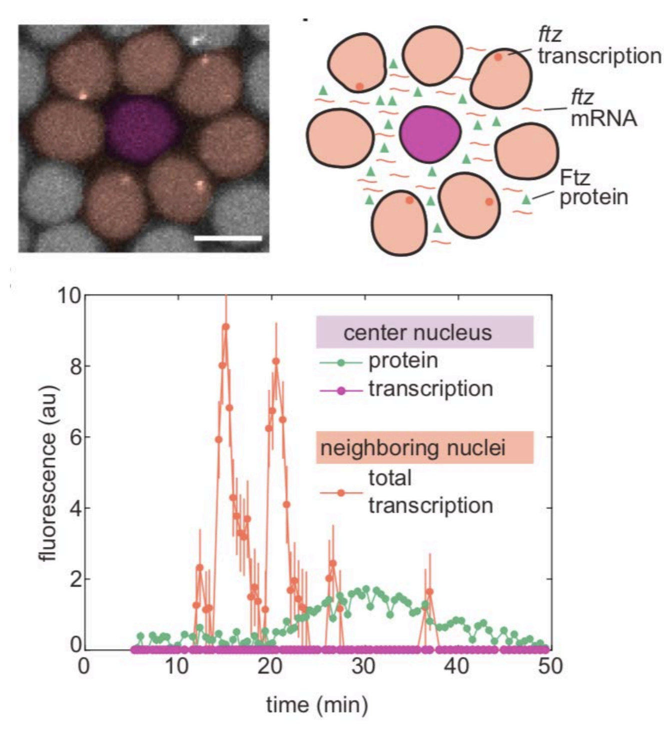
Simultaneous visualization of *ftz* transcription with an mCherry-MCP fusion and Llama-Tagged Ftz with eGFP



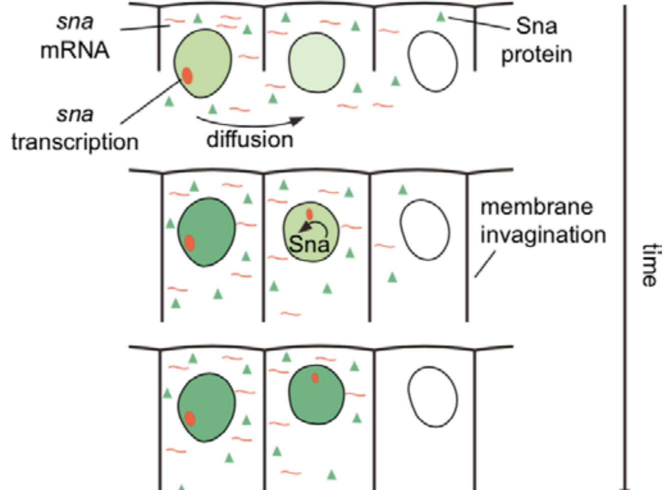
Revealing correlations among transcriptional bursts and protein bursts



Revealing inter-nuclear coupling



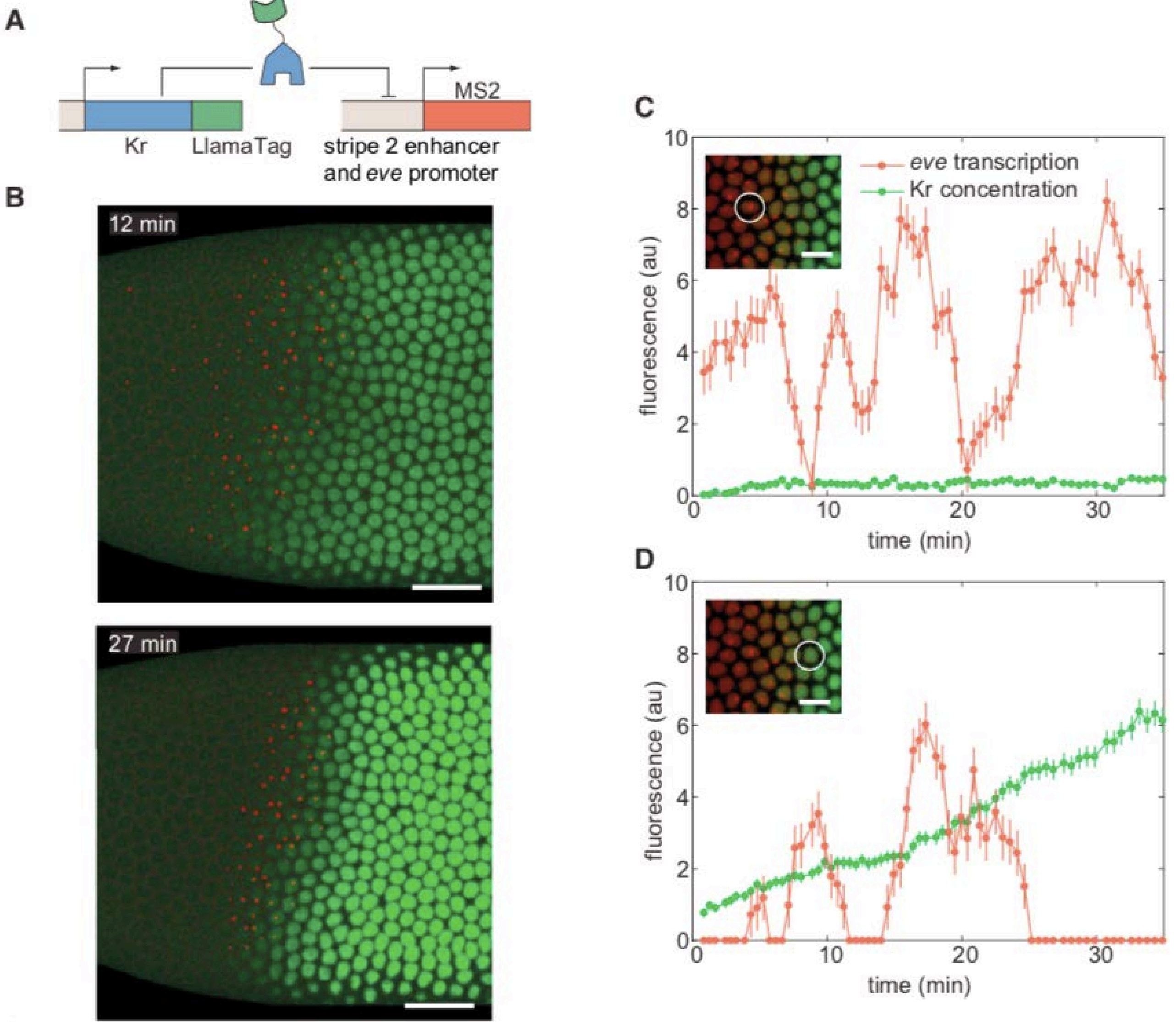
Coupling between transcription and protein concentration in neighboring nuclei provides evidence of **inter-nuclear communication** in the fly syncytium, where protein in one nucleus can originate from mRNA transcribed in adjacent nuclei.



Strong inter-nuclear coupling could "**kick-start**" transcriptionally inactive nuclei by engaging positive-feedback loop.

As cellularization progresses, inter-nuclear coupling ceases, effectively locking each cell into its final fate.

Uncovering the Dynamics of Transcription-Factor Input and Transcriptional Output at the Single-Cell Level



Comparison to other tagging approaches for the visualization of transcription factors

Comapred to other tagging systems, LlamaTag-GFP complex adds **<45 kDa** to the tagged protein, and does not affect endogenous protein function

LlamaTags are fully **encoded genetica**lly, circumventing the need for injections of organic dyes.

Multiplexing capabilities nanobodies raised against a fluorescent protein do not cross-react with fluorescent proteins originating from other species.

Creation of new **nanobodies that recognize a specific transcription factor**. This nanobody could then be **fused to a fluorescent protein** and expressed uniformly throughout the embryo. A transcription factor binds the nanobody-fluorescent protein fusion and transports it into the nucleus.
This approach can also be utilized to distinguish between phosphorylation states of the same TF, a regulatory feature that is pervasive in developmental programs.

Reporter–nanobody fusions (RANbodies) as versatile, small, sensitive immunohistochemical reagents

Masahito Yamagata[a,b] and Joshua R. Sanes[a,b,1]

2126–2131 | PNAS | February 27, 2018 | vol. 115 | no. 9

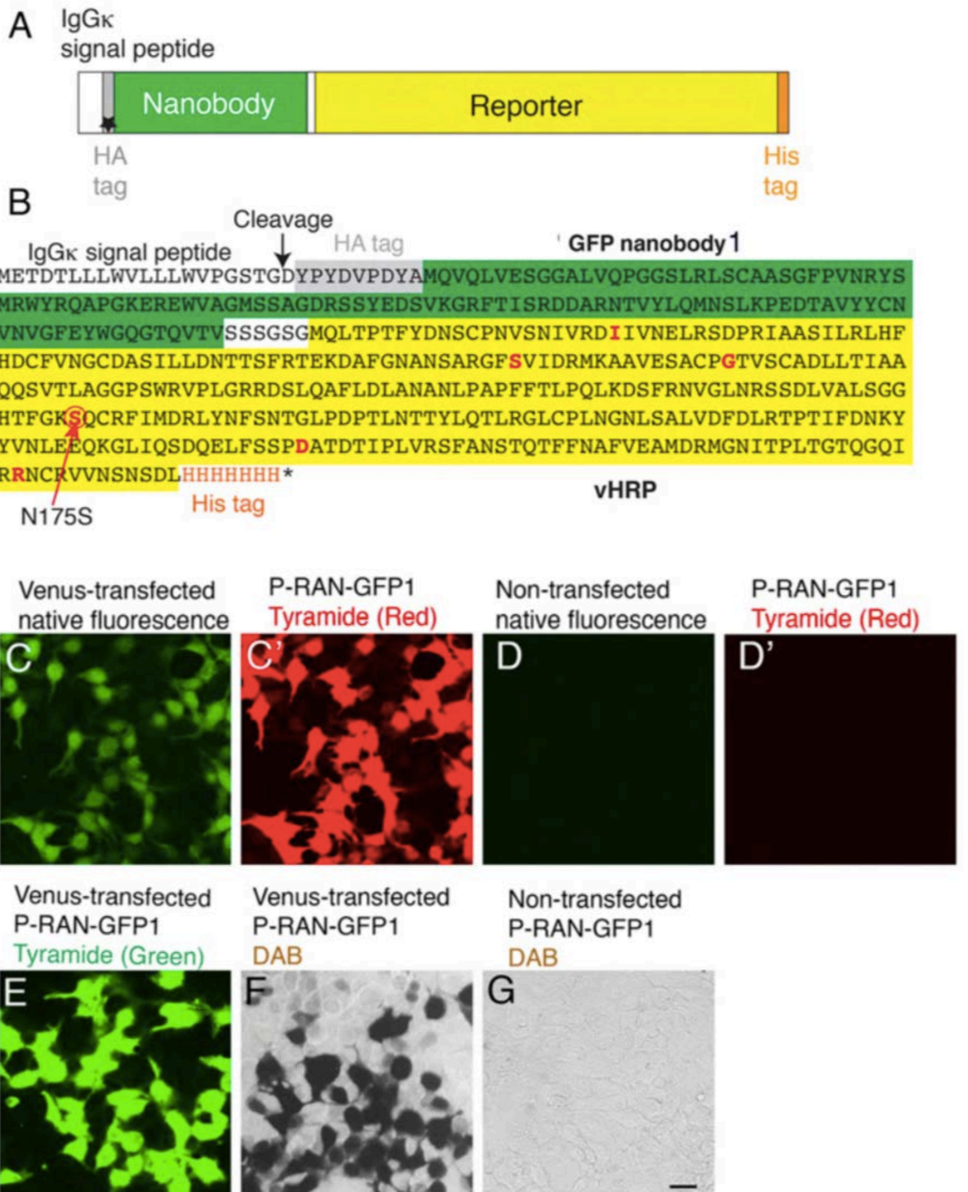
An immunohistochemical platform based on nanobodies

Nanobodies are used to prepare sensitive unimolecular **detection reagents** by genetically **fusing cDNAs encoding nanobodies to enzymatic or antigenic reporters**.

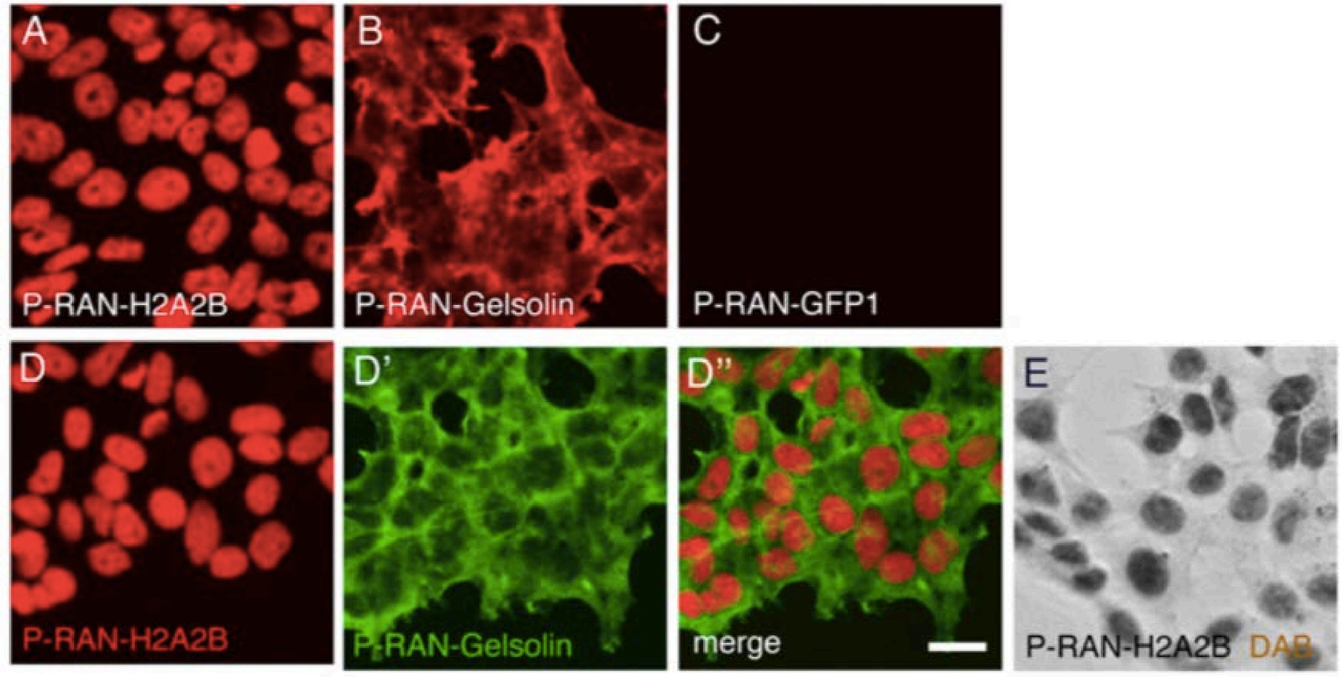
Can be used to **localize epitopes** and to **amplify signals from fluorescent proteins**.

Can be generated and purified simply and in unlimited amounts and can be preserved safely and inexpensively in the form of DNA or digital sequence.

Structure and use of RANbodies



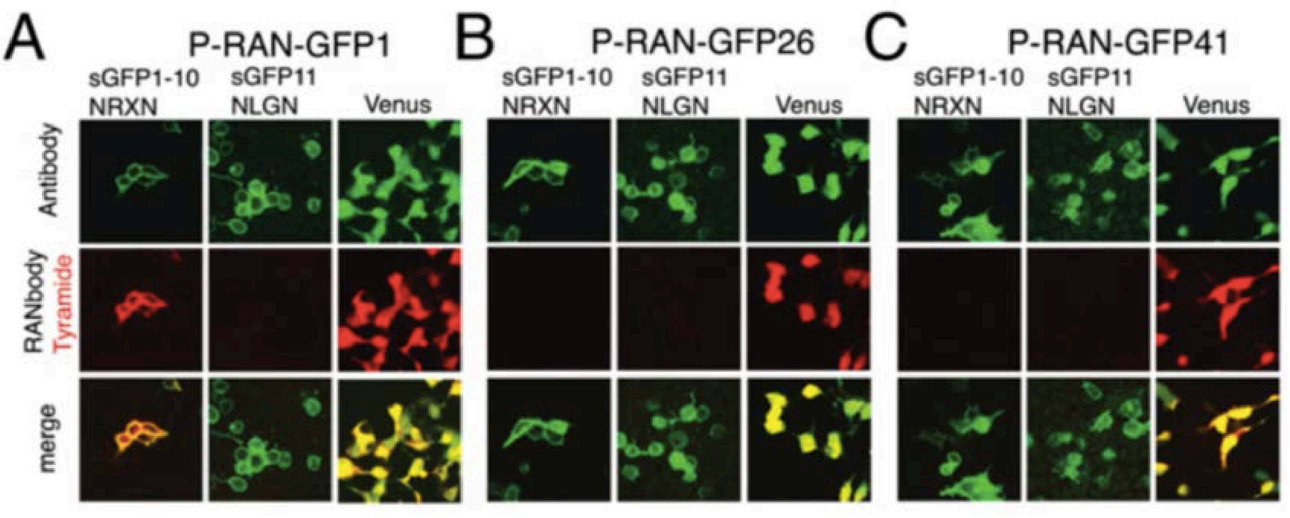
Detection of endogenous proteins with RANbodies

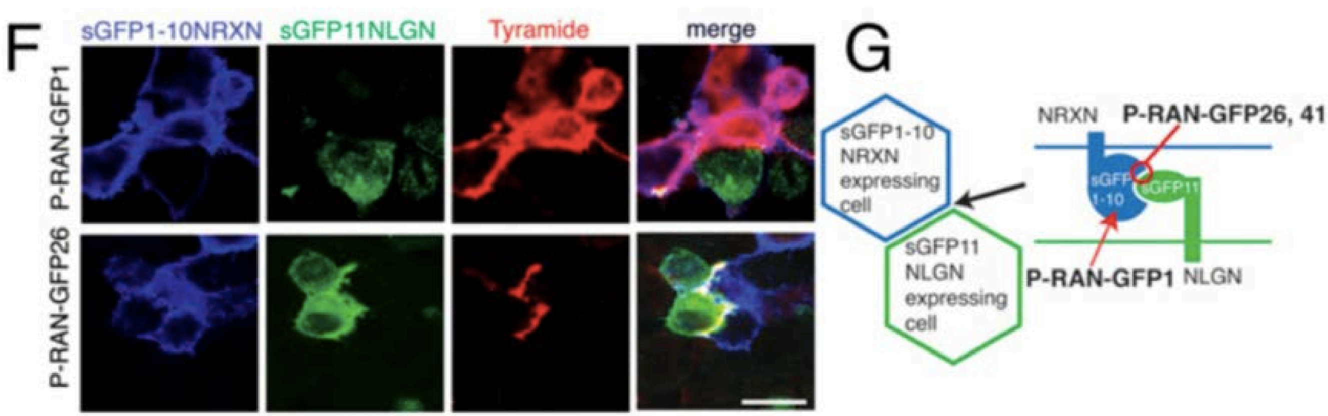


Generation of RANbodies against **endogenous protein** using **public nanobody sequences**

Sequencial staining of different antigens

RANbodies-GFP distinguish reconstituted GFP from GFP fragments





Advantages of RANbodies

They are **sensitive**, because of the amplification provided by the reporters (enzymatic for HRP and incorporation of multiple epitopes in the spaghetti monsters)

specific, because of the monoclonal nature of nanobodies

inexpensive, because they can be produced by standard methods in most laboratories

eternal because they can be regenerated based on information contained in a simple digital sequence file.

They can be used to detect a **variety of antigens** in both **cultured cells and tissue sections**

Nanobody for vDISCO technology

nature neuroscience
TECHNICAL REPORT
https://doi.org/10.1038/s41593-018-0301-3

Panoptic imaging of transparent mice reveals whole-body neuronal projections and skull–meninges connections

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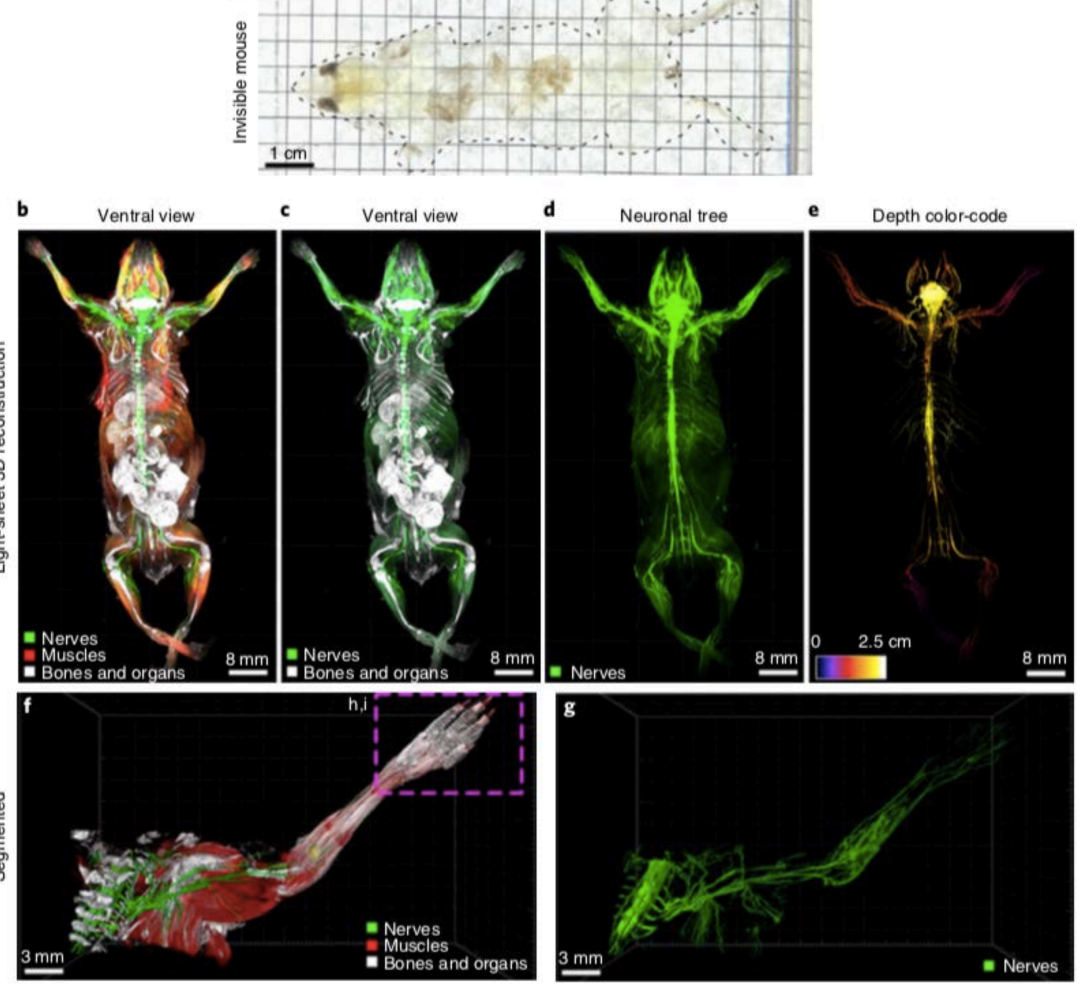
A pressure-driven nanobody-based whole body immunolabeling technology to enhance the signal of fluorescent proteins

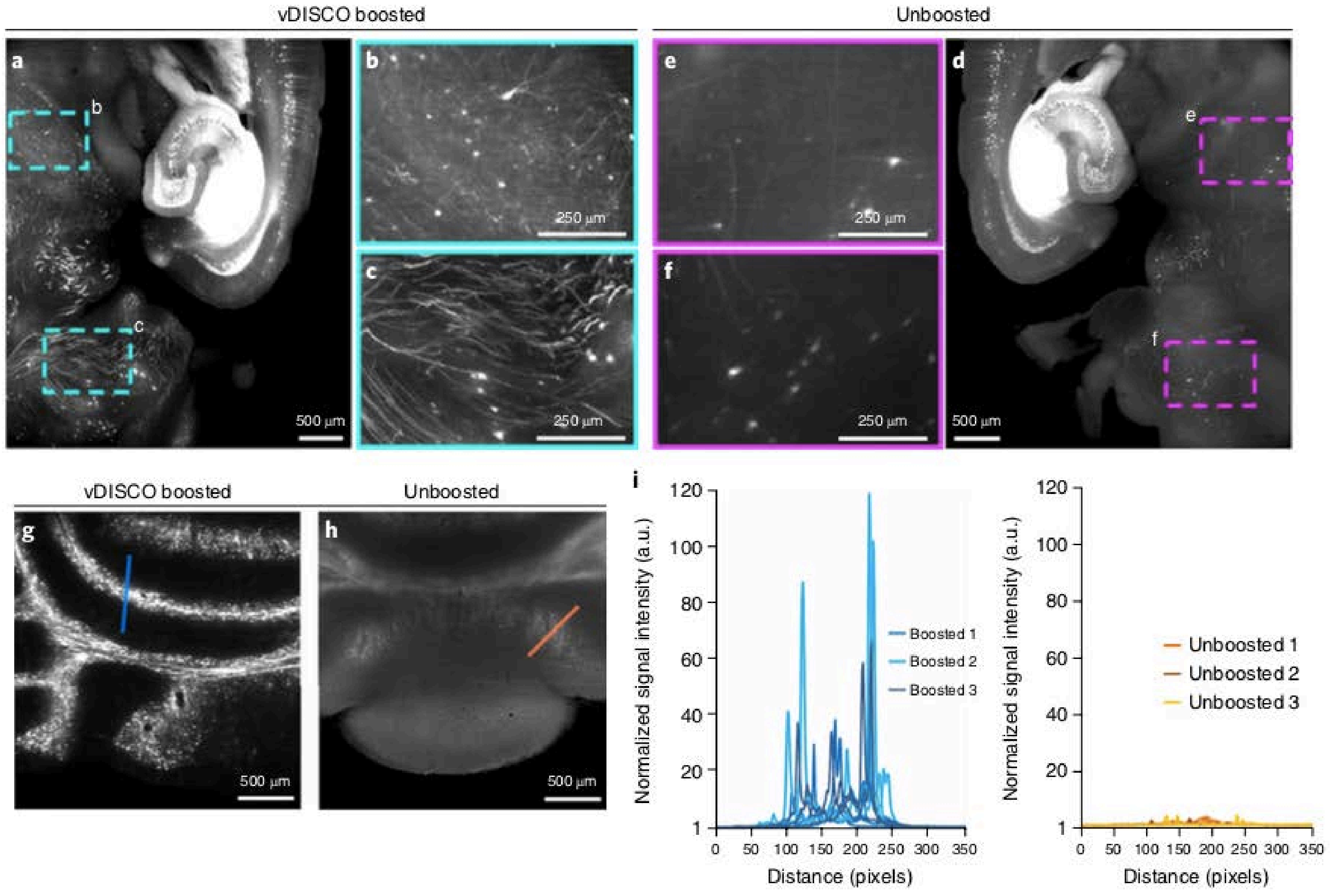


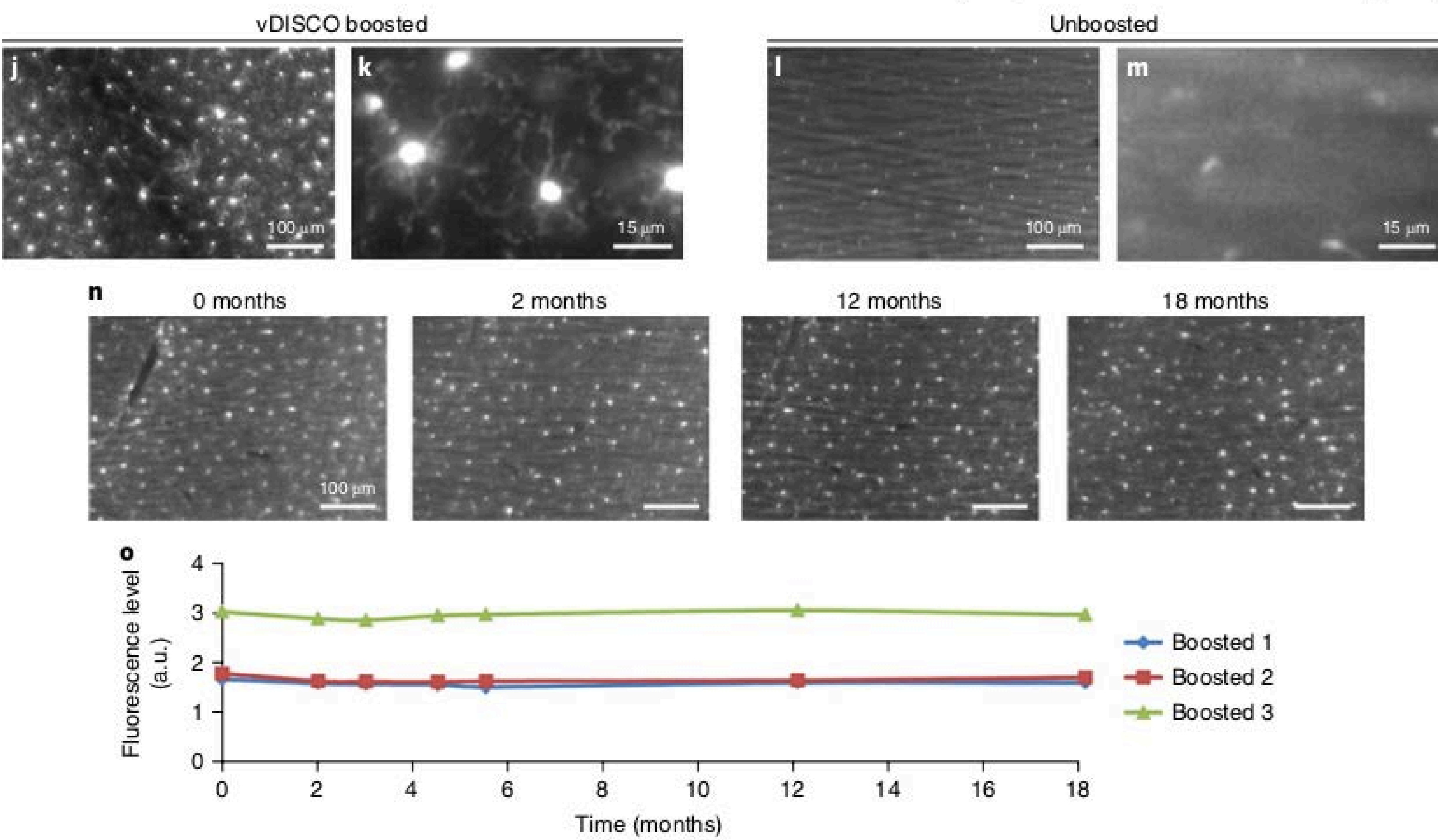
Thank you!

Trimer (Codon) Phosphoramidites

Protein mutagenesis can be used to fine tune a variety of properties, such as improved stability to high temperatures, denaturants, or non-aqueous solvents; higher affinity binding to a target molecule; increased rates of enzymatic reactions; or changes of specificities. However, generating and finding these improved proteins can be a difficult task. One of the most popular methods is to make pools of degenerate oligonucleotides, which can be incorporated into the genes as cassettes or by PCR by using the degenerate oligo as a primer.[1] Degenerate oligonucleotides are synthesized as a mixture of A/C/G/T phosphoramidites (N) at the site of the codons to be mutated. Problems arise, though, from using an equimolar solution of each base. First there is a coding bias. Out of the 64 possible codon combinations of A, C, G and T, 18 code for leucine, arginine or serine, but only 2 for tryptophan or methionine. As a result, only 3% of the mutagenic oligonucleotides will contain methionine or tryptophan, and over 28% will contain either leucine, arginine or serine. In addition, the three nonsense codons will lead to chain termination in 4.7% of the sequences. There are ways to improve this situation. For instance, using two degenerate mixes of bases, N and G/C, on the DNA synthesizer to insert NNG/C into the sequence will halve the number of the most degenerate codons, but still code for all 20 amino acids. However, still 59% of the clones will code for just eight amino acids and 3% will have a stop codon inserted.

The generation of redundant sequences and stop codons makes searching a clonal library inefficient. However, it is possible to improve the efficiency of this process by using a mixture of trinucleotide (trimer) phosphoramidites.[2–5] By synthesizing a set of trimers that cover all 20 amino acids, the mutation of a gene can be carried out at the codon level rather than at individual bases. Therefore, unlike other methods of mutagenesis, trimer phosphoramidites lead to no codon bias, no frame-shift mutations, and no production of stop codons, making them one of the most efficient tools to explore sequence space in protein regions that are important for function [6] – even in nonsaturating conditions.[7, 8]


vDISCO boosted
Unboosted
a
b
c
500 µm
250 µm
250 µm
e
f
d
250 µm
250 µm
500 µm
vDISCO boosted
Unboosted
g
h
500 µm
500 µm
i
Normalized signal intensity (a.u.)
Distance (pixels)
Boosted 1
Boosted 2
Boosted 3
Unboosted 1
Unboosted 2
Unboosted 3
0 50 100 150 200 250 300 350
1 20 40 60 80 100 120



vDISCO boosted
Unboosted
j
k
l
m
100 μm
15 μm
100 μm
15 μm
n
0 months
2 months
12 months
18 months
100 μm
o
Fluorescence level (a.u.)
0
1
2
3
4
0
2
4
6
8
10
12
14
16
18
Time (months)
Boosted 1
Boosted 2
Boosted 3