Universität Zürich[UZH]
UniversitätsSpital Zürich Klinik für Neurochirurgie
UniversitätsSpital Zürich Institute of Neuropathology

Journal Club

Production of versatile Nanobodies

Sandra Dias

Supervisor: Annika Keller

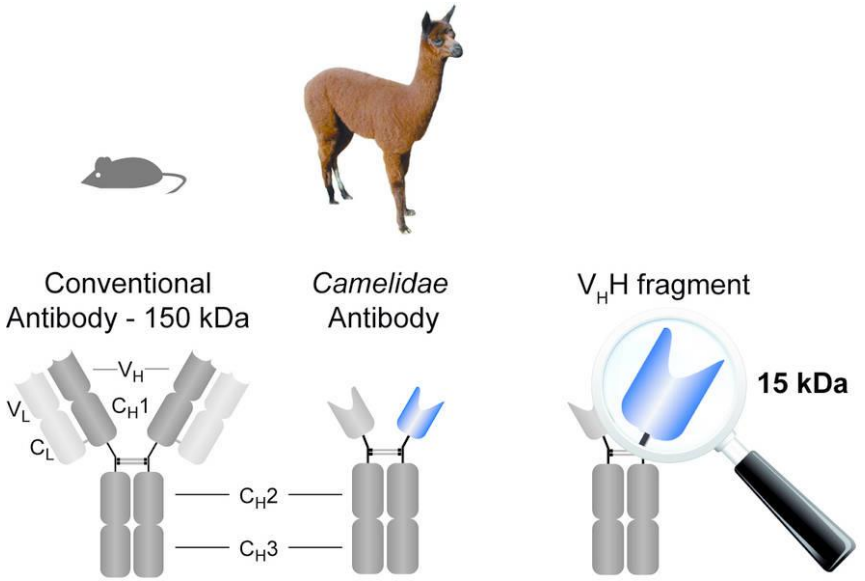
10 February 2015

Index

Introduction

Single-domain antibodies derived from variable regions of Camelidae atypical immunoglobulins.

Promise…



Chromotek®

Nanobodies: Natural Single-Domain Antibodies

Serge Muyldermans

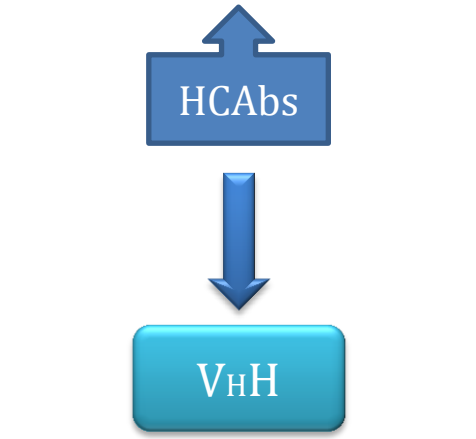
Research Group Cellular and Molecular Immunology, Vrije Universiteit Brussel, 1050 Brussels, Belgium; email: svmuylde@vub.ac.be

Department of Structural Biology, VIB, Vrije Universiteit Brussel, 1050 Brussels, Belgium

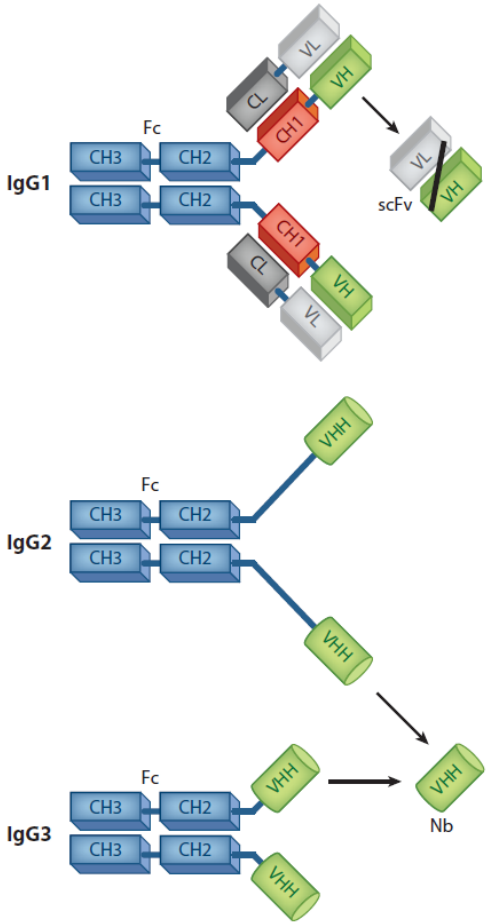
Annual Review of Biochemistry 2013. 82:775-97

Immunoglobulin-γ structure

A notorious exception to the conventional mammalian IgG structure is found in sera of Camelidae.



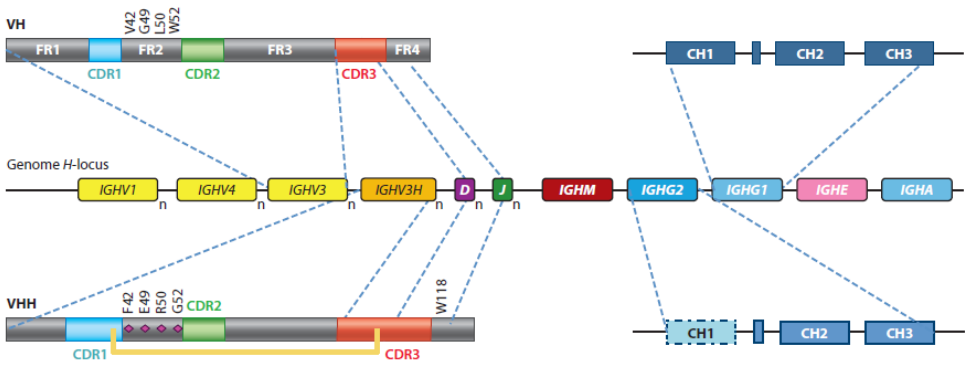
The structural and functional equivalent to the Fab fragment (antigen binding fragment) of conventional antibodies.



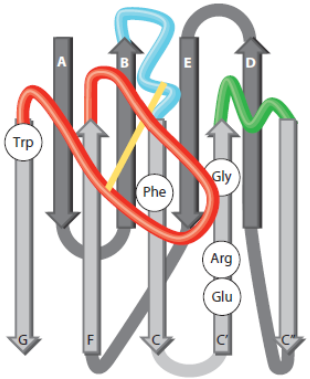
HCAb Subisotypes and VHH structure

- Homodimeric and heterodimeric antibodies are separated by differential affinity chromatography.

IgG2a
IgG2b



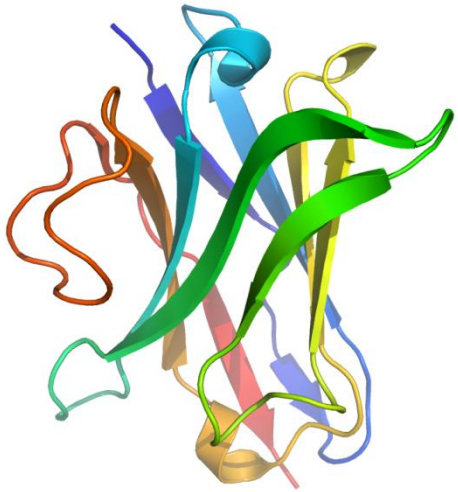
- The sequence variability within V domains is localized in three hypervariable (HV) regions surrounded by more conserved framework (FR) regions.



"Promiscuous VH genes contribute to the VHH repertoire"

Biochemical properties of Nanobodies (Nbs)

- Good quality of antigen specificity and affinity of Nbs from immune libraries
- Expressed to a high level in microorganisms
- Small size
- Easily concentrated by ultrafiltration to 1-10mg/mL in standard buffers.
- Stored for months at 4°C (longer -20°C)
- Robust



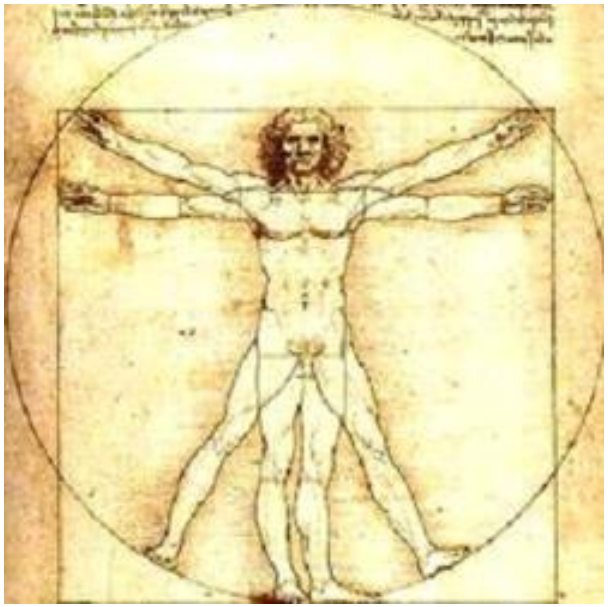
Wikipedia 2015

Human's match?

Disulphide bond-stabilized Nbs resistant to pepsin or chymotrypsin's degradation.

Low Immunogenicity

- Small size
- Stable behaviour
- Rapid clearance from blood (via kidney)
- Sequence sharing high degree of identity with human VH



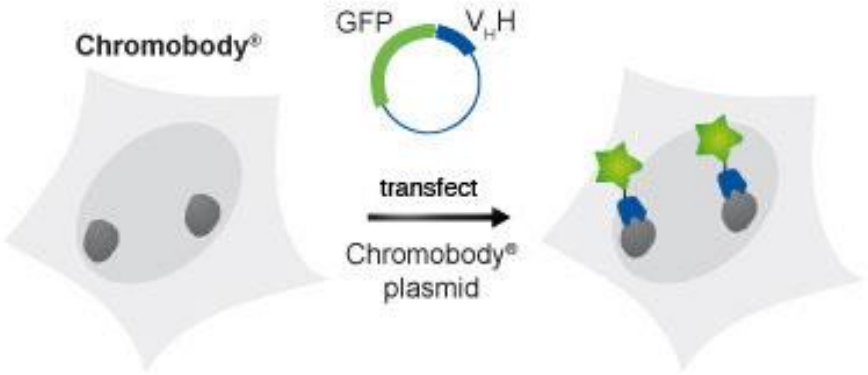
Applications with Nanobodies…

- ✓ Renewable source
- ✓ Economic production
- ✓ Small size
- ✓ Human(-ized) sequence
- ✓ Stable and soluble in aqueous solutions
- ✓ Reversible refolding
- ✓ Specific and high affinity for only one cognate target

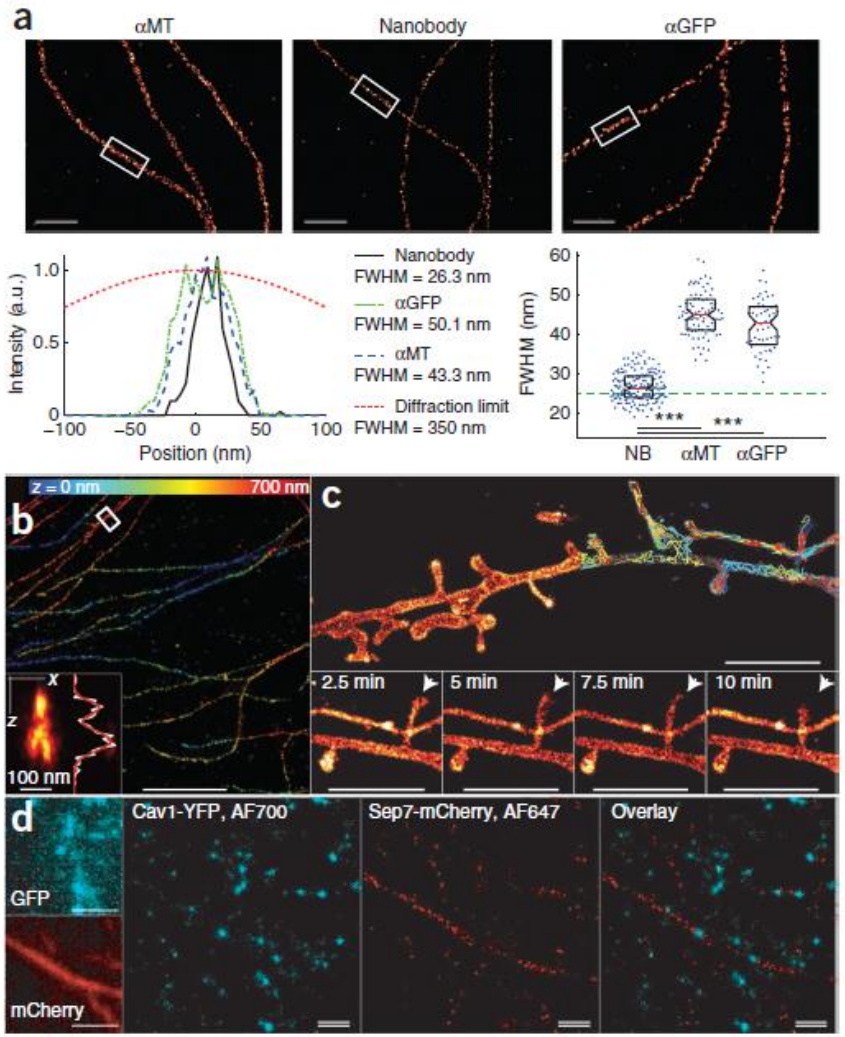
…as Research tools

Resource of well-characterized reagents for important targets

- Antigen tracing
- Imaging target protein expression, translocation and subcellular localization
- Difficult protein interactions
- Antigen depletion
- Crystallization chaperones



Chromobodies® - Chromotek



Ries J., Kaplan C, et al. *A simple, versatile method for GFP-based super-resolution microscopy via nanobodies*. 2012 Nature. June. Vol.9 No.6; 582-5587; doi:10.1038/NMETH.1991

…as Diagnostic tools

- Generate sensitive and selective biosensors
- Antigen-targeting photothermal therapeutic upon irradiation
- Nbs coupled to magnetic nanoparticles to capture and enrich analyte low concentrations → affinity adsorbents
- In vivo-imaging

…as Therapeutics

"A monoclonal Nb screened to recognize special epitopes involved in receptor recognition can reach an extremely high-neutralization potency."

- Able to access hidden and essential epitopes on pathogenic agents

Phase I and II: anti-IL6R, anti-TNFα, anti-von Willebrand factor, anti-RANKL

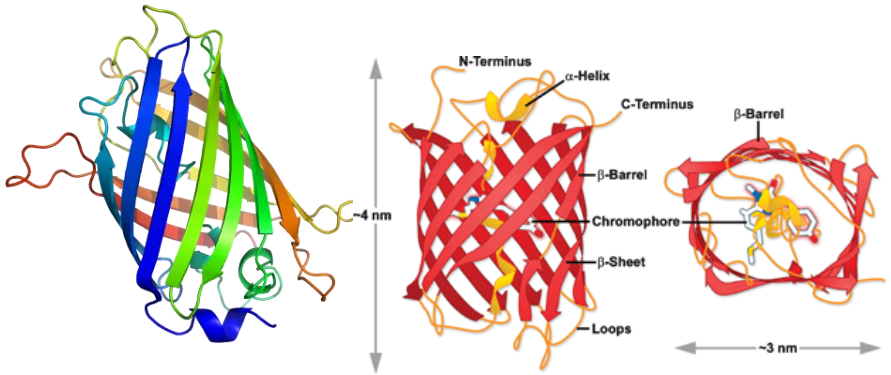
- Assessed in their capacity to deliver cargoes to tissues difficult to access.

A robust pipeline for rapid production of versatile nanobody repertoires

Peter C Fridy[1,8], Yinyin Li[2,8], Sarah Keegan[3], Mary K Thompson[1], Ilona Nudelman[1], Johannes F Scheid[4], Marlene Oeffinger[5,6], Michel C Nussenzweig[4,7], David Fenyö[3], Brian T Chait[2] & Michael P Rout[1]

Background

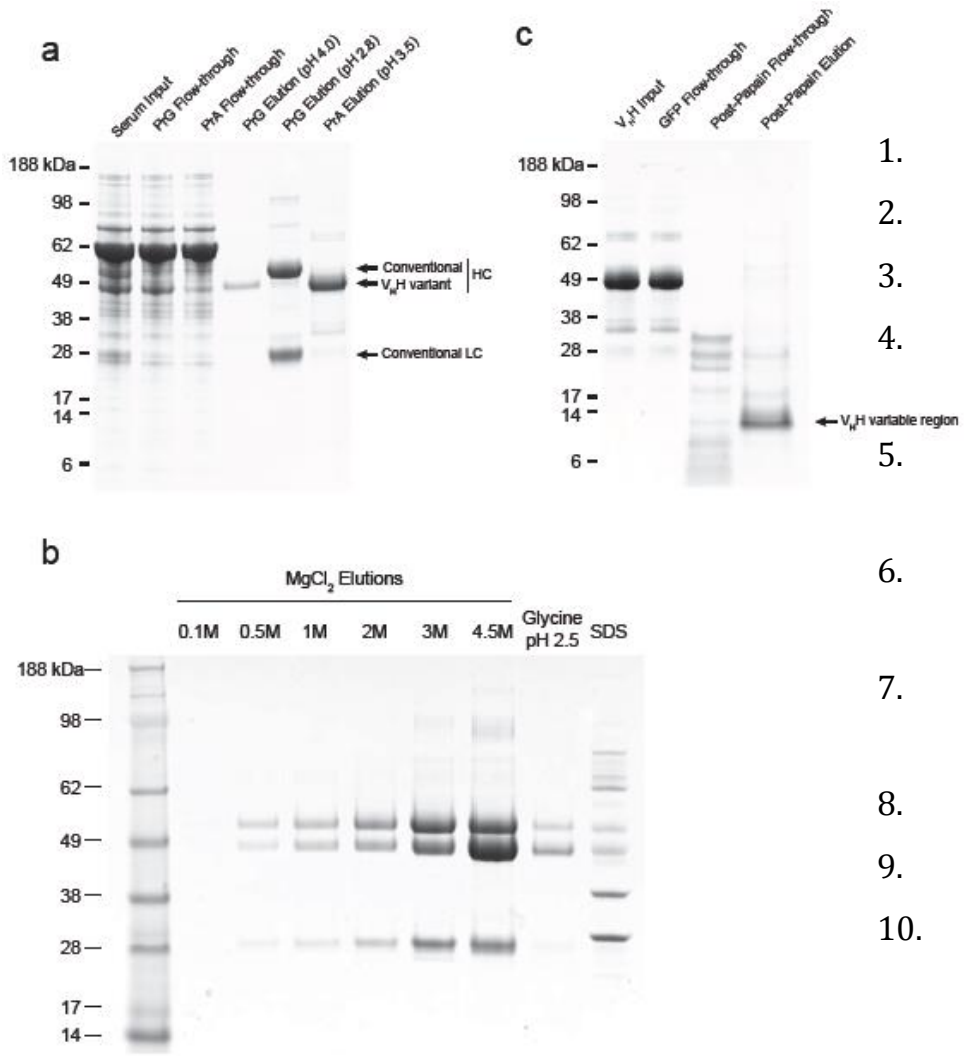
- Continual need for Ab that recognize target molecules with high affinity and specificity
- Common protein tags: GFP, mCherry, Flag
- Ab limitations: large size, availability and batch to batch variation



Wikipedia 2015 Royal Society of Chemistry 2015

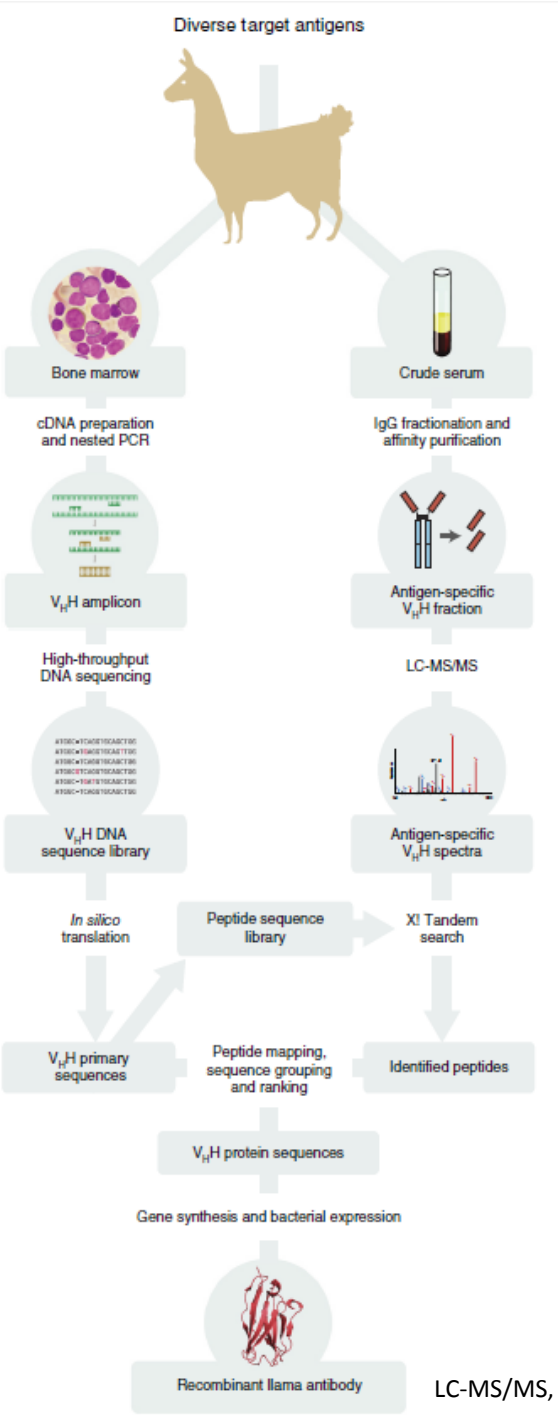
Emerge of single-domain antibodies – nanobodies - as an alternative.

"However rapid and robust techniques for isolating extensive repertoires of high-affinity nanobodies have proven elusive…"



Target antigen: GFP and mCherry

1. DNA sequence database from the same animal
2. Immunization of individual llamas with antigens
3. Confirmation of immune response
4. Serial fraction of serum bleeds to extract only VHH-containing heavy-chain antibodies
5. Purification of the VHH-containing fraction by washing with MgCl2
6. Digest with papain on resin → leave behind the minimal fragments of VHH-variable region
7. Elution and separation by SDS-PAGE of the antigen-bound VHH fragments → ~15kDa
8. Trypsin digestion of gel-purified bands
9. Analysis by liquid chromatography-MS and tandem MS
10. Recovery of highest-affinity VHH fragments (highest-stringency washes).



Animal-specific antibody sequence database

1. Lymphocyte RNA samples from individual immunized llamas
2. Isolate mononuclear cells from bone marrow aspirates
3. Generate cDNA form lymphocyte RNA
4. PCR to amplify sequences encoding the VHH variable regions

GFP ~800,000
mCherry ~ 3,000,000

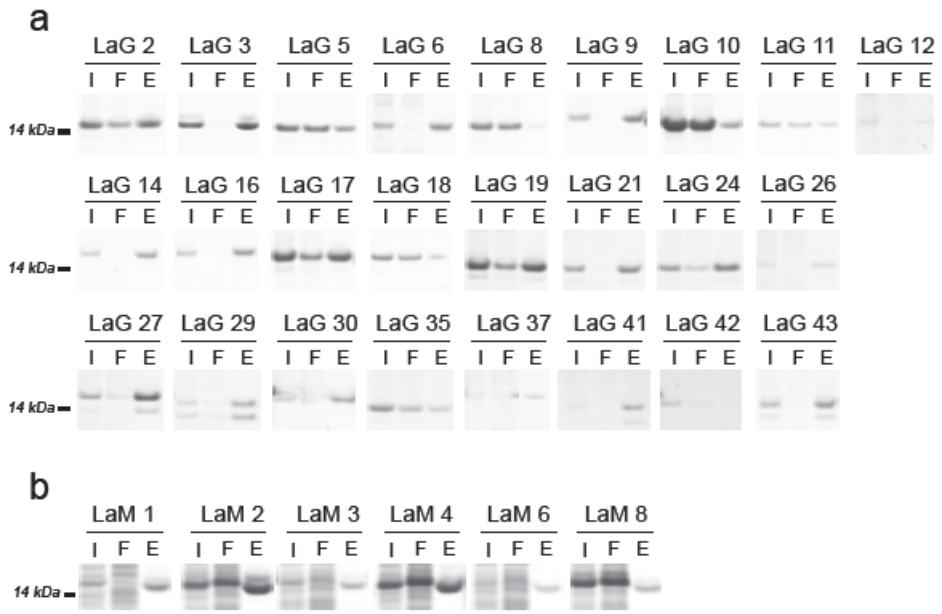
5. Reads are translated, filtered and trypsin digested *in silico* → searchable peptide database for MS analysis

LC-MS/MS, liquid chromatography-tandem mass spectrometry

“Llama Magic”

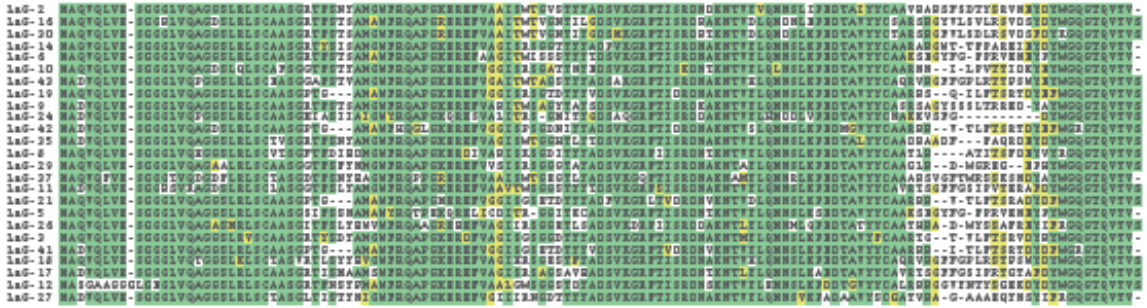
www.llamamagic.org

- V_HH sequences ranked by a metric based on MS sequence coverage of CDR3
- As well CDR1 and CDR2 coverage
- Total V_HH coverage, sequencing counts, mass spectral counts and expectation values of matched peptides



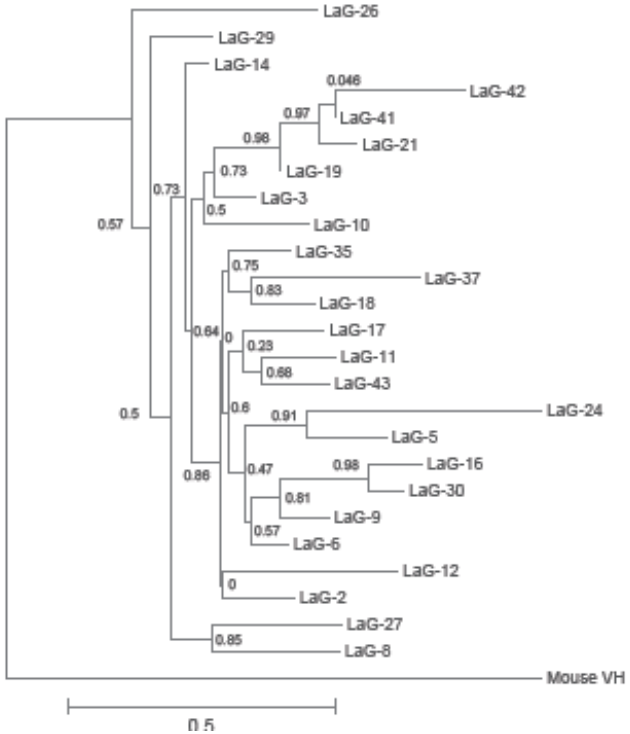
Automatic ranking pipeline + manual inspection → 44 LaG and 8 LaM

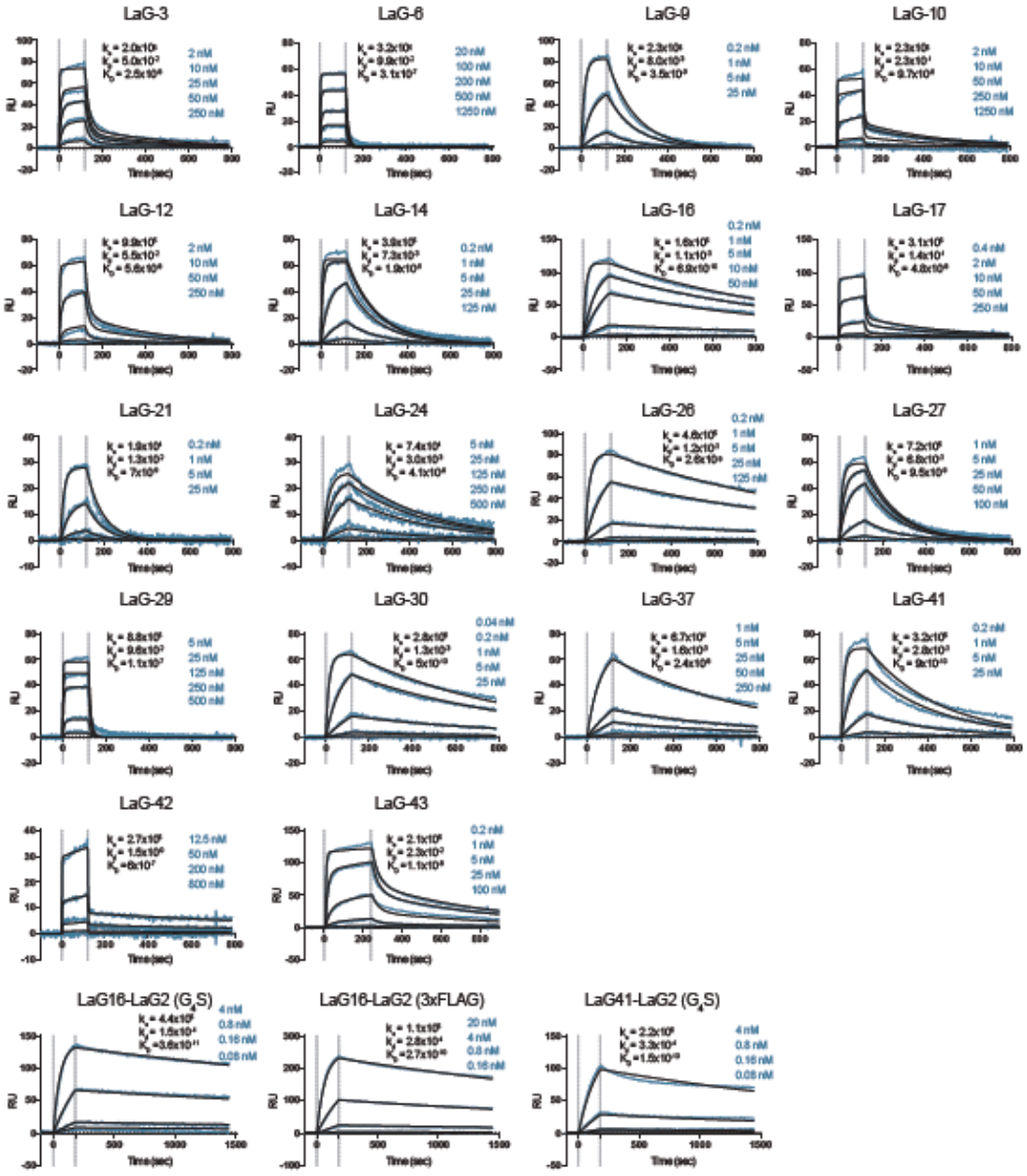
a



b

c


LaG-26
LaG-29
LaG-14
0.046
LaG-42
0.97
LaG-41
0.98
LaG-21
0.73
LaG-19
0.73
LaG-3
0.5
LaG-10
0.57
LaG-35
0.75
LaG-37
0.83
LaG-18
0.64
LaG-17
0
0.23
LaG-11
0.68
LaG-43
0.5
0.6
0.91
LaG-24
LaG-5
0.98
LaG-16
0.47
LaG-30
0.81
LaG-9
0.57
LaG-6
0.86
LaG-12
0
LaG-2
LaG-27
0.85
LaG-8
Mouse VH
0.5


LaG-3
LaG-6
LaG-9
LaG-10
LaG-12
LaG-14
LaG-16
LaG-17
LaG-21
LaG-24
LaG-26
LaG-27
LaG-29
LaG-30
LaG-37
LaG-41
LaG-42
LaG-43
LaG16-LaG2 (G4S)
LaG16-LaG2 (3xFLAG)
LaG41-LaG2 (G4S)
RU
Time (sec)

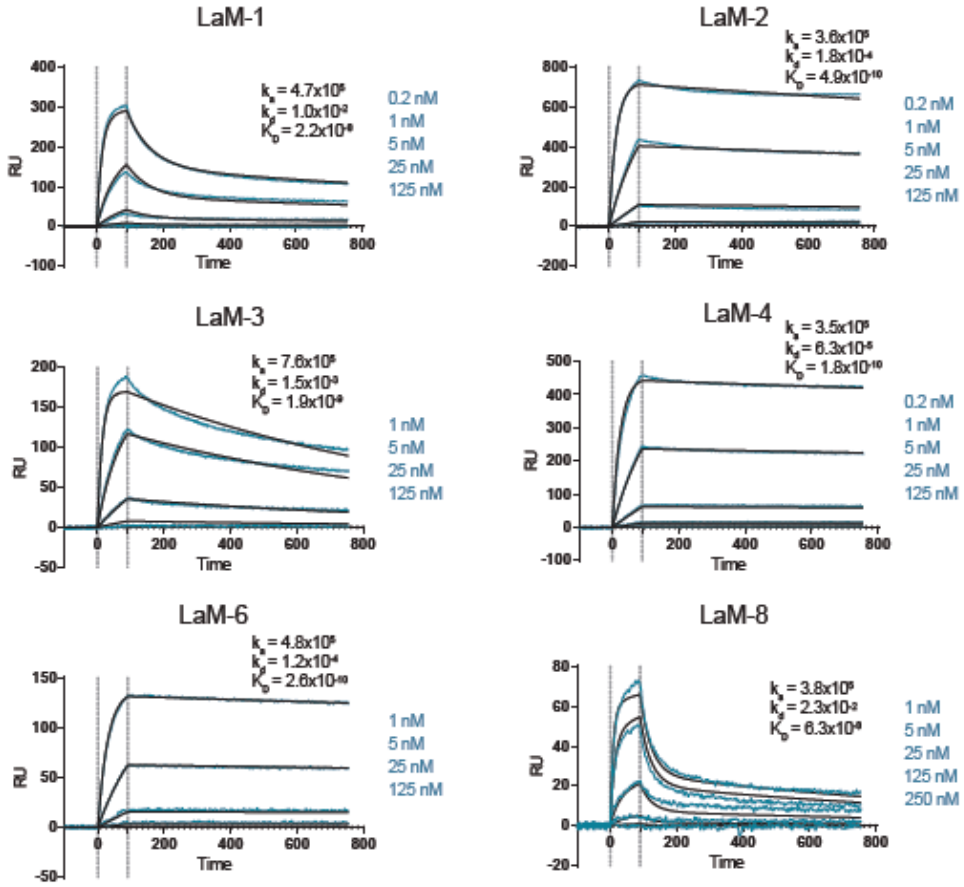
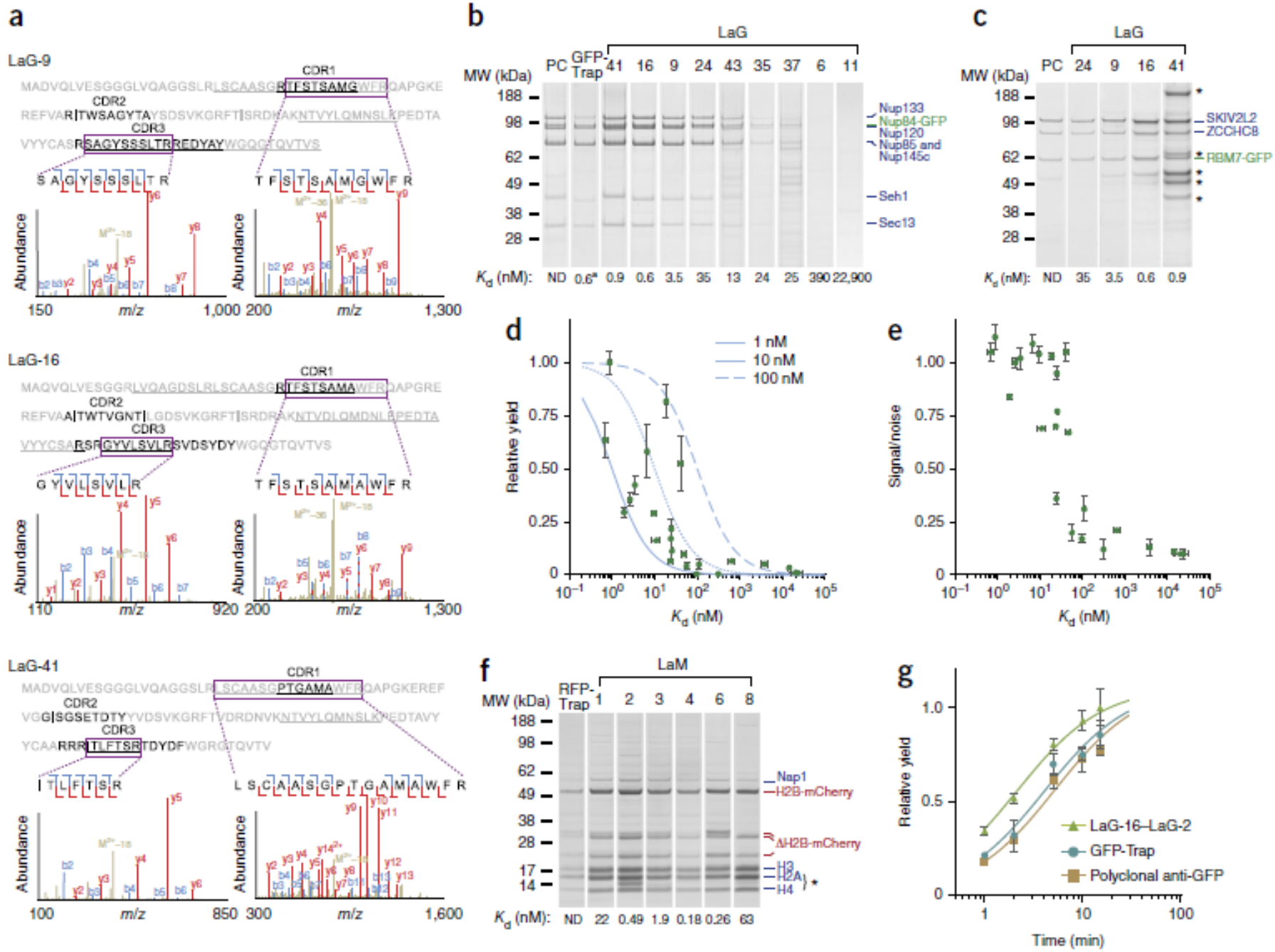
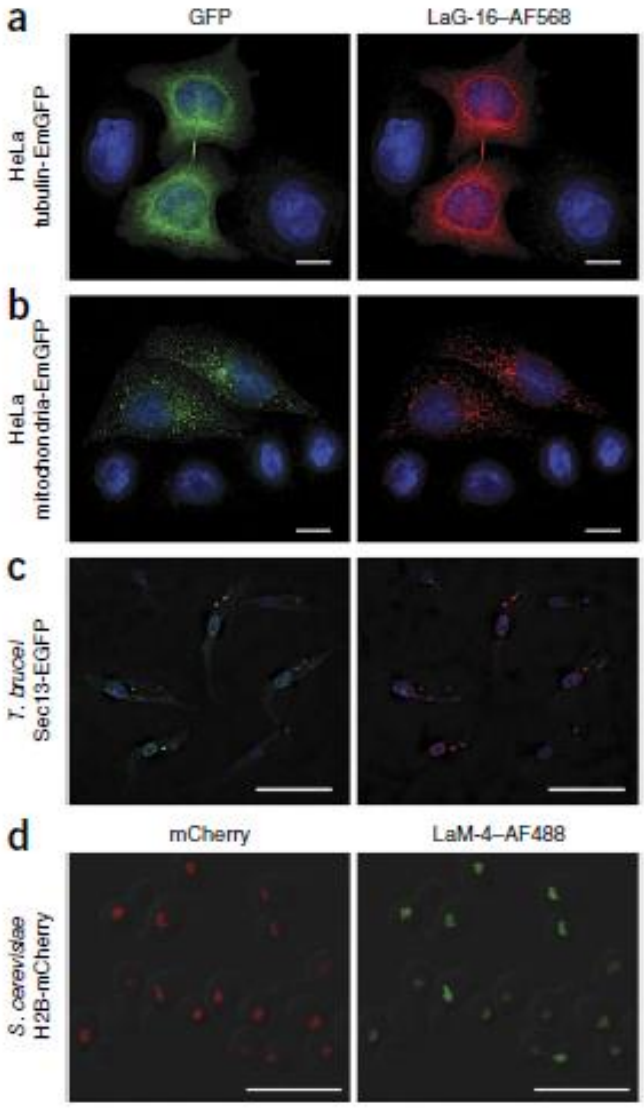

LaM-1
$k_a = 4.7x10^5$
$k_d = 1.0x10^{-2}$
$K_D = 2.2x10^{-8}$
0.2 nM
1 nM
5 nM
25 nM
125 nM
RU
Time
LaM-2
$k_a = 3.6x10^5$
$k_d = 1.8x10^{-4}$
$K_D = 4.9x10^{-10}$
0.2 nM
1 nM
5 nM
25 nM
125 nM
RU
Time
LaM-3
$k_a = 7.6x10^5$
$k_d = 1.5x10^{-3}$
$K_D = 1.9x10^{-9}$
1 nM
5 nM
25 nM
125 nM
RU
Time
LaM-4
$k_a = 3.5x10^5$
$k_d = 6.3x10^{-5}$
$K_D = 1.8x10^{-10}$
0.2 nM
1 nM
5 nM
25 nM
125 nM
RU
Time
LaM-6
$k_a = 4.8x10^5$
$k_d = 1.2x10^{-4}$
$K_D = 2.6x10^{-10}$
1 nM
5 nM
25 nM
125 nM
RU
Time
LaM-8
$k_a = 3.8x10^5$
$k_d = 2.3x10^{-2}$
$K_D = 6.3x10^{-8}$
1 nM
5 nM
25 nM
125 nM
250 nM
RU
Time

Table 1 | Characteristics of LaG, LaG dimer, and LaM proteins

Clone ID	MW (Da)	K_d (nM)	Nup84-GFP S:N	RBM7-GFP S:N	Binds *A. macrodactyla* CFP (LaG) or DsRed (LaM)	GFP epitope group	No. of binding-site residues	ASA of binding-site residues (Å^2)
LaG-2	15,919	19[a], 16	1.03	0.42	–	III	55	2,204
LaG-3	15,329	25	0.77	1.13	+	ND	ND	ND
LaG-6	15,700	310	0.12	ND	+	ND	ND	ND
LaG-9	16,062	3.5	1.02	1.04	+	I	62	2,551
LaG-10	15,748	97	0.17	ND	+	ND	ND	ND
LaG-12	16,090	56	0.20	ND	+	ND	ND	ND
LaG-14	16,002	1.9	0.84	0.58	+	I	66	2,519
LaG-16	16,306	0.7	1.05	0.92	+	I	60	2,605
LaG-17	15,823	50	0.67	ND	+	I	60	2,543
LaG-19	15,528	24.6[a]	0.95	1.06	+	II	54	2,404
LaG-21	15,452	7	1.09	ND	+	II	56	2,340
LaG-24	14,763	41	1.05	1.09	–	ND	ND	ND
LaG-26	16,221	2.6	1.00	ND	+	II	53	2,070
LaG-27	15,565	9.5	1.04	ND	+	II	57	2,216
LaG-29	15,449	110	0.31	ND	+	ND	ND	ND
LaG-30	16,159	0.5	1.04	ND	+	ND	ND	ND
LaG-35	16,010	23.5[a]	0.70	ND	+	ND	ND	ND
LaG-37	16,329	24	0.36	ND	+	ND	ND	ND
LaG-41	15,471	0.9	1.12	0.41	+	II	53	2,091
LaG-42	15,490	600	0.21	ND	+	ND	ND	ND
LaG-43	16,167	11	0.69	ND	+	I	55	2,381
LaG-5	15,589	14,200[a]	0.11	ND	ND	ND	ND	ND
LaG-8	15,953	20,000[a]	0.10	ND	ND	ND	ND	ND
LaG-11	16,221	22,900[a]	0.10	ND	ND	ND	ND	ND
LaG-18	16,459	3,800[a]	0.13	ND	ND	ND	ND	ND
LaG-16–G_4S–LaG-2	30,791	0.036	ND	ND	ND	ND	ND	ND
LaG-16–3×Flag–LaG-2	32,972	0.268	ND	ND	ND	ND	ND	ND
LaG-41–G_4S–LaG-2	29,956	0.150	ND	ND	ND	ND	ND	ND
LaM-1	15,380	22	N/A	N/A	–	N/A	N/A	N/A
LaM-2	15,151	0.49	N/A	N/A	–	N/A	N/A	N/A
LaM-3	15,196	1.9	N/A	N/A	+	N/A	N/A	N/A
LaM-4	14,866	0.18	N/A	N/A	+	N/A	N/A	N/A
LaM-6	14,428	0.26	N/A	N/A	–	N/A	N/A	N/A
LaM-8	14,666	63	N/A	N/A	–	N/A	N/A	N/A


a
LaG-9
CDR1
CDR2
CDR3
MADVQLVESGGGLVQAGGSLRLSCAASGRTFSTSAMGWFRQAPGKE
REFVARITWSAGYTAYSDSVKGRFTISRDNAKNTVYLQMNSLKPEDTA
VYYCASRSAGYSSSLTRREDYAYWGQGTQVTVS
S A G Y S S S L T R
T F S T S A M G W F R
Abundance
m/z
150
1,000
200
1,300
LaG-16
CDR1
CDR2
CDR3
MAQVQLVESGGRLVQAGDSLRLSCAASGRTFSTSAMAWFRQAPGRE
REFVAATWTVGNTLGDSVKGRFTISRDRAKNTVDLQMDNLKPEDTA
VYYCSARSRGYVLSVLRSVDSYDYWGQGTQVTVS
G Y V L S V L R
T F S T S A M A W F R
110
920
200
1,300
LaG-41
CDR1
CDR2
CDR3
MADVQLVESGGGLVQAGGSLRLSCAASGPTGAMAWFRQAPGKEREF
VGGISGSETDTYYVDSVKGRFTVDRDNVKNTVYLQMNSLKPEDTAVY
YCAARRRTLFTSRTDYDFWGRGTQVTV
I T L F T S R
L S C A A S G P T G A M A W F R
100
850
300
1,600
b
LaG
PC
GFP-Trap
41
16
9
24
43
35
37
6
11
MW (kDa)
188
98
62
49
38
28
Nup133
Nup84-GFP
Nup120
Nup85 and Nup145c
Seh1
Sec13
Kd (nM):
ND
0.6*
0.9
0.6
3.5
35
13
24
25
390
22,900
c
LaG
PC
24
9
16
41
MW (kDa)
188
98
62
49
38
28
SKIV2L2
ZCCHC8
RBM7-GFP
Kd (nM):
ND
35
3.5
0.6
0.9
d
1 nM
10 nM
100 nM
Relative yield
1.00
0.75
0.50
0.25
0
Kd (nM)
e
Signal/noise
1.00
0.75
0.50
0.25
0
Kd (nM)
f
LaM
RFP-Trap
1
2
3
4
6
8
MW (kDa)
188
98
62
49
38
28
17
14
Nap1
H2B-mCherry
ΔH2B-mCherry
H3
H2A
H4
*
Kd (nM):
ND
22
0.49
1.9
0.18
0.26
63
g
Relative yield
1.0
0.5
0
LaG-16–LaG-2
GFP-Trap
Polyclonal anti-GFP
1
10
100
Time (min)

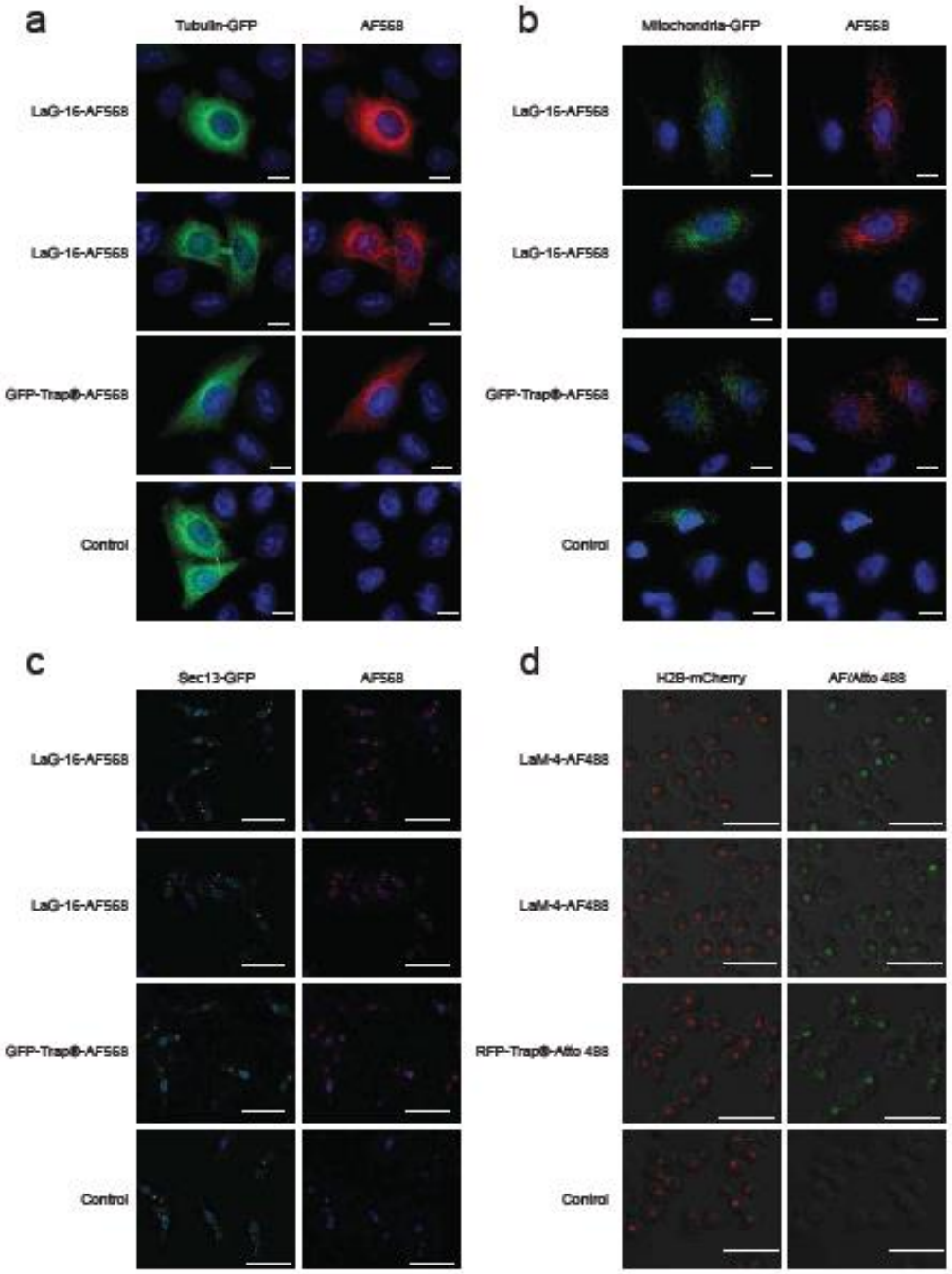
Tools for Fluorescence Microscopy

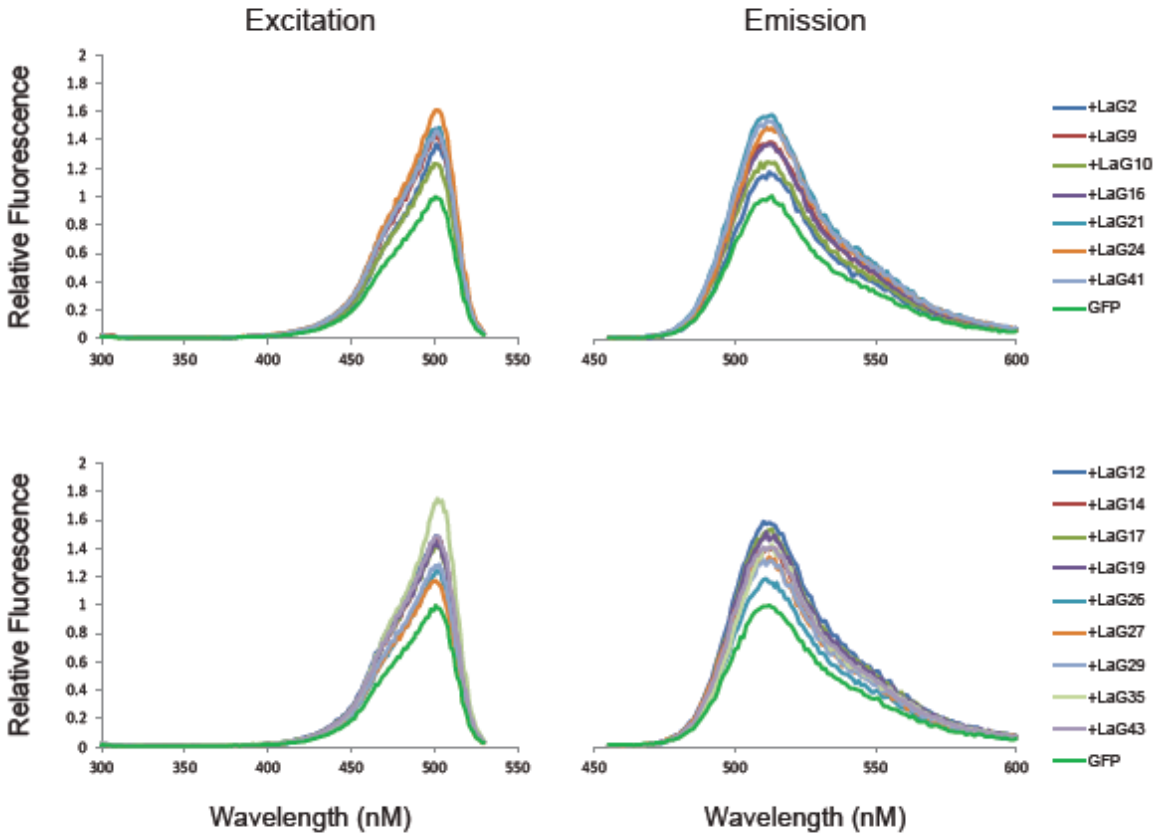


- Standard and Super Resolution Microscopy

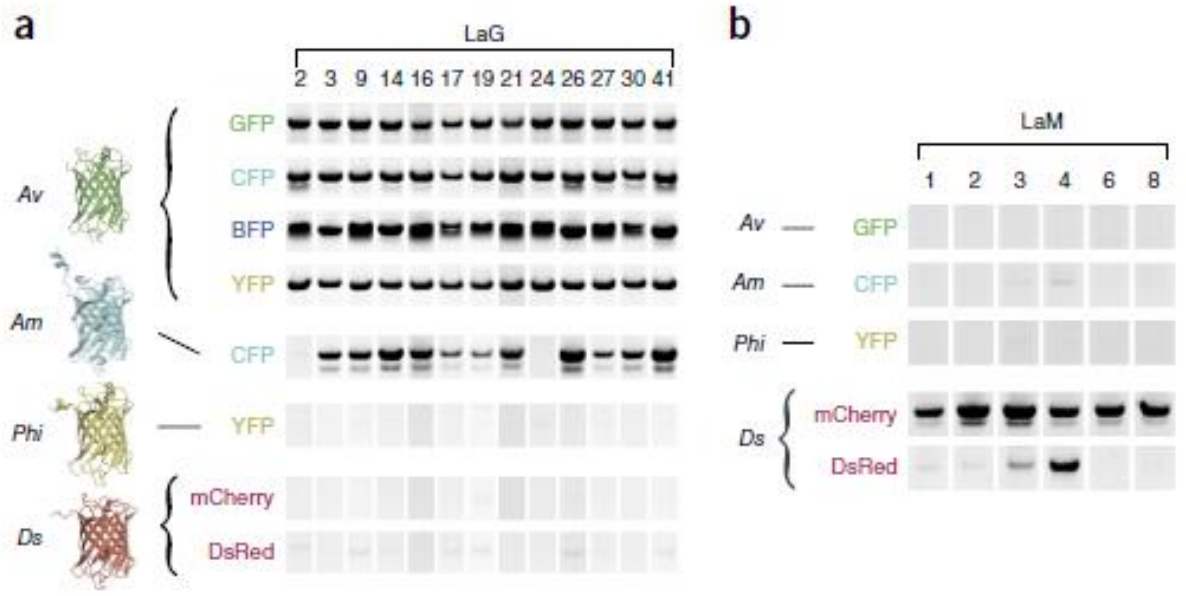
Target Proteins	Nanobodies
Tubulin- Emerald GFP Mitochondria- Emeral GFP	LaG-16 – Alexa Fluor 568 (Life Technologies)
Sec13-EGFP*	LaG-16 – Alexa Fluor 568
H2B- mCherry	LaM-4 – Alexa Fluor 488

* Localizes the nuclear pore complex and COPII coated vesicle


a
Tubulin-GFP
AF568
LaG-16-AF568
LaG-16-AF568
GFP-Trap®-AF568
Control
b
Mitochondria-GFP
AF568
LaG-16-AF568
LaG-16-AF568
GFP-Trap®-AF568
Control
c
Sec13-GFP
AF568
LaG-16-AF568
LaG-16-AF568
GFP-Trap®-AF568
Control
d
H2B-mCherry
AF/Atto 488
LaM-4-AF488
LaM-4-AF488
RFP-Trap®-Atto 488
Control

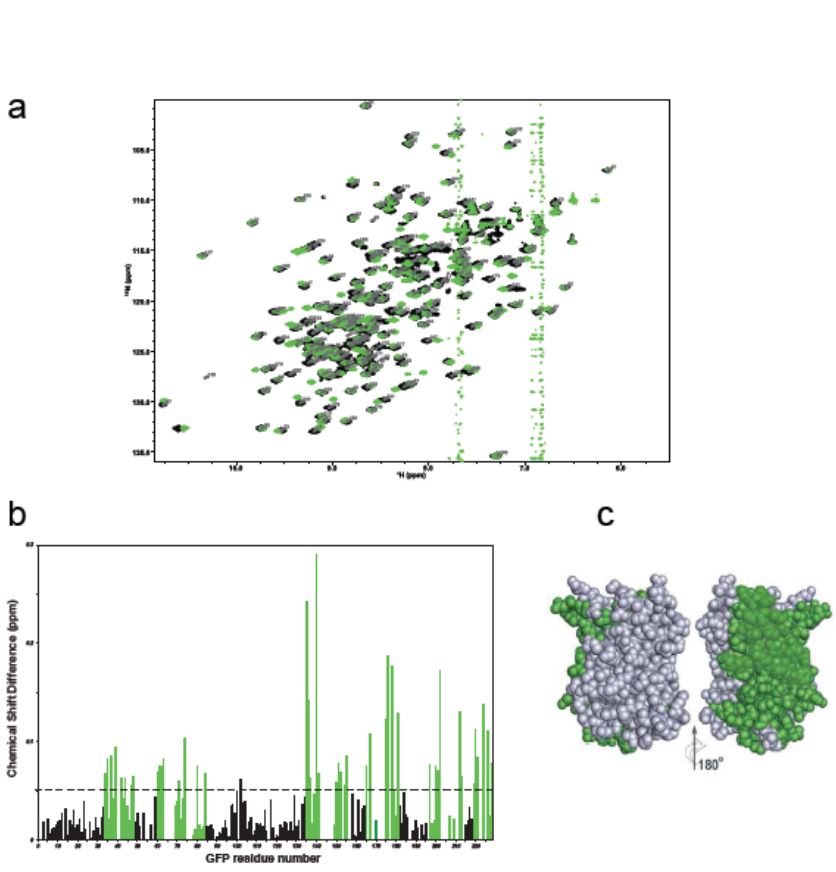

Excitation
Emission
Relative Fluorescence
Relative Fluorescence
Wavelength (nM)
Wavelength (nM)
+LaG2
+LaG9
+LaG10
+LaG16
+LaG21
+LaG24
+LaG41
GFP
+LaG12
+LaG14
+LaG17
+LaG19
+LaG26
+LaG27
+LaG29
+LaG35
+LaG43
GFP

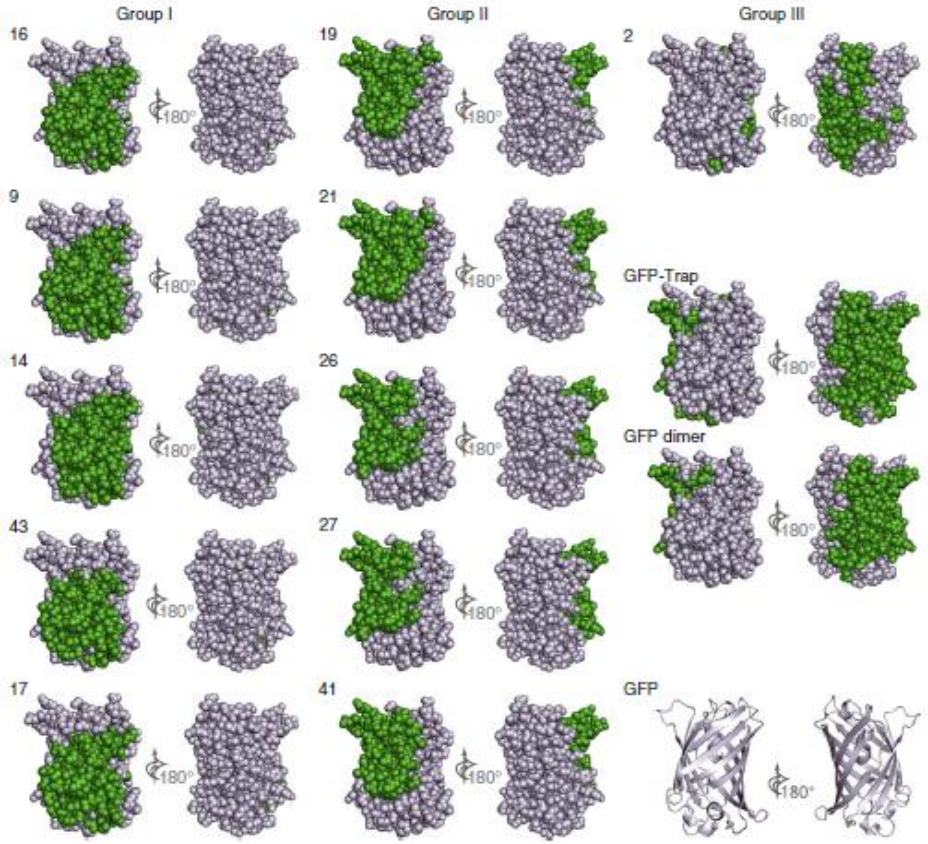
Homologs recognition?



"(…) although the identified LaGs bind specifically to fluorescent proteins that have high identity to EGFP, differential binding activities can be obtained though use of variants from other species."

Mapping of Nbs epitope on EGFP



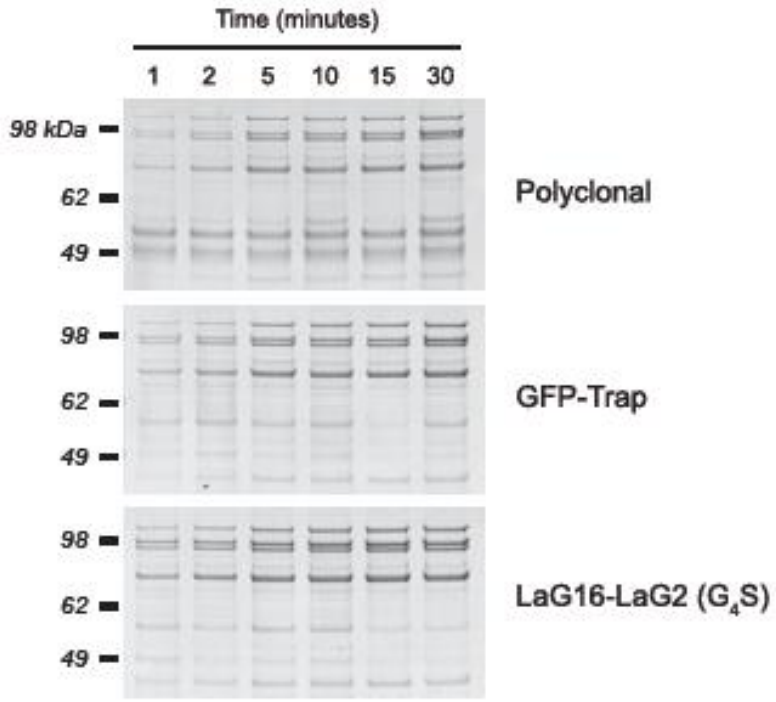


Ultrahigh-affinity reagents

Heterodimers of LaGs with non-overlapping binding sites on GFP

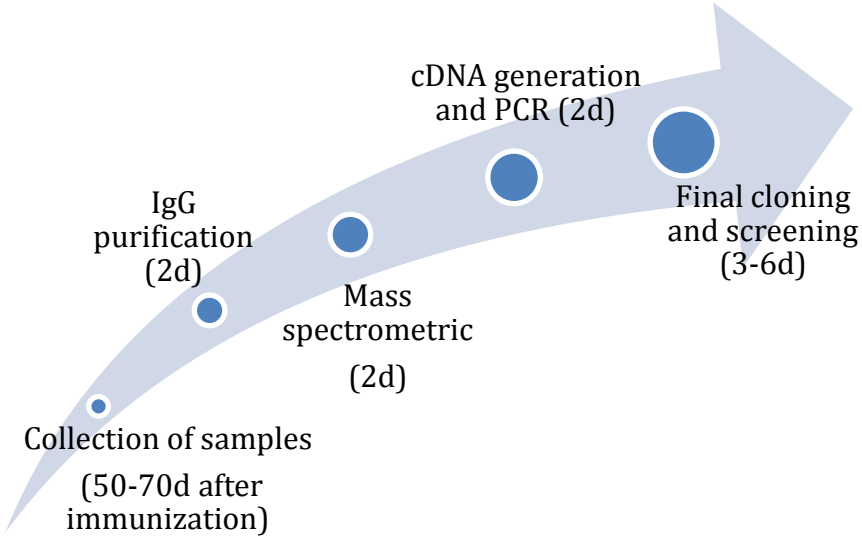
Can they potentially bind with higher affinity?

- ✓ Increasingly rapid affinity isolations
- ✓ Capture of weakly or transient associated complex components
- ✓ Detection of low abundance antigens



Summary

- Rapid production of a comprehensive repertoire of specific, high-affinity Nbs for use in the characterization of target molecules
- Identification of high-affinity Nb sequences directly from animal serum
- Outsourced: animal handling, high-throughput sequencing, mass spectrometric and gene synthesis
- Entire process can take 4-6 weeks



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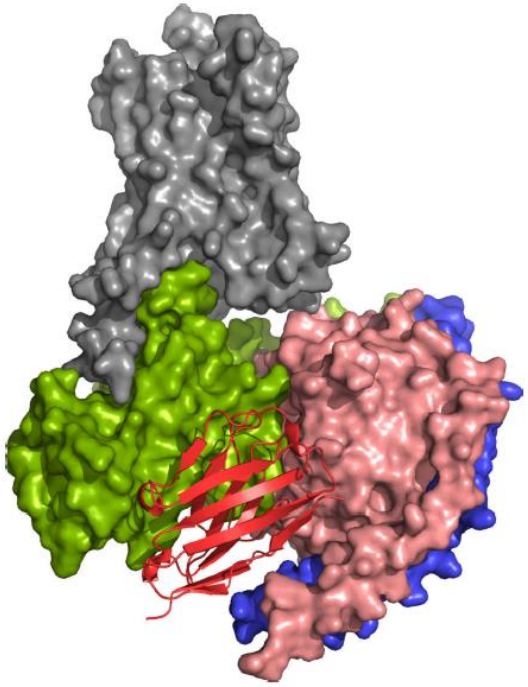
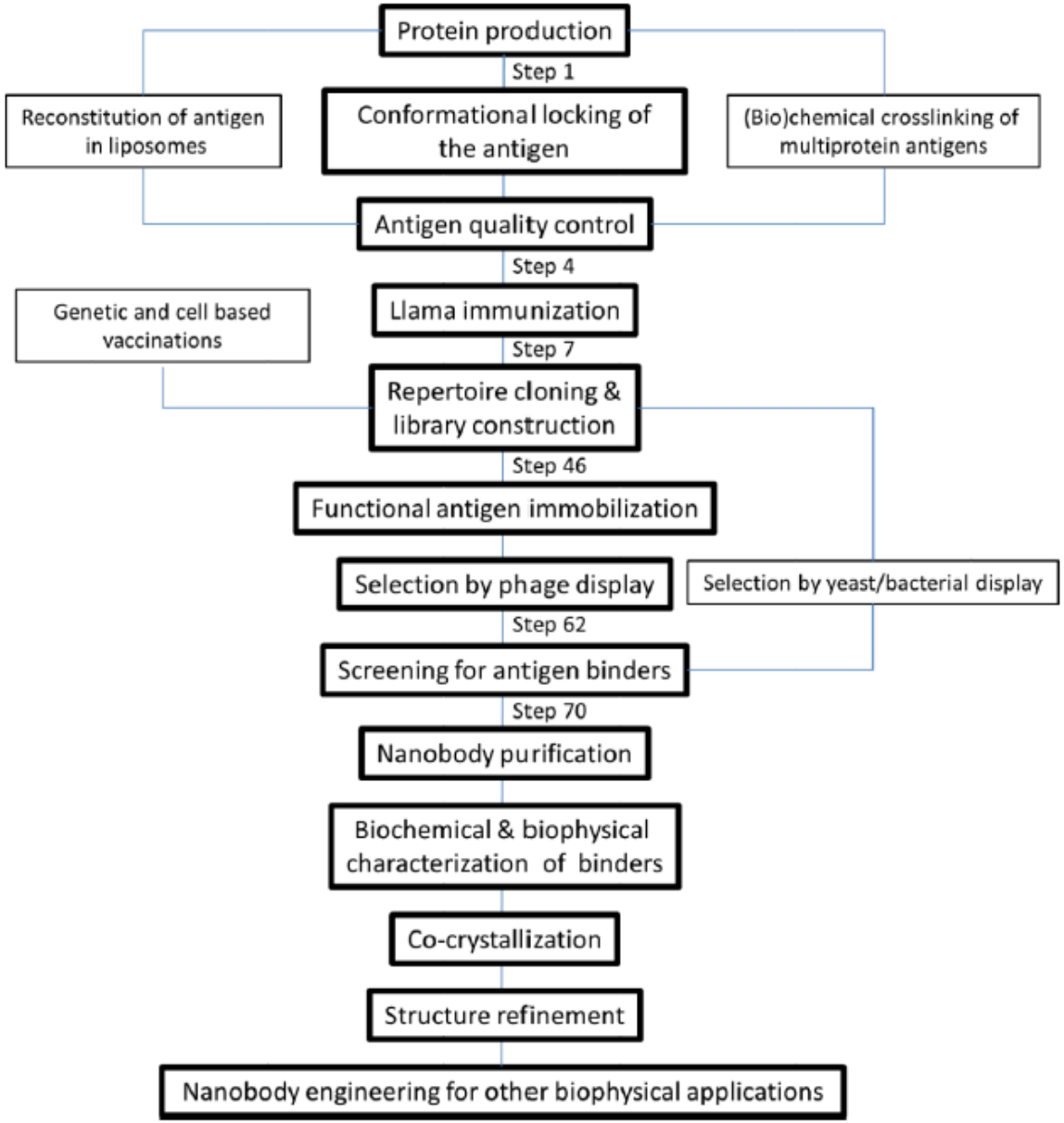
A general protocol for the generation of Nanobodies for structural biology

Els Pardon[1,2], **Toon Laeremans**[1,2], **Sarah Triest**[1,2], **Søren G. F. Rasmussen**[3], **Alexandre Wohlkönig**[1,2], **Armin Ruf**[4], **Serge Muyldermans**[2,5], **Wim G. J. Hol**[6], **Brian K. Kobilka**[7], and **Jan Steyaert**[1,2]

Protocol for generation, selection and purification of recombinant in vivo matured Nanobodies for structural biology.



Structure of the β2AR•Gs complex solved by Nanobody-enabled X-ray crystallography.


Protein production
Step 1
Reconstitution of antigen in liposomes
Conformational locking of the antigen
(Bio)chemical crosslinking of multiprotein antigens
Antigen quality control
Step 4
Genetic and cell based vaccinations
Llama immunization
Step 7
Repertoire cloning & library construction
Step 46
Functional antigen immobilization
Selection by phage display
Selection by yeast/bacterial display
Step 62
Screening for antigen binders
Step 70
Nanobody purification
Biochemical & biophysical characterization of binders
Co-crystallization
Structure refinement
Nanobody engineering for other biophysical applications

frontiers in
CELLULAR NEUROSCIENCE

PERSPECTIVE ARTICLE
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Nanobodies as modulators of inflammation: potential applications for acute brain injury

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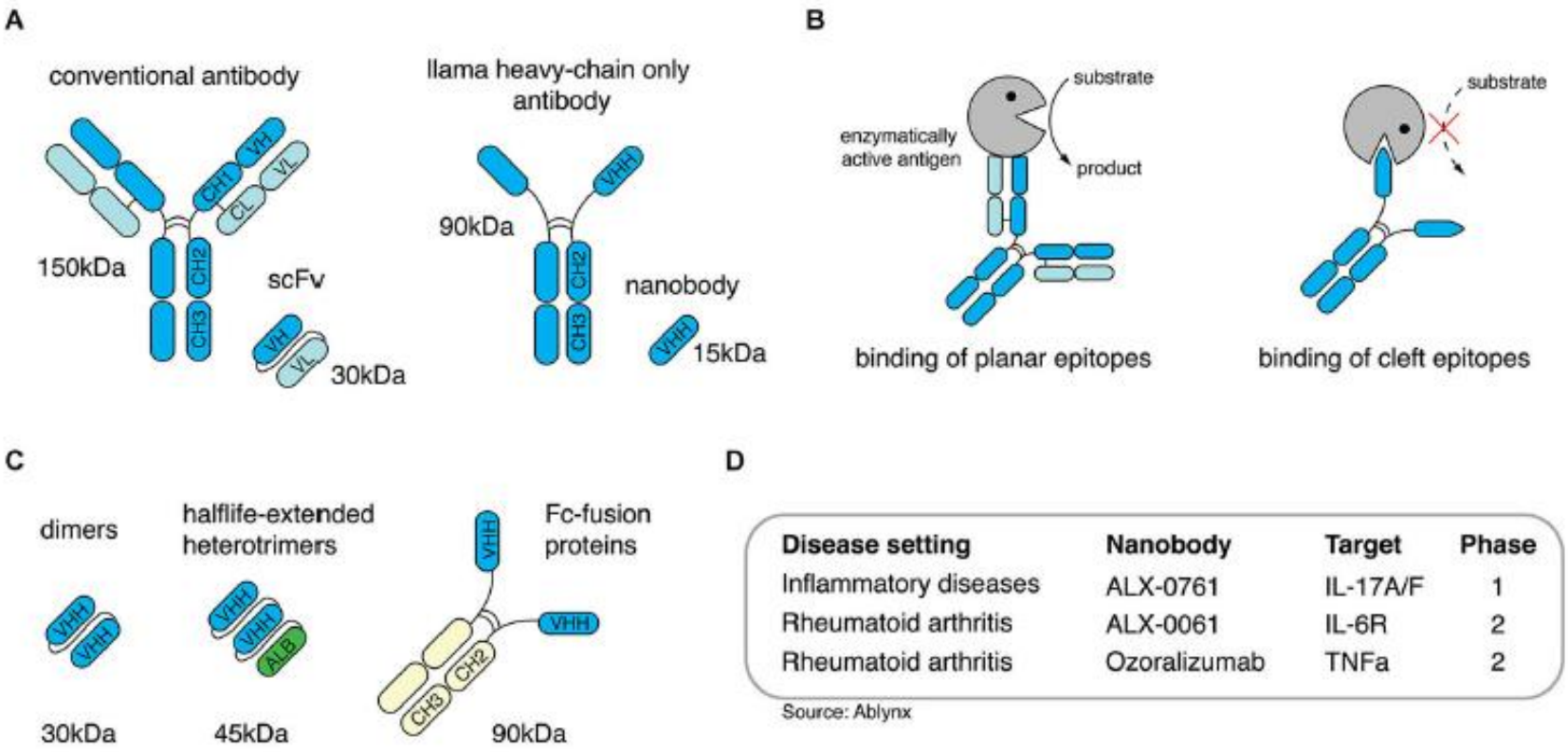
[2] *Department of Immunology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany*



Disease setting	Nanobody	Target	Phase
Inflammatory diseases	ALX-0761	IL-17A/F	1
Rheumatoid arthritis	ALX-0061	IL-6R	2
Rheumatoid arthritis	Ozoralizumab	TNFa	2

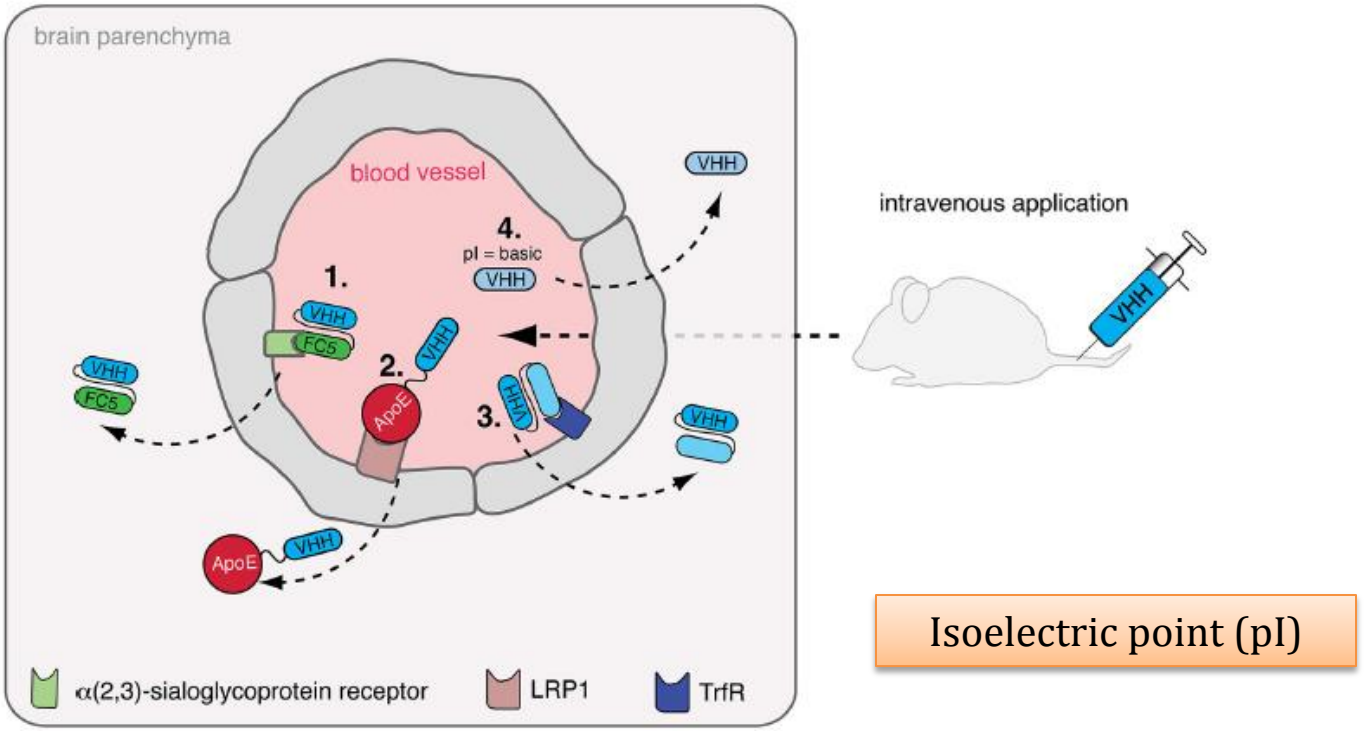
Source: Ablynx

Agonistic anti-Fc-γ-RIIIA nanobodies ➔ CEA^{+} tumor cells ➔ *in situ* NK activation

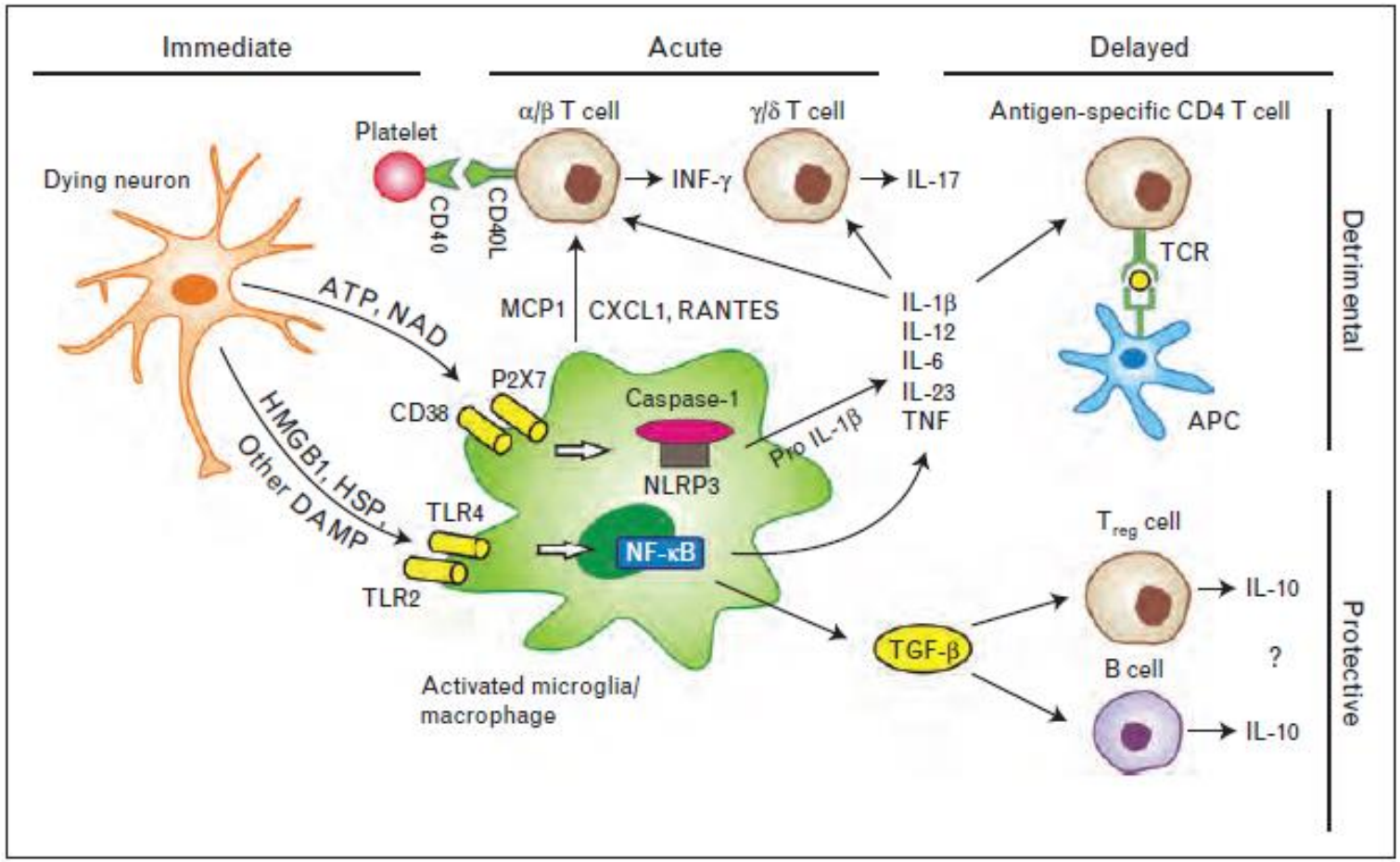
FC5 – Dal

↓

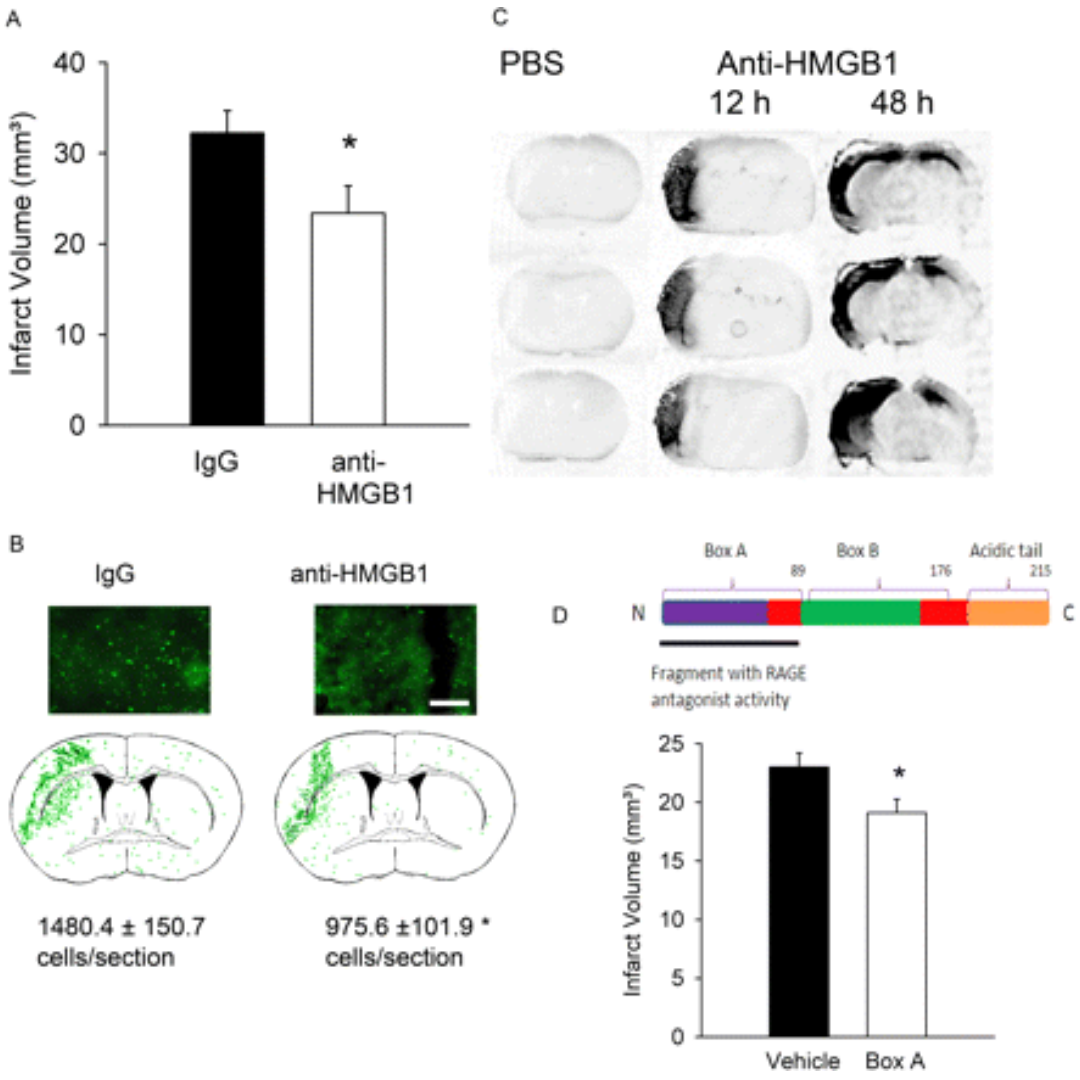
Significative Analgesic response



Isoelectric point (pI)



Magnus, T. et al. *Immune mechanisms of stroke.* 2012 Curr. Opin. Neurol. 25, 334-340.
doi: 10.1097/WCO.0b013e328352ede6



Muhammad, S. et al. *The HMGB1 receptor RAGE mediates ischemic brain damage.* 2008. J. Neuroscience 28, 12023-12031. doi: 10.1523/JNEUROSCI.2435-08.2008

Final Disclosures

Expectations

- "Nbs generation" will create access to unique locations, such as those that cross Blood-Brain Barrier or even by penetrating own cell's membranes and delivering .

- Additional developments for Nbs as highly specific capturing agents; in chromatin immunoprecipitations; intracellular imaging; and in specific degradation, retention or translocation of intracellular targets.

- Usage in next generation therapeutics and its commercialization.

Final Disclosures

Limitations

- Sometimes camelids immunization response to antigen in the HCAb is low.

- Steel need to elucidate the ontogeny of functional HCAbs in B cells of camelids.

- Nanobodies perform poorly at binding peptides or intrinsically unfolded parts of proteins.

- The tool-box of Nb-based products still needs further development for target validation and to facilitate its application.


Thank you for your atention!