

Module 2 overview

lecture

1. Introduction to the module
2. Rational protein design
3. Fluorescence and sensors
4. Protein expression

lab

1. Start-up protein eng.
2. Site-directed mutagenesis
3. DNA amplification
4. Prepare expression system

SPRING BREAK

5. Review & gene analysis
6. Purification and protein analysis
7. Binding & affinity measurements
8. High throughput engineering

5. Gene analysis & induction
6. Characterize expression
7. Assay protein behavior
8. Data analysis

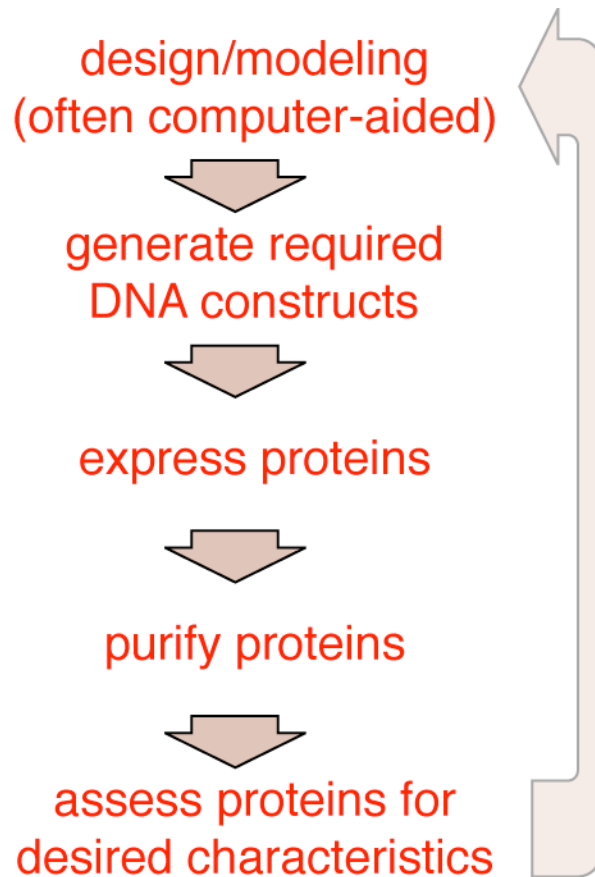
Lecture 8: High throughput engineering

- I. General requirements for HT engineering
 - A. High throughput vs. rational design
 - B. Generating libraries

- II. Selection techniques
 - A. Phage display and related techniques
 - B. Selection for properties other than affinity

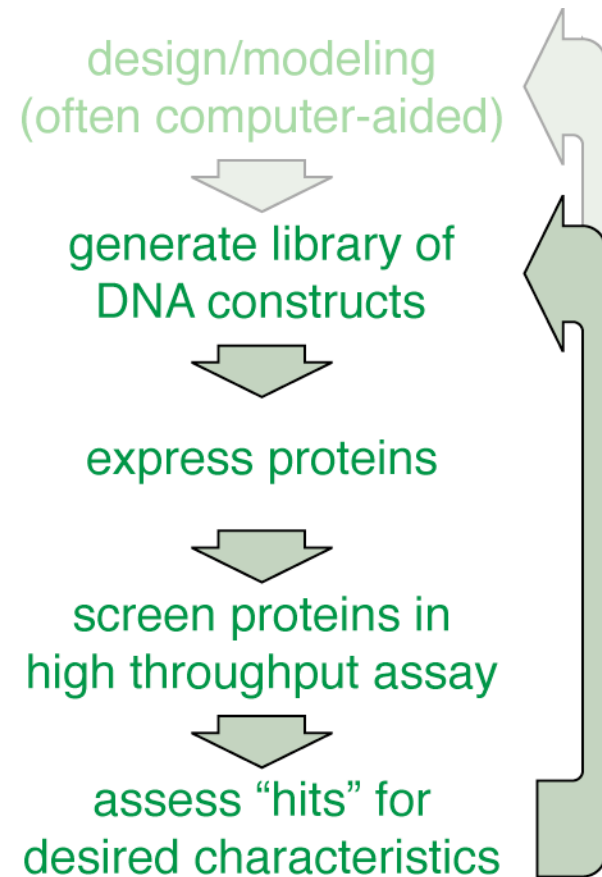
Rational protein design:

Knowledge-based, deterministic engineering of proteins with novel characteristics



“Irrational” high throughput protein engineering:

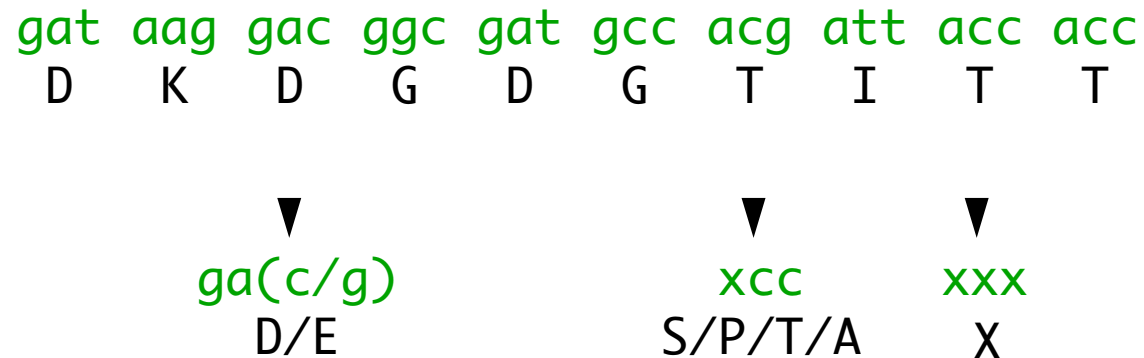
Selection for desired properties from libraries of random variants



Methods for generating mutant protein libraries:

- site-directed mutagenesis with degenerate primers
- error-prone PCR
- gene shuffling

Degenerate primers



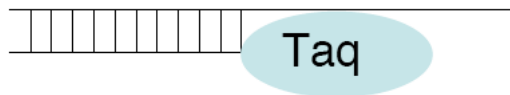
- not all combinations of AA's possible at each position
- number of combinations expands exponentially
- degenerate primers synthesized by split-pool method
- standard primer design criteria must be considered

PCR polymerase and conditions may be chosen to promote mutations

Polymerase	Template doublings (d) ^a	<i>lacI</i> ⁻ plaques ^b (% \pm SD)	Mutation load ^c (per kilobase) (\pm SD)	Error rate ^d (per base) ($\times 10^{-6} \pm$ SD)
Pfu-Pol (exo ⁺)	12.3	0.61 \pm 0.09	0.017 \pm 0.002	1.4 \pm 0.2
Pfu-Pol (exo ⁻)	11.8	20 \pm 1.7	0.58 \pm 0.05	49 \pm 4
Taq-Pol	11.6	3.9 \pm 0.16	0.12 \pm 0.006	10 \pm 0.5

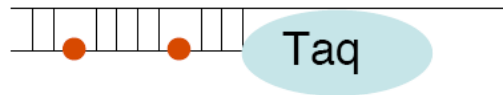
error rate = mutation load \div template doublings

normal PCR



dCTP, dTTP
dGTP, dATP
Mg²⁺

error-prone PCR



dCTP, dTTP \uparrow
dGTP, dATP \downarrow
Mg²⁺ \uparrow
Mn²⁺

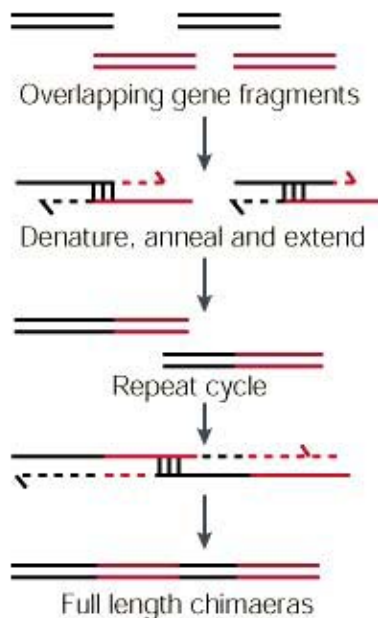
some mutations are more likely than others

Mutation	Pfu-Pol(exo ⁻) D473G ^a	Taq-Pol (Mn ²⁺ / unbalanced dNTPs) ^b	Taq-Pol (Mn ²⁺ / unbalanced dNTPs) ^c	Taq-Pol (unnatural mutagenic bases) ^d
A \rightarrow T/T \rightarrow A	28	40.9	11.4	0.2
A \rightarrow C/T \rightarrow G	7.4	7.3	3.3	8.4
A \rightarrow G/T \rightarrow C	19.2	27.6	60.9	78.3
G \rightarrow A/C \rightarrow T	22	13.6	18.1	13.2
G \rightarrow C/C \rightarrow G	7.3	1.4	4.3	0.7
G \rightarrow T/C \rightarrow A	10.3	4.5	1.8	0.0
Insertion	2.9	0.3	Not given	\sim 0
Deletion	2.9	4.2	Not given	\sim 0

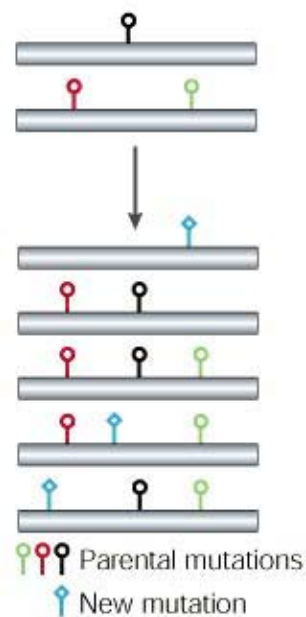
Gene shuffling techniques mimic diversity due to meiotic recombination:

- fragments of homologous genes combined using “sexual PCR”
- diversity may arise from error prone PCR or multiple genes

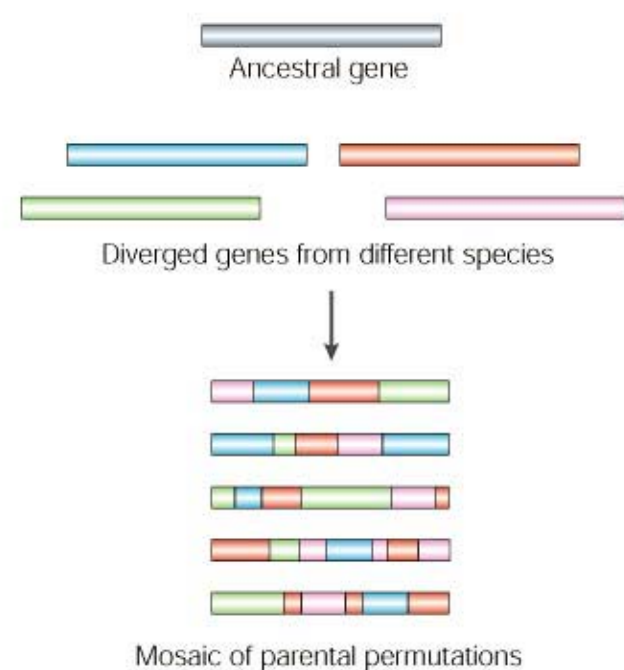
a Recursive PCR and gene assembly



b Single-gene shuffling



c Family shuffling



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Source: Brannigan, J. A., and A. J., Wilkinson. "Protein Engineering 20 Years on."

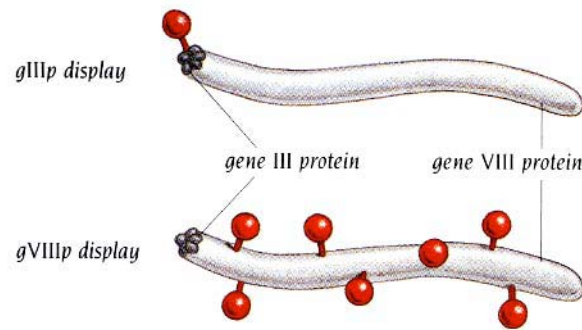
Nat Rev Mol Cell Biol 3 (2002): 964-70. © 2002.

How are libraries of mutant proteins screened?

All major methods include a strategy to keep DNA sequence info associated with the proteins that are being screened.

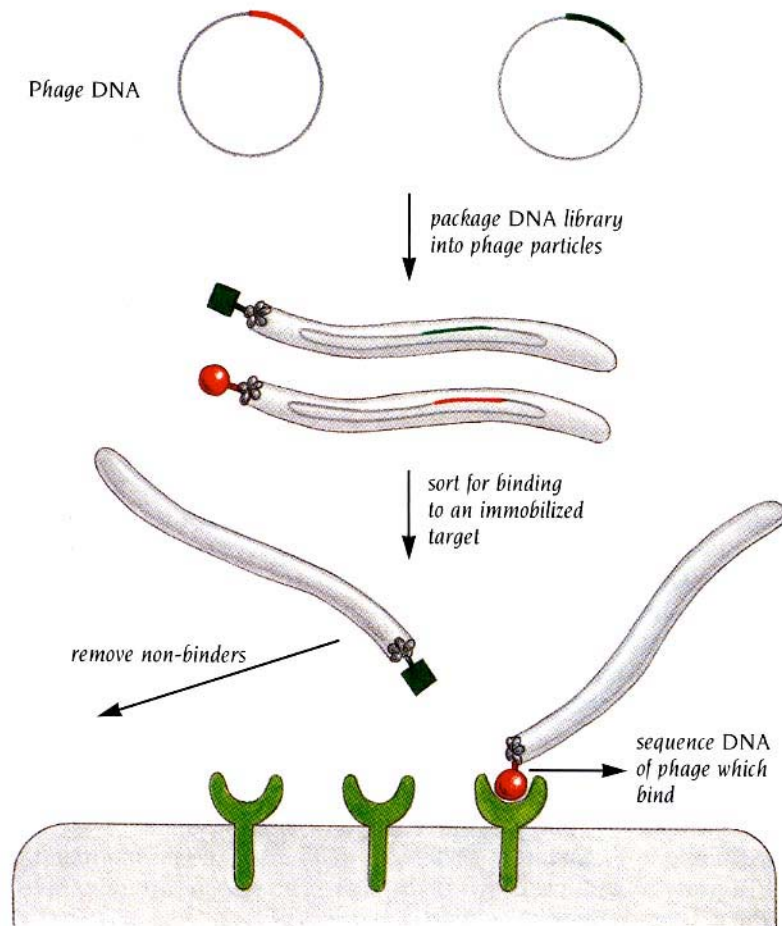
Phage display is a versatile high throughput method to do this:

protein “displayed” on the coat of a bacteriophage, by fusing to a natural phage coat protein



**M13 phage
(contains DNA)**

Image from Branden, C., and J. Tooze. *Introduction to Protein Structure*. 2nd ed. New York, NY: Garland Science, 1999. © Garland Science. All rights reserved. This content is excluded from our Creative Commons license. For more information, see <http://ocw.mit.edu/fairuse>.

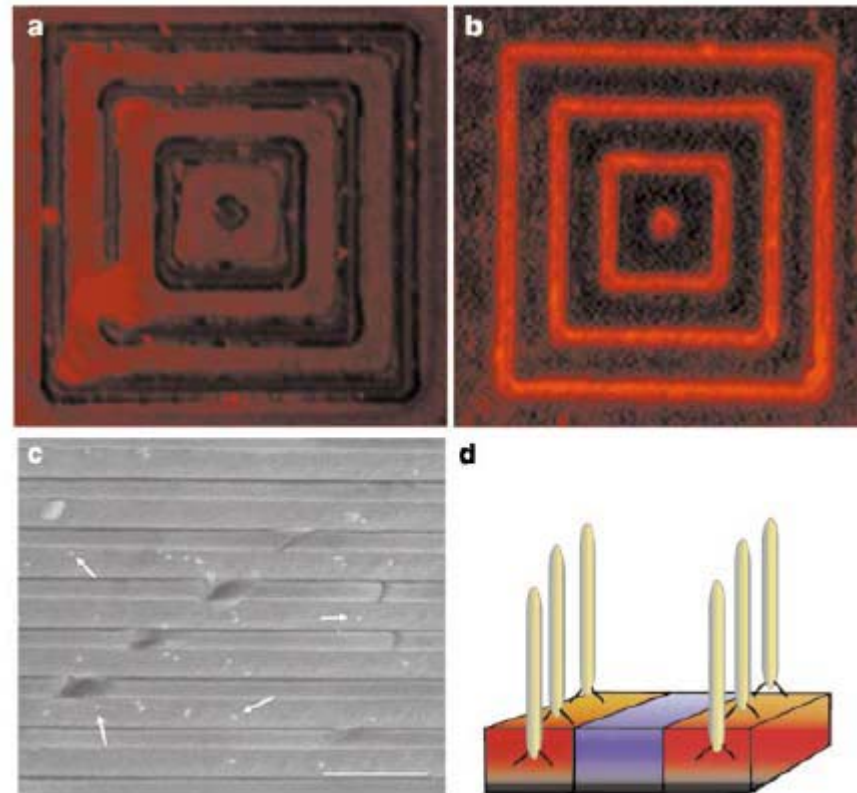


Application: phage-displayed peptides that bind to GaAs

selected sequences

G13-5	V	T	S	P	D	S	T	T	G	A	M	A
G12-5	A	A	S	P	T	Q	S	M	S	Q	A	P
G12-3	A	Q	N	P	S	D	N	N	T	H	T	H
G1-4	A	S	S	S	R	S	H	F	G	Q	T	D
G12-4	W	A	H	A	P	Q	L	A	S	S	S	T
G14-3	A	R	Y	D	L	S	I	P	S	S	E	S
G7-4	T	P	P	R	P	I	Q	Y	N	H	T	S
G15-5	S	S	L	Q	L	P	E	N	S	F	P	H
G14-4	G	T	L	A	N	Q	Q	I	F	L	S	S
G11-3	H	G	N	P	L	P	M	T	P	F	P	G
G1-3	R	L	E	L	A	I	P	L	Q	G	S	G

phages patterned on target substrate



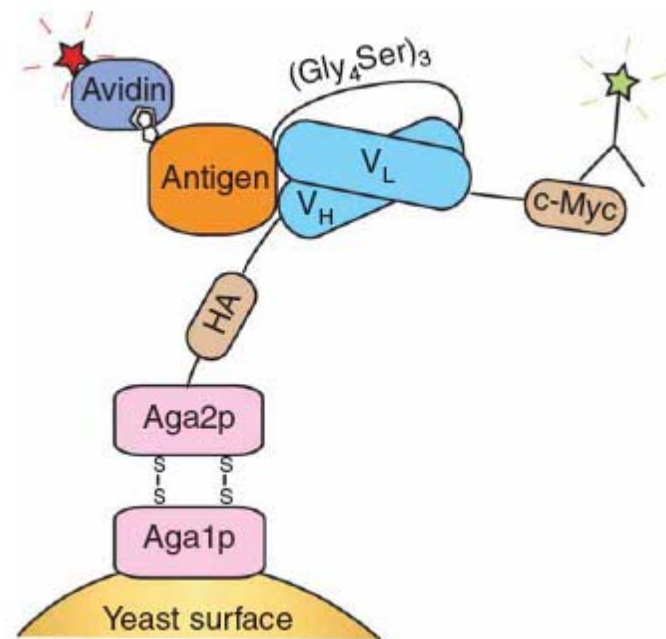
Whaley *et al.* (2000) *Nature* 405: 665-8.

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Source: Whaley, S. R., et al. "Selection of Peptides with Semiconductor Binding Specificity for Directed Nanocrystal Assembly." *Nature* 405 (2000): 665-668. © 2000.

Yeast display: similar to phage display, but with proteins fused to a *Saccharomyces* cell wall protein (DNA in yeast)

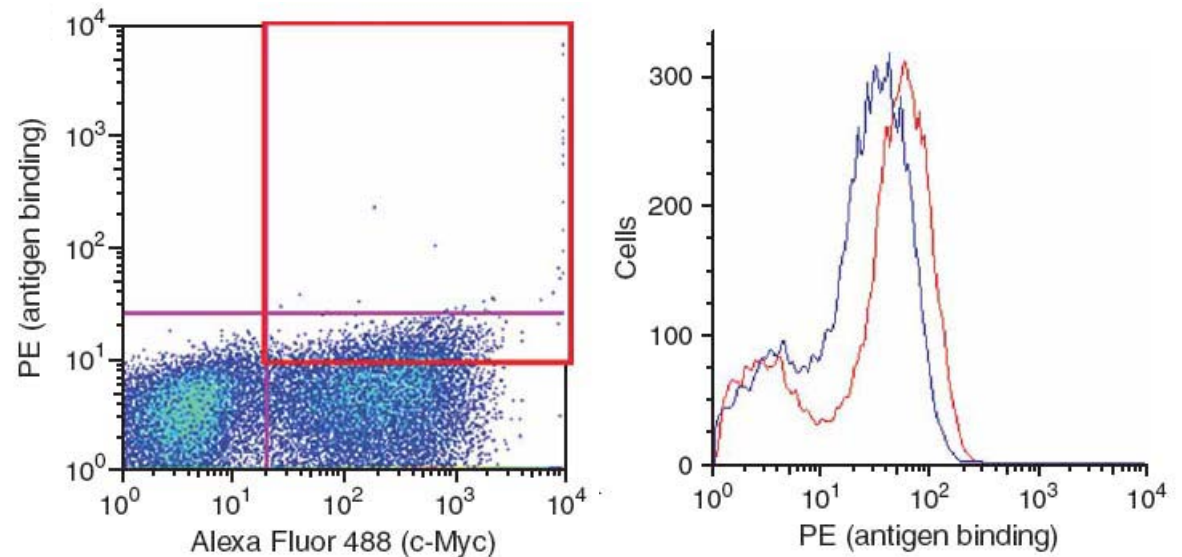
What would you expect advantages to be, compared with phage display?



In this example, a population of scFvs was screened for binding to an antigen

left: selection criterion for FACS assay

right: comparison of wt (blue) and selected (red) scFv binding



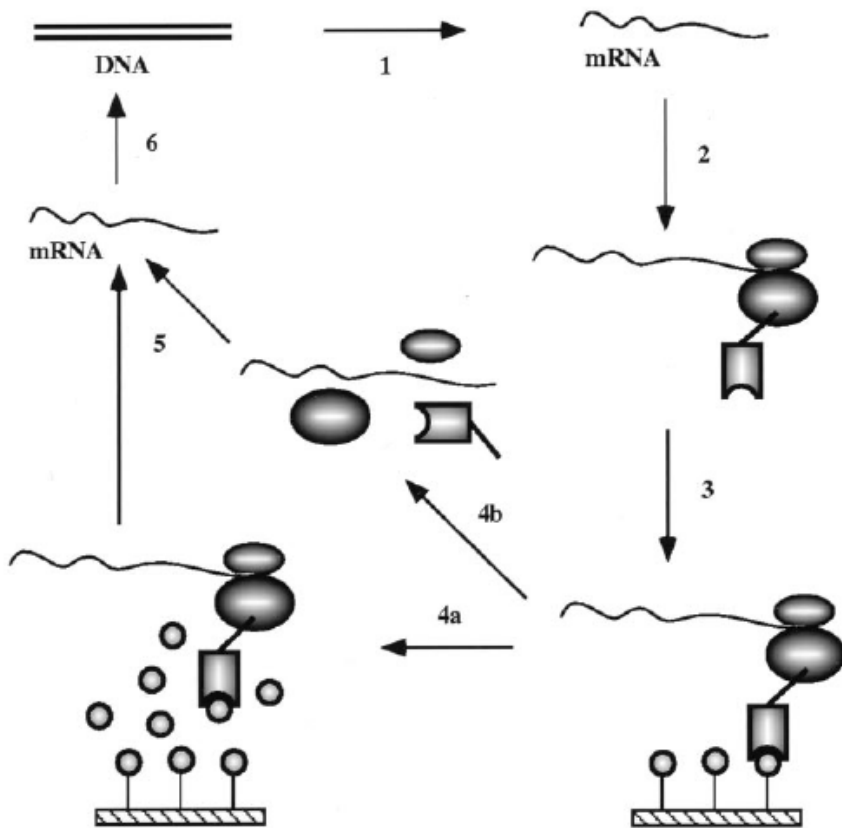
Chao *et al.* (2006) *Nat. Protoc.* 1: 755-68

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Source: Chao, G., et al. "Isolating and Engineering Human Antibodies Using Yeast Surface Display."

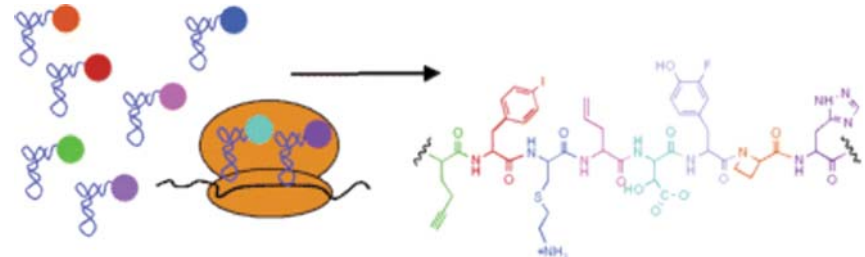
Nature Protocols 1(2006): 755-768. © 2006.

Ribosome display: mRNA and synthesized proteins held together non-covalently on a ribosome



What are advantages of this technique over phage/yeast display methods?

- screening not in the presence of large particles
- incorporation of unnatural amino acids



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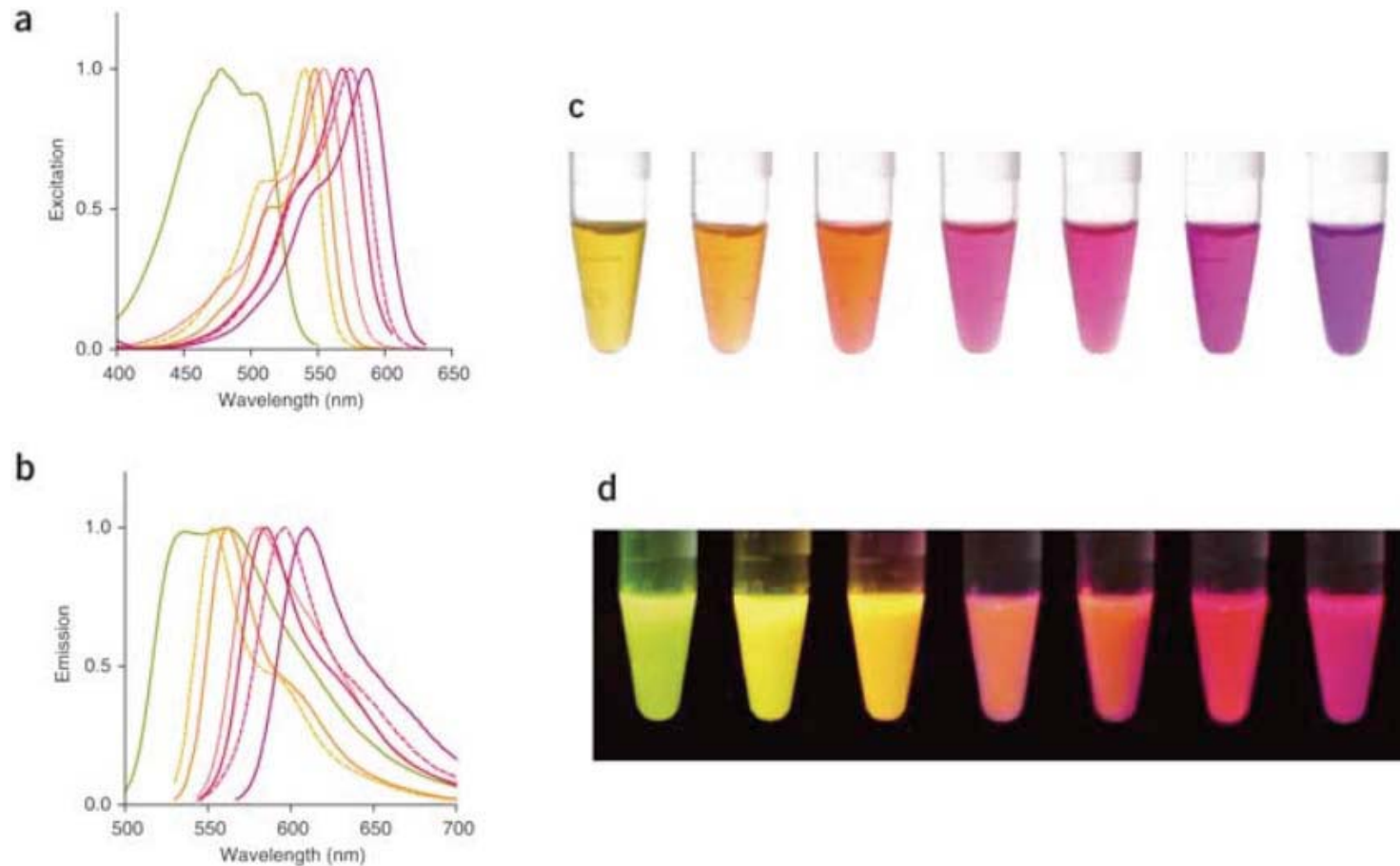
Left: Hanes & Plückthun (1997) *Proc. Natl. Acad. Sci. USA* 94: 4937-42

Right: Josephson *et al.* (2005) *J. Am. Chem. Soc.* 127: 11727-35

Courtesy of National Academy of Sciences, U. S. A. Used with permission.
 Source: Hanes, J., and A. Plückthun. "In *Vitro* Selection and Evolution of Functional Proteins by Using Ribosome Display." *PNAS* 94, no. 10 (1997): 4937–4942. Copyright © 1997 National Academy of Sciences, U.S.A.

What about properties other than affinity?

A simple example: screen for dsRed variants with different excitation and emission wavelengths—how could this be done?



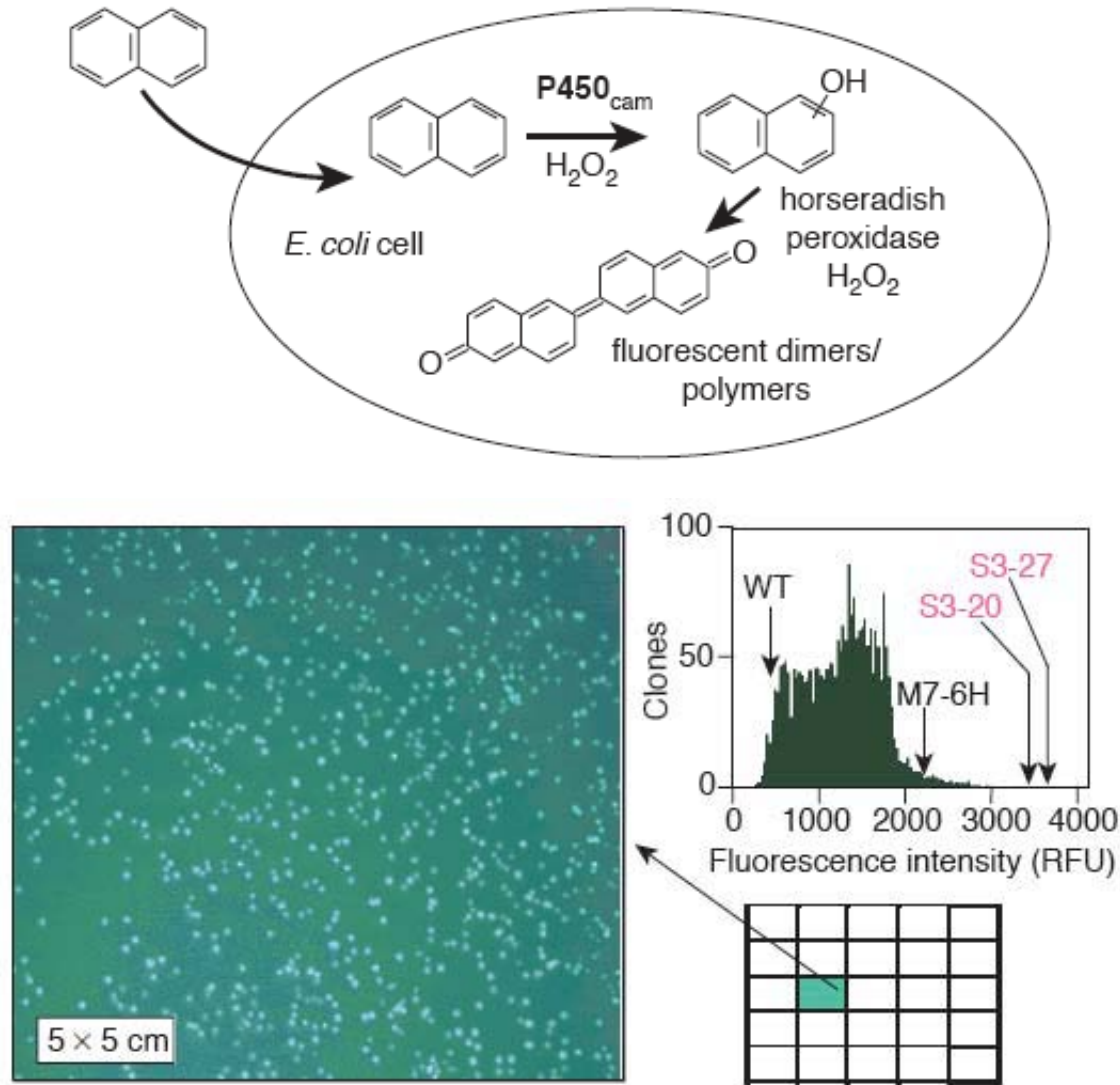
Shaner *et al.* (2004) *Nat. Biotechnol.* 22: 1567-72

Reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology. Source: Shaner, N. C., et al.

"Improved Monomeric Red, Orange and Yellow Fluorescent Proteins Derived from *Discosoma* sp. red Fluorescent Protein."

Nature Biotechnology 22 (2004): 1567-1572. © 2004.

Directed evolution of enzymatic activity: screen is a fluorescence assay



Joo et al. (1999) *Nature* 399: 670-3

Reprinted by permission from Macmillan Publishers Ltd: Nature. Source: Joo, H., et al.

"Laboratory Evolution of Peroxide-Mediated Cytochrome P450 Hydroxylation." *Nature* 399 (1999): 670-673. © 1999.

Which type of screening method to use?

<u>screen method</u>	<u>throughput</u>	<u>other notes</u>
SELEX	10^{15}	selection of DNA/RNA
ribosome display	10^{15}	<i>in vitro</i> protein synthesis
phage display	10^{11}	best for small proteins/peptides
yeast display	10^8	compatible w/eukar. proteins
plate assays	$< 10^5$	versatile but more complex

number of variants in a protein library

x residues = 20^x possible variants

12 residues = 4×10^{15} variants

lesson: impossible to cover sequence space except with short sequences (or few positions) and only the most high throughput techniques

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