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Authors
Basu, Koli
Green, Evan M
Cheng, Yifan
et al.

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Why recombinant antibodies — benefits and applications

Koli Basu[#a], **Evan M. Green**[#b], **Yifan Cheng**[b,c], **Charles S. Craik**[a]

[a]Department of Pharmaceutical Chemistry, University of California, San Francisco

[b]Department of Biochemistry and Biophysics, University of California, San Francisco

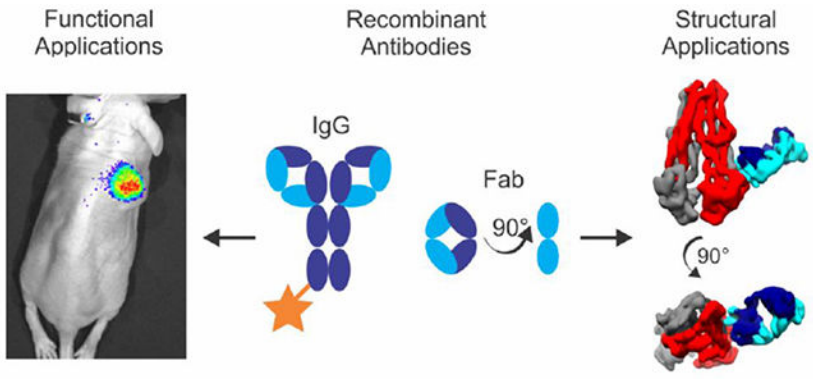
[c]Howard Hughes Medical Institute, University of California, San Francisco

[#] These authors contributed equally to this work.

Abstract

Antibodies (Abs) are ubiquitous reagents for biological and biochemical research and are rapidly expanding into new therapeutic areas. They are one of the most important probes for determining how proteins function under normal and pathophysiological conditions. Abs are required for quantification of targets, detection of temporal and spatial patterns of protein expression in cells and tissues, and identification of interacting partners and their biological activities. Their remarkable specificity and unique binding properties can facilitate three-dimensional structure determination using X-ray crystallography and electron cryomicroscopy. While hybridoma technology that involves animal immunization is often productive, many antigen targets do not generate useful Abs. This is particularly true if unique states of the target or critical non-immunogenic target sequences need to be recognized by the Abs. By using the methods of recombinant antibody generation, identification, and engineering, these 'hybridoma-refractory' antigens can be readily targeted. Specific, reproducible, and renewable recombinant Abs are proving to be invaluable reagents in applications ranging from biological discovery to structure determination of challenging macromolecules.

Graphical Abstract



Comparing hybridoma and recombinant antibodies

Monoclonal, target-specific antibodies (Abs) are routinely developed through hybridoma technology[1] or by biopanning with recombinant antibody libraries [2,3]. For hybridoma antibody development, B-cells from animals are harvested several weeks after they have been inoculated with the antigen. Immortalized hybridoma cells are generated through the fusion of B-cells with a myeloma cell line. Each hybridoma cell normally secretes a single species of immunoglobulin G (IgG) and the secreted IgGs are used in binding assays, such as ELISA or FACS, to select for cell lines producing antigen binders[4,5]. Since the antibody generation and affinity maturation occurs *in vivo* through the animals immune response, these Abs can have high specificity and affinity[6].

A common theme in any antibody campaign is that the investigator will 'get what they screen for'. A significant drawback of hybridoma antibody campaigns is that the antigen will undergo proteolytic degradation to initiate the antibody-generating immune response (Fig 1) and the derived Abs may not recognize the native form of the antigen. Another disadvantage is that the Fragment of antigen-binding domain (Fab) (Fig 2) obtained by IgG proteolysis[7] may not retain the same antigen-binding properties as the IgG. A polypeptide sequence that does not generate an immune response or three-dimensional epitopes can make antigens particularly hybridoma-refractory. Furthermore, the utility and reliability of numerous commercial Abs, many of which are generated through hybridoma technology, can be limited since the specificity and cross reactivity of Abs vary from vendor-to-vendor and lot-to-lot[8].

Many of the pitfalls of hybridoma antibody generation are resolved through biopanning with recombinant antibody libraries[9,10]. These libraries are generated by cloning synthetic or naive Fabs, single-chain variable fragments (scFvs), or nanobodies (Nbs) (Fig 2) into phage or yeast display vectors[11–14]. Antigen-binding Abs are enriched through several rounds of selection by solid-phase[2] or by flow cytometry[15,16] (Fig 1). Biopanning parameters can be selected for the desired antibody-antigen binding conditions. The ability to counter select against non-desired epitopes is a particularly powerful advantage of the biopanning approach. Counter selection can be accomplished using different conformation states, homologues, or specific domains[14,17]. Since the entire process is done *in vitro*, temperature, buffer conditions, antigen ligand concentrations, and oligomeric assembly can be readily controlled. With proper selection conditions, the antigen has a much lower chance of being altered through the recombinant biopanning process compared to the hybridoma approach of animal inoculation. Recently, advances in automating the recombinant antibody biopanning process has greatly reduced the effort, time, and cost required[18].

Another advantage of using a recombinant antibody library is that binders from the biopanning process can be easily characterized through DNA sequencing. Once the sequence of the antibody is known it is essentially immortalized, renewable, and can be produced in recombinant expression systems. Recombinant expression of Fabs, scFVs, and Nbs in *Escherichia coli* is a fraction of the cost of hybridoma Abs generation[19]. Recombinant Abs can be engineered to have additional functionalities including tags for

purification or immunoprecipitation, conjugation sites to increase chemical space, or mutagenesis to map the determinants of antigen recognition. The antibody sequence allows for *in vitro* affinity maturation, mimicking an immune response, to select Abs with higher affinity and lower off-rate[20]. Abs derived from recombinant affinity maturation campaigns often have affinities 10-50 times higher than their parent[21]. Engineering of therapeutic antibody candidates can improve their pharmaceutical effects, such as prolongation of half-life to increase their efficacy[22]. Further efforts to develop therapeutic Abs that take advantage of recombinant technologies have recently been reviewed[23].

Functional applications of recombinant antibodies

Since the advent of biopanning by phage and yeast display, recombinant Abs have become invaluable reagents for therapeutics, imaging, and understanding protein-protein mediated mechanisms of action (Fig 2). Therapeutic Abs typically prevent binding of ligands to receptors by either blocking the ligand or the receptor[24]. Abs against the immune checkpoint protein programmed death (PD-1) and its ligand (PD-L1) are recent examples[25]. Currently, there are several antibody therapeutics against these two targets for various cancer treatments that are either approved or in clinical trials[26], indicating the importance of this growing field. And with this, the development of tools for preclinical assessment of patient responses to immunotherapy is also emerging, such as immunopositron emission tomography (immunoPET)[27] where Abs towards the immune checkpoint proteins are conjugated with a radiotracer and used for *in vivo* imaging.

Recombinant Abs that recognize specific states of a protein can potentially be used as diagnostic tools. An example of this is a recombinant active-site-specific Fab developed for the transmembrane serine protease, matriptase[17]. Active matriptase is a biomarker for tumorigenesis. The recombinant Fab distinguished cancer from non-cancer cell lines *in vitro* and in colon cancer sections from human tissue micro arrays. *In vivo* imaging of colon cancer patient-derived xenograft models showed tumor uptake of the radio-labelled antibody, indicating that this Fab could be used for noninvasive tumorigenesis evalution[28].

Abs are also used for exploring the roles of specific intercellular and extracellular proteins. Blocking protein-protein interactions by Abs can help dissect the mechanism of action of the target of interest. By converting Abs to scFvs, intracellular signaling pathways can be probed since scFvs are properly folded in the reducing cytoplasmic environment, whereas IgGs and Fabs are not. An example of this is the intracellular expression of an scFv which was generated against (β-arrestins (βarrs), an important regulator of GPCR (G protein-coupled receptor) signal transduction[29]. This scFv selectively disrupts βarrs/clatherin interaction and inhibits agonist-induced endocytosis of GPCRs without affecting other molecular pathways. Another such example of Fab-based modulation is of the Vif protein complex, which is an HIV protein complex that counteracts the antiviral effects of the host apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3 (APOBEC3) immune proteins[30]. *In vitro* experiments with the Fabs, and *in vivo* experiments with transiently expressed scFvs, showed that Vif uses a multi-pronged approach involving both degradation-dependent and - independent mechanisms to suppress APOBEC3-innate immunity.

Cell classification and profiling, using a technique referred to as phage-antibody next generation sequencing (PhaNGS)[31], demonstrated yet another innovative use of recombinant Abs. In this technique, a collection of defined Fabs that bind specifically to previously identified targets of interest are displayed on phage and pooled to make a customized library. Fab-phage bound to cells are identified through next-generation sequencing of a barcode region in the Fab. It is expected that PhaNGS will be useful for observing surface protein changes in disease-state cells and for identification of new combinatorial biomarkers and drug targets.

Recombinant antibodies in X-ray crystallography

Abs are used extensively in structural biology to help determine high-resolution structures of antibody-antigen complexes owing to their ability to facilitate crystal packing of challenging targets, act as a fiducial marker to aid in particle orientation in electron microscopy (EM), and trap specific conformational states (Fig. 2). In the following sections, we highlight the broad utility of Abs for structural studies with a particular emphasis on recombinant Abs.

In order to determine the three-dimensional structure of a protein using X-ray crystallography, it is necessary to generate well-ordered crystals that diffract to high resolution. For this to occur, crystal contacts must be made between individual macromolecules to generate a three-dimensional lattice. Formation of crystal contacts can be impeded by a lack of suitable exposed surface area or by conformational flexibility. This is particularly true of detergent-solubilized membrane proteins as the detergent micelle often precludes crystal contacts between transmembrane helices[32]. Abs have been used to assist in the crystallization of membrane proteins since it was observed that the lattice contacts of membrane-protein crystal structures are often mediated by polar surfaces[33]. In the case of the potassium channel KcsA co-crystallization with a Fab was necessary to achieve the resolution required to assign potassium ion coordination in the central pore[34].

While early antibody-assisted crystallization studies used hybridoma derived Abs, there are a number of significant advantages for recombinant Abs. Since full length IgGs are typically too flexible to generate well-ordered crystals, Fabs must be generated through proteolytic cleavage, which is not required with recombinant Abs. For most labs, purification of the milligram quantities of recombinant Abs required for a crystallography campaign is more straightforward and reproducible compared to hybridomas-based expression and purification. Conformationally-specific Abs are easier to generate with recombinant approaches given the *in vitro* nature of the biopanning method making it possible to select for Abs in the presence of ligands or binding-partners. Recombinant Abs have been used extensively in GPRC structural biology making it possible to trap active states and complexes with interaction partners[35,36]. Use of recombinant Abs is not restricted to membrane proteins as they have been used for co-crystallization of a number of soluble proteins including a module of the polyketide synthetase 6-deoxyethronolide B synthase[37] and matriptase[38,39]. To increase the utility of recombinant Abs for X-ray crystallography, recent work has been done to engineer the scaffold with desired properties, such as the use of ‘elbow locking’ mutations to rigidify the link between the constant and variable domains to increase crystallizability[40].

Recombinant antibodies in cryoEM

Recent advances in electron cryomicroscopy (cryoEM) have made it possible to solve near-atomic resolution structures of proteins[41]. While several well-behaved samples have achieved resolutions better than 2 Å, achieving the resolution required to build *de novo* models for new samples is still challenging[42]. Before the 'resolution-revolution', Abs were used as fiducial markers for EM by immunogold labeling[43] but more recently the use of recombinant Fabs as fiducial markers for single-particle cryoEM of small and challenging targets was demonstrated[44]. This technique has been used to determine a number of high resolution structures that were recalcitrant to high-resolution structure determination without a Fab [45,46]. Fabs have a number of advantages for this task. Unlike other methods of increasing molecular weight, such as green fluorescent protein or maltose-binding protein fusions[47], Fabs can specifically recognize and bind rigid three-dimensional epitopes thereby facilitating high-resolution refinement[44].

The characteristic shape of Fabs in both negative stain and cryoEM provides a means for assessing the quality of medium-low resolution structures in addition to high-resolution alignment[44]. As is the case with X-ray crystallography, recombinant Abs have also been crucial for the structure determination of several GPCR complexes[48–51], integrin[52], and ABC transporters[53] by trapping specific conformations. In addition to conformational selectivity, Fabs can alter the orientation distribution of vitrified samples. In the recent structure of TMEM16A, a calcium-activated chloride channel, the sample in detergent had a significant preferred orientation bias resulting in a structure with anisotropic resolution[54]. By merging datasets with and without a Fab, it was possible to increase the resolution from 3.8 to 3.4 Å, as the two samples had different orientation distributions resulting in a final structure that was more isotropic and higher-resolution.

The low signal-to-noise ratio of cryoEM data makes refinement of psuedosymmetric complexes particularly challenging as protomers may not align properly if differences between them are small. Fabs have been used to overcome this problem in several systems as a symmetry-breaking fiducial mark. For example, Fabs against the heterodimeric ABC transporter TmrAB were used to increase the molecular weight of the relatively small membrane protein while simultaneously breaking a pseudo two-fold symmetry allowing for the unambiguous assignment of the individual protomers[55]. In the case of TmrAB, the estimated angular accuracy was significantly higher for the TmrAB-Fab complex relative to the apo sample. This same approach has been applied to several heteromeric ion channels[56,57]. Future engineering of antibody scaffolds may specifically tailor them to the unique requirements of single particle cryoEM with reduced flexibility and increased desired orientation distributions[58]. Since the theoretical minimum molecular weight required for high-resolution alignment is estimated to be around 50 kDa, approximately the same molecular weight of a Fab, it is conceivable that any Fab-antigen complex will be suitable for cryoEM in the future[59].

Conclusions

The lack of high-quality Abs for many macromolecules and their complexes limits our ability to determine protein structure, to detect proteins in pathology samples, and to image and treat diseases. The availability of potent, specific, reproducible, and readily renewable probes for the entire proteome, including hybridoma-refractory antigens, could transform the study of biology and enable the discovery of new therapeutics. Recombinant antibody technology, in addition to existing hybridoma techniques, provides a robust pipeline for the generation of these essential reagents.

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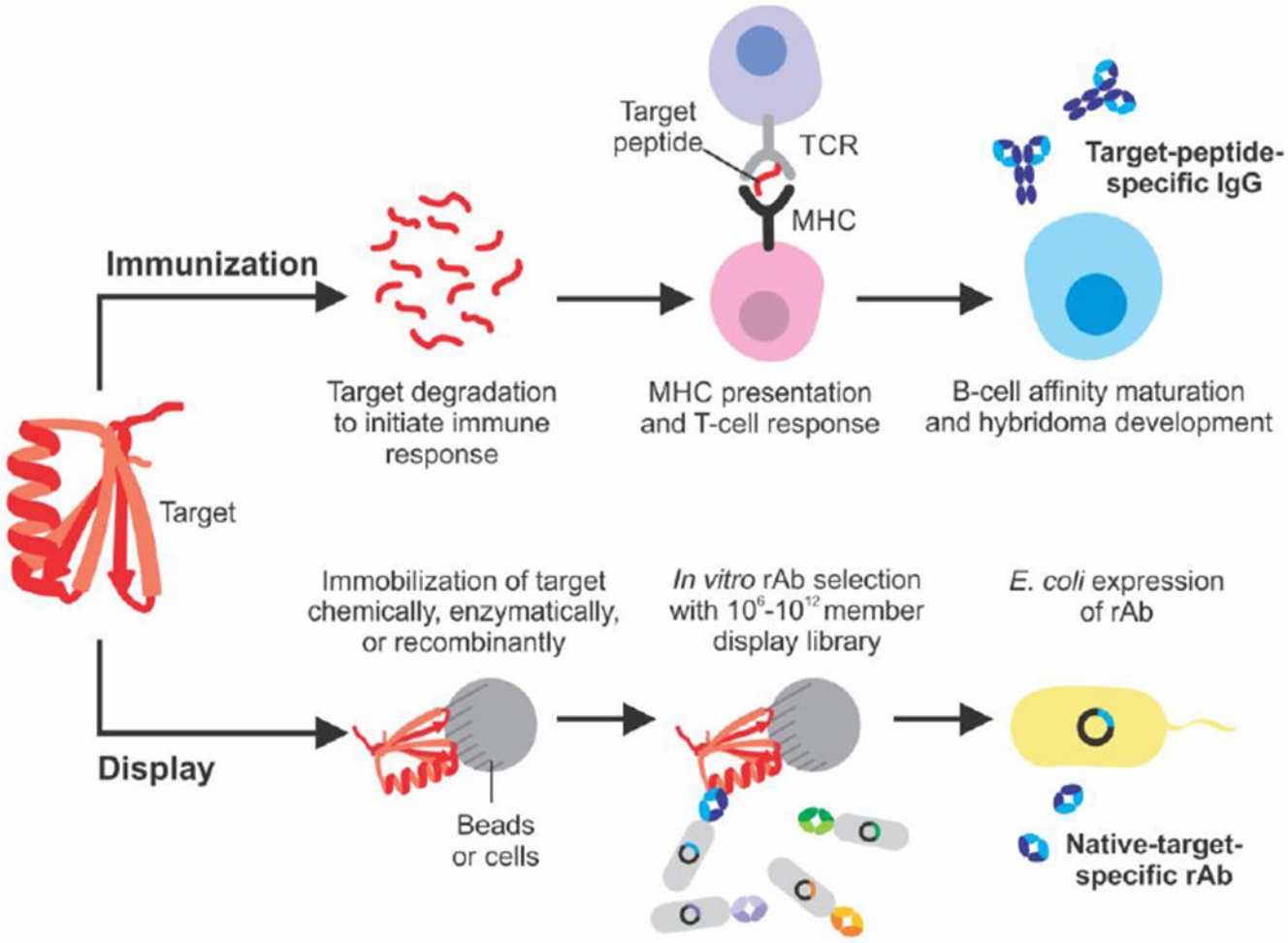

Figure 1: Antigen presentation in hybridoma antibody generation versus recombinant antibody display.

Target antigen (red) follows hybridoma immunoglobulin G generation path (top) or recombinant antibody (rAb) generation path (bottom). In top path, the pink cell is a major histocompatibility complex (MHC) presenting cell, the purple cell is a T-cell, the blue cell is a B-cell, the black receptor is a MHC, and the grey receptor is a T-cell receptor (TCR). In bottom path, the grey oval particles are display library members (yeast or phage) with colored displayed rAb and the yellow cell is *Escherichia coli*.

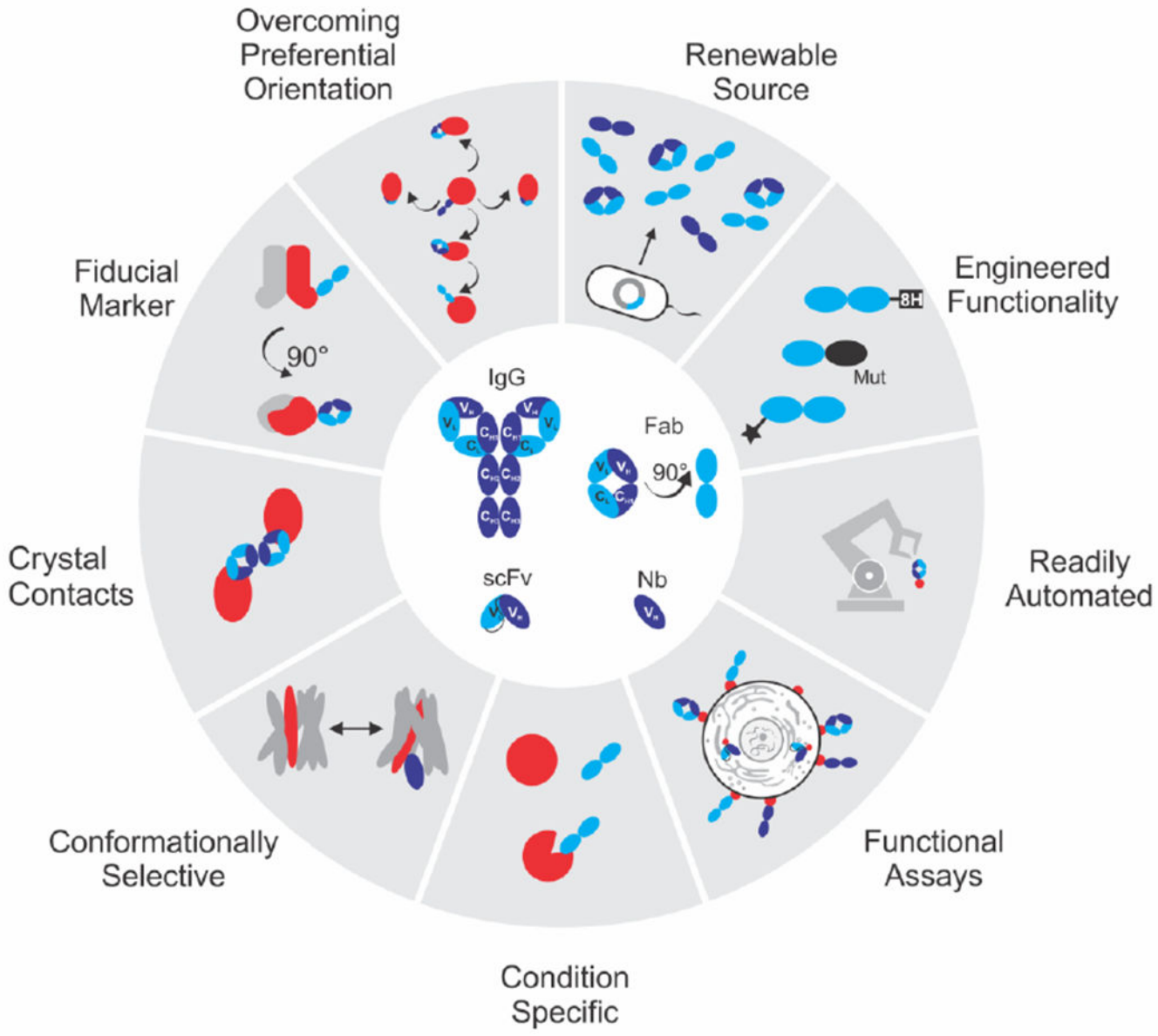

Figure 2: Different formats and important applications of recombinant antibodies.

Various antibody formats are shown in the center of the circle: immunoglobulin G (IgG), fragment of antigen-binding domain (Fab), single-chain variable fragment (scFv), and nanobody (Nb). IgGs are composed of two unique polypeptide chains, a heavy chain (H) and a light chain (L) based on their molecular weights. Each chain has one variable region (V_H, V_L). The light chain has one constant region (C_L) and the heavy chain has three constant regions (C_{H1-3}). Two heavy chains and two light chains come together to make an IgG. A Fab is composed of V_H, V_L, C_{H1} and C_L. An scFv is a single polypeptide chain composed of both V_H and V_L connected with a flexible linker. A Nb consists of only a V_H. Heavy chains are colored in dark blue, light chains are colored in cyan, and antibody targets are colored in red.