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Recombinant Antibodies for Academia: A Practical Approach

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Abstract: After several decades of optimization, phage display technology enables the routine isolation and production of recombinant monoclonal antibodies *in vitro*. As such it has the potential to provide the academic community with a vast, inexpensive and renewable supply of well-characterized reagents, reducing bottlenecks in basic science, helping increase reproducibility of experiments, and phasing out the use of animals for production and discovery of antibodies. Yet the overwhelming majority of fundamental research laboratories still use incompletely characterized antibodies developed in animals. In order to promote increased use of recombinant antibodies in academia, we have recently initiated an open source recombinant antibody facility in Geneva (*http://www.unige.ch/antibodies*). Here we describe our experience at the Geneva Antibody Facility: the various techniques involved in isolation and production of antibodies, the strategic choices that we have made, and what we hope will be a bright future for this project as part of a growing movement in the scientific community to replace all animal-derived antibodies with recombinant antibodies.

Keywords: Recombinant antibodies

The 3R strategy concerning animal experiments[1] has three principles: experiments involving animals should be *refined* to decrease animal suffering and increase the scientific value of the results obtained. In doing so, the number of animals should be *reduced* to the minimum necessary to obtain useful data. Finally, where possible, animal experiments should be *replaced* with alternative non-animal models or technologies.

In the field of toxicology, replacement has recently gathered a great deal of momentum: it is likely that integrated *in vitro* testing strategies will in the near future replace much of the mandatory animal tests currently performed.[2] For basic science in academia, much progress has been made in refining animal experiments to minimize suffering and increase the quality of the data generated. Replacement strategies have proven more difficult to implement, however, because experimental procedures require constant adaptation as fundamental research questions evolve. In this respect, animal immunization for antibody discovery and production is a rare exception.

Antibody generation in animals is no longer either necessary or desirable. Over the last few decades, new techniques have been developed to isolate and produce antibodies entirely *in vitro*.[3] This provides the academic community with a unique opportunity to demonstrate its willingness to apply replacement strategies when they are available.

This article has two main goals. First, to provide a simple description of the main approaches available for generating antibodies, addressed to non-experts, with a specific focus on antibodies generated *in vitro*. Second, to describe the new Geneva facility dedicated to the *in vitro* discovery, production and archiving of antibodies for the academic community, stressing the technical choices that guided this project and outlining the steps that could be taken to develop its future scalability and sustainability in the context of other similar initiatives around the world.

First Part: Antibodies Come in Three Flavors

When challenged with a pathogen, innate immunity represents the first line of defence against infections. This is followed by adaptive immunity, which includes the generation of antibodies against the foreign antigen. It has been known for centuries that pathogen challenge can be readily mimicked by immunization, and the natural antibody response in animals has been adopted as a tool to generate antibodies against a wide range of antigens. The usual strategy is to inject the antigen (protein, peptide, or, for non-peptidic antigens, protein conjugates) into the animal, formulated with adjuvants in order to stimulate a potent immune response. Notably, the adjuvants generally used for animal immunization are not deemed acceptable for use in humans because of the side-effects they induce.

Following immunization, the immune system of the animal scans its natural repertoire of different antibody-producing cells (10^7–10^9, according to the size of the animal) in order to (i) select rare cells that produce antibodies which recognize the antigen, (ii) induce these cells to multiply, (iii) stimulate them to evolve higher affinity binding to the antigen, and (iv) trigger them to produce large quantities of the resulting antibodies.

From a practical point of view, there are three main methods to generate specific antibodies. They are referred to here as polyclonal, monoclonal or recombinant antibodies (Table 1).

Polyclonal Antibodies

Polyclonal antibodies represent the antibody mixture that can be purified directly from an animal's serum a few months after immunization. The term 'polyclonal' is used because at this time the serum will contain a mixture of different antibodies directed against the antigen, resulting from

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Table 1. Relative advantages of different types of antibodies

	POLYCLONAL	MONOCLONAL	RECOMBINANT
Time to isolate and produce	6 weeks	3–6 months	6 weeks
Animals required	Yes	Yes	No
Selection	*in vivo*, by immune system	*in vivo*, by immune system	*in vitro*, independent of immune system
Defined molecular species	No	Yes	Yes
Reformating possible	No	No	Yes
Storage volume	Largest	Smaller, requires maintenance	Smallest, no maintenance
Sustainable production	No	Yes, unless problems with storage	Yes
Sequence available	No	No	Yes

the combined production of a number of different antibody producing cell clones that were amplified during the antibody response. Because more blood can be drawn from larger animals, typical sources of polyclonal antibodies are rabbits, sheep and goats rather than rats or mice. Polyclonal antibodies are relatively inexpensive to produce, but the total amount that can be produced is limited by the amount of blood that can be drawn from the immunized animal during its lifetime. Once a supply of serum from an animal is exhausted, a new immunization must be performed, necessitating a new animal experiment and resulting in a polyclonal mixture that, because of the stochastic nature of the adaptive immune response, cannot possibly be identical to the previous one.

Monoclonal Antibodies

In 1975, Köhler and Milstein discovered a method to immortalize mouse antibody-producing cells.[4] This led to the development of a new strategy to produce monoclonal antibodies, with such far-reaching consequences that they were awarded the Nobel Prize for Physiology and Medicine in 1984. To generate monoclonal antibodies, antibody-producing cells are collected from an immunized animal and fused to a tumour cell line to generate immortalized antibody-producing cell lines called hybridomas. Each hybridoma can be cultured as an individual clone, enabling unique (*i.e.* monoclonal) antibodies with the desired characteristics to be isolated and produced. Hybridoma technology has not been widely adopted in species other than rats and mice, so almost all available monoclonal antibodies are of rodent origin.

Monoclonal antibodies are technically more difficult to produce than polyclonal antibodies, but as single molecular species of defined structure they generally represent more valuable research reagents. Furthermore, immortalized hybridoma cells outlive the animals from which they were obtained: they can be stored frozen for many years and then, if needed, expanded in culture to produce monoclonal antibody in amounts greatly in excess of what the immunized animal could naturally produce in its lifetime.

Recombinant Antibodies

To generate recombinant antibodies, the whole process of antibody selection and production is reconstituted *in vitro*. In the most widely used technology, antibody phage display,[3] a large collection of filamentous bacteriophages (typically in the range 10^9–10^{10}) are engineered to encode and display a repertoire of different antibodies. This phage repertoire is then incubated with the chosen antigen *in vitro*. Phages displaying antibodies capable of binding to the antigen are purified in a 'panning' step, then the genes encoding the selected antibodies can be amplified. Over several rounds of panning and amplification, extremely rare antigen-specific monoclonal antibodies can be isolated. These antibodies are referred to as recombinant antibodies, as a reference to the recombinant DNA technology used to engineer the initial collection of bacteriophages. Recombinant antibodies are initially selected as DNA antibody-coding sequences, and can be readily produced in cultured cells, in a variety of formats, as described below.

What Constitutes a Useful Antibody for Research?

A perfect antibody is one that would be suitable for all of the different applications used by the academic research community, (*e.g.* ELISA, Western blot, immunofluorescence microscopy, *etc.*). According to these criteria there are very few, if any, perfect antibodies. All antibodies, whether polyclonal, monoclonal or recombinant, are initially characterized by testing their ability to bind the antigen or antigen fragment that is typically presented in a purified and/or concentrated form. Further characterization is then necessary to determine, for example, (i) whether an antibody raised against a peptide fragment recognizes the full-length target protein, (ii) if it is capable of detecting the antigen at its endogenous level (usually low), and (iii) with which detection procedures it is compatible. For this reason, catalogs of research antibodies for sale typically specify for which application or applications they are best suited.

By the same criteria, very few adequately characterized antibodies are worthless: for example (i) many antibodies that do not recognize their target protein by Western blot recognize it in immunofluorescence experiments, (ii) an antibody that recognizes a peptide epitope within a protein but is unable to recognize the full-length protein may be useful for certain applications, such as helping to determine the extent to which a protein is folded or degraded, or to report on the post-translational modifications it has received. For this reason, all adequately characterized antibodies with definable specificity and utility should ideally be kept in a searchable archive that is available for use by the scientific community. Additionally, calls have been made for sequence information, which uniquely defines each monoclonal antibody, to be deposited as part of the characterization procedure.[5]

Second Part: Recombinant Antibodies Are Better

In addition to ethical considerations, recombinant antibodies exhibit numerous practical advantages over animal-derived antibodies. These are outlined below.

Control of Selection Conditions

When an antigen is used to immunize an animal, it is subject to whatever modification and degradation that might take place before and during its encounter with the animal's adaptive immune system. Furthermore, the immune system has

evolved complex tolerance mechanisms that prevent it from generating antibodies directed against structures that resemble self-antigens, and that reduce its capacity to generate antibodies against many antigens.

These problems are bypassed in the generation of recombinant antibodies.[3] Selection of the antibodies is achieved *in vitro* under conditions that can be tightly controlled by the user. For example, enzyme inhibitors can be used to ensure that a peptide antigen remains intact and retains any user-added modifications (*e.g.* phosphorylation) throughout the selection procedure. The use of large naïve or synthetic antibody libraries bypasses the requirement to use *in vivo* immune systems, enabling the selection of antibodies against self-antigens and against non-peptidic antigens that generally show low immunogenicity.

Additionally, it is possible to use *in vitro* selection approaches to direct the isolation of recombinant antibodies towards specific structures within an antigen, for example by alternating positive panning steps against the antigen with negative panning steps against a structural variant of the antigen lacking the target structure. Such approaches are impossible using immunized animals.

Finally, once an antibody with promising binding affinity has been identified, using recombinant antibody technology it is possible to generate a second-generation repertoire using targeted mutagenesis to modify its sequence, and then perform further selection on this repertoire in order to isolate new antibodies with improved affinity and/or specificity for the target.

Formatting

Antibody proteins have a modular structure (Fig. 1A) featuring variable regions, responsible for engaging antigens, and constant regions (Fc), which do not engage antigen but whose physiological roles are (i) to increase the valency of the antigen-binding variable domains, (ii) to increase the *in vivo* stability of the antibody molecule, and (iii) to link antigen binding to effector functions in the immune system. From a practical point of view, the constant domains are also the targets of secondary reagents used to reveal the presence of an antibody. In antibody phage display, the smallest functional antigen binding fragment, known as a single chain Fv (scFv) is most commonly used, with its small format facilitating the cloning of large antibody gene libraries and the production of recombinant phage in bacteria (Fig. 1B). After isolation, the DNA encoding scFvs selected by phage display can be readily reformatted for soluble (*i.e.* no longer attached to phage) expression in bacteria (Fig. 1C).[6] However, since monovalent scFv fragments do not benefit from the avidity gain of multimerized immunoglobulins, they are often reformatted into constructs that provide dimerization and facilitate detection with secondary reagents. This can be achieved by producing a fusion protein composed of an scFv fused to an Fc domain, a format often referred to a minibody (Fig. 1D).[7] In most *in vitro* experimental settings, minibodies are equivalent in performance to intact immunoglobulins,[8] although for *in vivo* work it is sometimes necessary to reformat into a full immunoglobulin in order to take advantage of the enhanced circulatory lifetime and effector functions provided by the intact antibody structure. A significant advantage of recombinant antibodies is that during reformatting into minibodies the Fc fragment can easily be interchanged between that of different species, *e.g.* human, rabbit or mouse, allowing the use of a wide range of secondary antibodies. One disadvantage of minibody reformatting is that for efficient production, minibodies require expression in mammalian cells, involving procedures that are more complex and expensive than production in bacteria.

Finally, the range of possibilities for reformatting recombinant antibodies is not restricted to variations on natural antibody formats. This presents a wide range of attractive research options (*e.g.* fusion with enzymes, linkage of several different antigen-binding domains, *etc.)*. One such application, where scFv are fused to fluorescent proteins for expression in the cytosol of a mammalian cell as intrabodies, has been successfully used to enable detection of the corresponding proteins during live cell imaging.[9]

Production and Storage

All characterized antibody samples are potentially valuable, and should in principle be stored in a searchable archive, but there are costs and difficulties associated with archiving, and these vary significantly according to the manner in which the antibody sample was originally produced.

For polyclonal antibodies, the available serum, though limited (typically 100 to 200 ml), must be kept frozen, and requires significant freezer space. For example a maximum of 100 typically sized serum samples can be kept in a small –20 °C freezer.

For monoclonal antibodies, production relies on a stock of hybridoma cells kept frozen in liquid nitrogen. In addition, each hybridoma cell line must be thawed and refrozen every few years, and occasionally recloned and retested. Many hybridoma cells have been lost due to improper handling or storage, or following accidents. Because of the required time and effort for conservation, when a researcher retires his/her stock of polyclonal or monoclonal antibodies is often lost.

Storing recombinant antibodies requires significantly less space and resources. The gene encoding a given antibody can be stored in the form of highly stable plasmid DNA, or even *in silico*, in a database of recombinant antibody genes that can readily be resynthesized at a later date and at a different site to enable production of the encoded antibody. There are essentially no limits to the number of recombinant antibodies that can be stored in this way, and accidental loss is easily avoided.

In summary, because recombinant antibodies bypass the need for animal experimentation, they can be selected in a more controlled and less biased manner than animal-derived antibodies. Like monoclonal antibodies they are unique and defined molecular species that can be produced in unlimited amounts, but they can be stored safely in much higher numbers, readily sequenced and can be produced in a wide variety of formats according to the intended use. With all of these key advantages, we believe that the widespread replacement of animal-derived antibodies with recombinant antibodies is inevitable: no longer a question of if, but when and how?

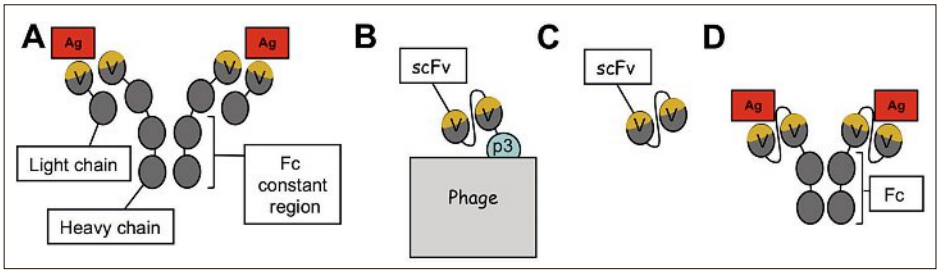

Fig. 1. Formatting recombinant antibodies. A. Natural IgG antibodies are made of two heavy chains and two light chains. In the variable domains (V), hypervariable regions (orange) form the antigen-binding sites. B. To select recombinant antibodies, the variable domains of the heavy and light chains are connected by a linker to form a single-chain Fv (scFv) fused to the p3 protein at the surface of a phage. C. Selected antibodies can be produced as monovalent scFv. D. The minibody format (an scFv fused to the Fc portion of a heavy chain) is dimeric and its Fc portion can be recognized by classical secondary detection reagents.

Third Part: Recombinant Antibodies for Academia

In the pharmaceutical industry, recombinant antibody technology has been used extensively to generate therapeutic antibodies for the treatment of a variety of pathologies, including inflammatory disease, autoimmune disease and cancer. Today a sizable portion of the new treatments approved each year is based on the use of therapeutic recombinant antibodies.[10]

There is a recognized need to adopt recombinant antibodies in academia.[5] Antibodies have been defined as one of the most variable reagent classes used in fundamental research laboratories, accounting for a significant part of the low reproducibility of many experiments.[11] Recombinant antibodies have the potential to become a huge, reliable and well-characterized source of reagents for basic science,[5] but this has not happened so far, and currently use of recombinant antibodies in academia is rare.

The initial development and implementation of recombinant antibody technology was strongly driven by industrial incentives. Technology for generating and using recombinant antibody repertoires was protected by patents, and for almost two decades recombinant antibody activity and expertise was largely confined to the industrial sector. It is still very challenging for academic research groups to gain access to high quality naïve antibody libraries.

Recent years have seen a much wider uptake of phage antibody technology, leading to a broader base of scientists qualified to use it, together with the movement of some of the early leaders in the field from industry to academia.[12–14] This has created an environment where, for the first time, recombinant antibody technology is poised to start catering to the academic community: *reliable binding agents for all*.[5]

Antibodies Against Any Target: The Value of the Long Tail

The Geneva Antibody Facility selects and produces new recombinant antibodies for the academic community. The Geneva Antibody Facility is not focused on the generation of research antibodies that are commercially available, nor was it set up to compete with private companies in the generation of therapeutic antibodies. Instead, its vision is to help create a sustainable system to generate a vast searchable archive of antibodies against any antigen in any species, for the use of the academic research community.

Unlike some of the other recombinant antibody initiatives,[15] the Geneva Antibody Facility's scope is not restricted to antigens of human origin. Necessarily, many of the antibodies generated will be used very infrequently, once a year worldwide or even less. There is an enormous academic value in generating and maintaining such a universal archive, but selection, production, storage, and even funding strategies must be thought out in advance in order to set up a framework potentially capable of handling hundreds of thousands of antibodies.

Why We Select Antibodies on Behalf of Users

We recognize that at present most research laboratories have not mastered the technology to select and produce recombinant antibodies, and many will never do so. In addition, high-quality standard naïve antibody libraries are not easily accessible. A huge step forward in the creation of the Geneva Antibody Facility was to gain access to libraries from NovImmune SA, on the understanding that the main aim of the facility is to generate fundamental research reagents for the academic community.

Consequently, the primary aim of the Facility is to perform selection and production of recombinant antibodies for the academic community. This is clearly not scalable, however, and we believe that the construction of a truly universal recombinant antibody archive will not be possible without the input of many different groups, and will therefore depend upon further spread of recombinant antibody expertise, high quality libraries and know-how into the academic sector.

Why We Only Do Initial Characterization

The antibodies selected by the Geneva Antibody Facility have all been shown to specifically recognize their target antigen (often a protein fragment) by ELISA. Although it has been proposed that recombinant antibody characterization could be standardized by specially funded laboratories,[5] we believe a more sustainable and scalable solution would be to ensure that further characterization is performed and reported by the scientist end-users themselves, using an open access knowledgebase attached to the Facility's website. This knowledgebase would also list publications describing the use of a particular antibody.

Why We Use Minibodies

Currently, our experience with Facility users suggests that most researchers wish to obtain recombinant antibodies that can be used experimentally in the same way as intact, animal-derived antibodies. Researchers would be reluctant to use unusual antibody formats that would necessitate the use of different procedures or different secondary reagents. Consequently, the Geneva Antibody Facility produces antibodies exclusively in the minibody format. Three variants (human, mouse or rabbit Fc) are currently available.

How We Store and Produce Antibodies

Long-term storage of huge numbers of antibodies can only be achieved in plasmid or *in silico* form. This requires that, on demand, antibodies must be produced by transient transfection of mammalian cells. The Facility has developed plasmids that allow us to reach antibody concentrations of 100 μg/ml in serum-free cell supernatants. Alternatively, more simple and cheaper production procedures typically yield cell supernatants containing approximately 1 μg/ml of antibodies. Finally, users are welcome to bypass the Facility's production process by either (i) obtaining a sample of the expression plasmid for their own use or (ii) synthesizing the gene encoding an antibody of interest and incorporating it into their own expression vector. Here again, we envisage that the open source model is the most appropriate way to provide scalability and sustainability to a large scale recombinant antibody initiative.

Incorporation of Previously Published Recombinant Antibodies

Many recombinant antibodies have already been selected and characterized by the academic community, but their sequences have not been centralized into a single database. We plan to launch a new initiative in 2017: the Facility will ensure reformatting and production, on demand, of any previously published recombinant antibody or sequenced hybridoma. For this, we will only request the sequence of the antibody, and where applicable a published reference in which it is described.

Conclusion

As much as possible we are trying to develop the Geneva Antibody Facility as an open-access, low cost and collaborative platform. It is complementary to the other large recombinant antibody initiatives that are currently underway[16] as well as those that were carried out in the past,[15] with the exception that we believe that a community-driven open source venture is more likely to have the scalability and above all the financial sustainability necessary for success. We hope that researchers worldwide will take advantage of this new resource and in doing so will contribute to its success, providing an important resource for increasing data reproducibility in biomedical research.

Finally, from an ethical, regulatory and political point of view, it is highly desirable to operate the switch from antibodies produced in animals to antibodies selected and produced entirely *in vitro*. In this perspective, this initiative is also aimed at fostering a climate of understanding and cooperation between academia and civil society.

Appendix: Glossary

Antigen: an element (protein, lipid, small molecule, *etc*.) against which an antibody can be raised

Antibody: a Y-shaped protein (see Fig. 1), also known as an immunoglobulin, whose two identical arms are responsible for antigen binding and whose stalk is responsible for interacting with effector components of the immune system. The antigen binding sites of different antibodies are highly variant in sequence.

Epitope: a small region of an antigen recognized by a specific antibody

Fc portion: the stalk region of the Y-shaped antibody molecule that, in contrast to the antigen-binding sites, is invariant in sequence (see Fig. 1). Most secondary antibodies recognize the Fc portion of antibodies from a defined species (*e.g.* anti-mouse Ig)

Immunization: Delivery of an antigen to an animal formulated in such a way as to elicit the generation of an antibody response

Immuno-fluorescence: detection of a protein by fluorescent antibodies, after fixation and permeabilization of a cell or tissue. Proteins are usually in a folded state in fixed cells.

Minibody: a fusion protein comprised of a scFv and an Fc portion (see Fig. 1)

scFv: single-chain fragment variable, a fusion protein consisting of the two immunoglobulin domains of an antibody that comprise the antigen binding site (VH and VL) joined *via* a flexible linker peptide (see Fig. 1)

Western blot: detection of a proteic antigen after a protein mixture has been fractionated on an acrylamide gel and transferred to a nitrocellulose membrane. Protein are usually in an unfolded state on the nitrocellulose.

Secondary antibodies: antibodies that recognize a large number of antibodies of a given species *via* the Fc portions, which are normally used in research as conjugates (fluorochrome, enzyme) to reveal the presence of a 'primary' (antigen-specific) antibody.

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