FLUOROBODIES

Mixing antibodies and the green fluorescent protein to unravel the genomic revolution

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Antibodies are proteins produced in animals and humans in response to infection. They bind tightly and selectively to infecting agents (antigens, such as surface proteins found on bacteria or viruses), providing the mechanism by which those agents can be destroyed. Because they bind so well to their targets (usually proteins), antibodies are used extensively in biological research to identify proteins. They also form the basis for many important diagnostic tests, such as the pregnancy test, as well as almost all tests involving infectious diseases.

We have combined the selective binding properties of antibodies with the extraordinary fluorescent properties of a protein found in a jellyfish to synthesize a new type of protein called a fluorobody. Like antibodies, fluorobodies bind tightly and selectively to antigens, but unlike antibodies, they glow a very visible green when illuminated by blue light. They will therefore become a powerful tool for biological research, for which seeing the location of proteins and having a means to track their interactions are most significant. Fluorobodies also have the potential for use in biosensors to detect infectious and biothreat agents.

Antibody Libraries

Antibodies bind to antigens and subsequently initiate immune responses as a result of their modular structure, which is basically the same for all antibodies (see Figure 1). A region found at one end of the antibody—the variable region—varies (as its name suggests) between different antibodies. This is the part responsible for recognizing and attaching to antigens. At the other end of the antibody is the so-called constant region. In humans, there are seven different types of constant regions, and these are the parts that activate the killing mechanisms once the variable region has latched onto its target.

Although antibodies are produced naturally by the immune system against infecting microorganisms, they can also be made artificially against almost any target if that target is injected into an animal. Such a process is called immunization and involves three or four injections of the target into the animal (usually mice or rabbits) until antibodies can be detected in the blood. The ability to artificially induce antibody production has been a major reason to use antibodies so heavily in biological research.

Whereas animals are very effective at generating antibodies by immunization and have been used for this purpose for decades, 10 years ago a method to create specific antibodies that did not require the use of animals was developed in a laboratory in Cambridge, the United Kingdom. At any one time, 100 million to one billion different antibodies are thought to be present in human blood. Each is produced by a specialized cell, called a B cell, which circulates in the blood. Each B cell has a different specific "antibody gene" (the gene is a section of DNA that describes how to make the antibody) that is made of a unique arrangement of variable gene regions (refer to Figure 1). Because of its unique antibody gene, each B cell makes antibodies of only one specificity.[1] By taking blood from many different human volunteers and harvesting all the B cells, the Cambridge researchers could extract millions of different antibody genes. Then they

[1] B cells will clone themselves; therefore a small number of B cells will produce the same antibody.

isolated the genes that made the variable portions of antibodies and separated them from the genes that made the constant region. In this way, they created a "library" of human variable-region genes.

It was hypothesized that any specific antibody could be produced if its antibody gene could be isolated from the library and inserted into bacteria, which would then synthesize the antibody encoded by the gene. The problem was that the library was simply a collection of unlabeled molecules. Although techniques were available to insert genes into bacteria, identifying the single, correct antibody-producing bacterium among millions and millions of different bacteria was extremely difficult.

The Cambridge researchers surmounted this difficulty by adopting a strategy of physically coupling the antibody genes to the antibodies they make. They carried out this coupling in the laboratory using a phage, which is a virus that infects only bacteria. The particular phage, called fd, has only five different proteins on its surface, one of which is called gene 3 protein. Using gene-splicing techniques, the researchers fused an antibody gene to gene 3 of the phage. The genetically engineered "antibody phage" was identical to the normal fd phage, except that it displayed an antibody on its surface, as seen in Figure 2. The important point is that phages can easily be made to replicate in the laboratory. By inserting the antibody gene into the phage, the Cambridge researchers had, in effect, created an antibody that could be replicated.

Then they created a phage antibody library consisting of millions of phages, each containing a different antibody gene and hence displaying a different antibody on its surface. It is possible to select specific antibodies from the library by mixing the entire library with a protein target of interest (see Figure 3). Some of the phages would bind to the target, whereas the nonbound ones could be removed by washing. The bound phages could then be eluted from the target and allowed to replicate in a controlled environment. Thus, the library could be used to produce antibodies that have specificity for the target of interest.

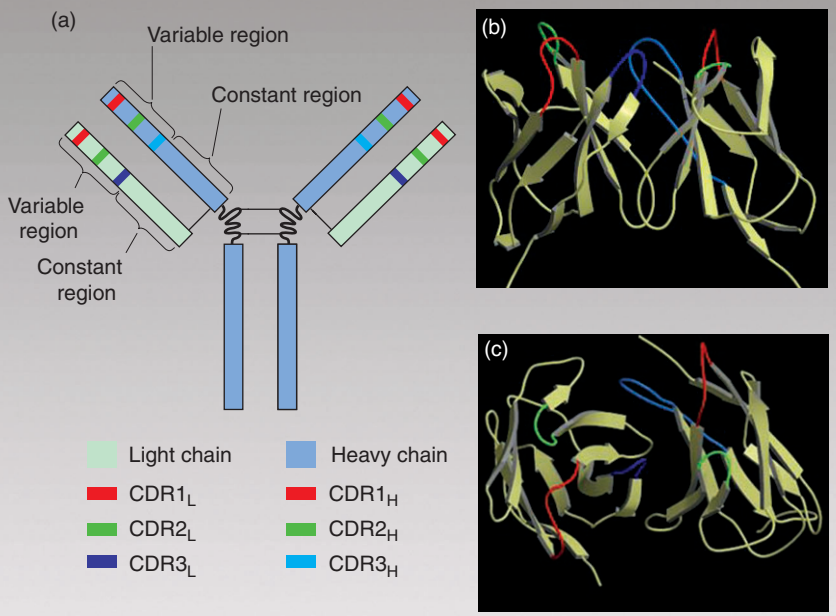

Figure 1. Antibody Structure and Binding

(a) All antibodies have the same basic structure: two heavy chains and two light chains covalently joined together to form a complex. The figure shows a linear representation of the amino acid sequence of the most common antibody type, immunoglobulin G (IgG), which has a Y-shaped structure. At the ends of the arms of the Y are the so-called variable regions of the heavy and light chains. This is the part responsible for recognizing infectious targets (antigens) and attaching to them. The recognition of such targets is largely mediated by the three complementarity-determining regions (CDRs), which are hypervariable regions found interspersed within each of the variable regions. (b) This ribbon diagram shows a side view of the three-dimensional structure of the variable regions, whereas (c) shows a top view. The CDRs form protruding looplike structures, creating a unique surface that will bind to a specific antigen with high affinity.

Fluorobodies

One problem with the antibodies generated by the library technique is that they tend to fall apart relatively easily. Furthermore, to be detected, they require the addition of other reagents—a problem common to all antibodies, even those derived from rabbits or mice. Our group at Los Alamos addressed these problems by inventing fluorobodies.

The underlying structure of the fluorobody is a remarkable protein called green fluorescent protein (GFP), which is obtained from the jellyfish *Aequorea victoria.* Expression of this protein alone will render fluorescent any tissue, cell, or animal when it is viewed under blue light. GFP has an extremely stable canlike structure, as seen in Figure 4.

Our working hypothesis was that the CDR3 hypervariable loop of an antibody (refer to Figure 1) could be spliced into GFP, thus creating a fluorescent protein with antigen-binding

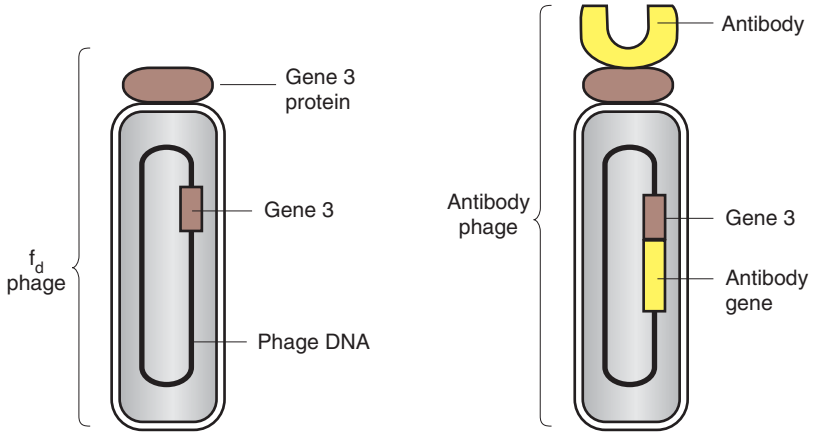

Figure 2. Phage Library Detection Technique
By fusing an antibody gene to one of the genes that produces a phage surface protein, we create an antibody phage that displays a specific antibody.

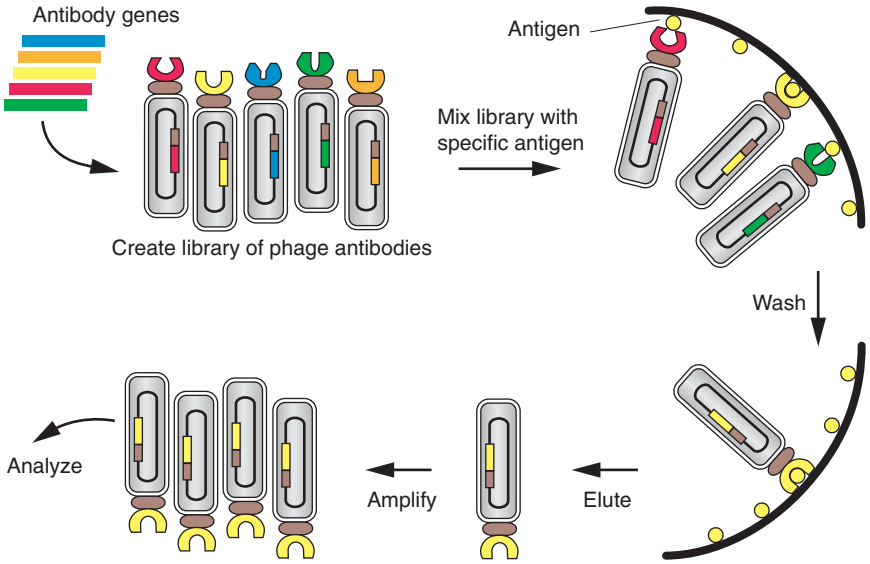

Figure 3. Selecting Specific Antibodies
One can select antibodies that bind to specific targets by mixing the entire phage antibody library with a target of interest. Only those phage antibodies that have an affinity for the target will bind; all others can be washed away. The selected phage antibodies can then be eluted from the target and cloned to large numbers in the laboratory for subsequent use.

capabilities. It was not at all obvious that such a splice would produce a viable product. Previous attempts at inserting amino acids (the constituents of proteins) into GFP had typically resulted in unstable, nonfluorescent molecules. But the CDR3 loop tends to be very "floppy." Our guess was that it could be inserted into the relatively exposed loops at the top of GFP and would not affect the canlike structure responsible for fluorescence.

We first developed a novel protocol that would insert CDR3 genes into the GFP gene at four different sites. The result was what we hoped would be a "fluorobody gene" that produced a modified GFP protein with four exposed antigen binding loops. Then using tens of millions of different CDR3 genes, we synthesized millions of different fluorobody genes. We next coupled the fluorobody to its own DNA by fusing each gene to gene 3 of the fd phage, thus creating a phage fluorobody library. We were gratified to see the library glowing brilliant green in its sample tube. Then following the same selection procedure described above, we obtained a number of highly specific fluorobodies that bound as tightly to their targets as antibodies. We had successfully created stable, easily produced, and easily detectable protein markers.

We can now use fluorobodies to track specific proteins and observe in which tissue, cells, or organelles they are expressed and with which molecules they interact. This capability is crucial for the next phase of the Human Genome Project, for which Los Alamos researchers are developing methods to select fluorobodies against hundreds of targets simultaneously. Fluorobodies will likely prove to be very powerful in the development of novel diagnostic tests and should accelerate drug development, because the ability to observe the effects of a drug in real time will allow very rapid screening against millions of compounds. ■

Acknowledgments

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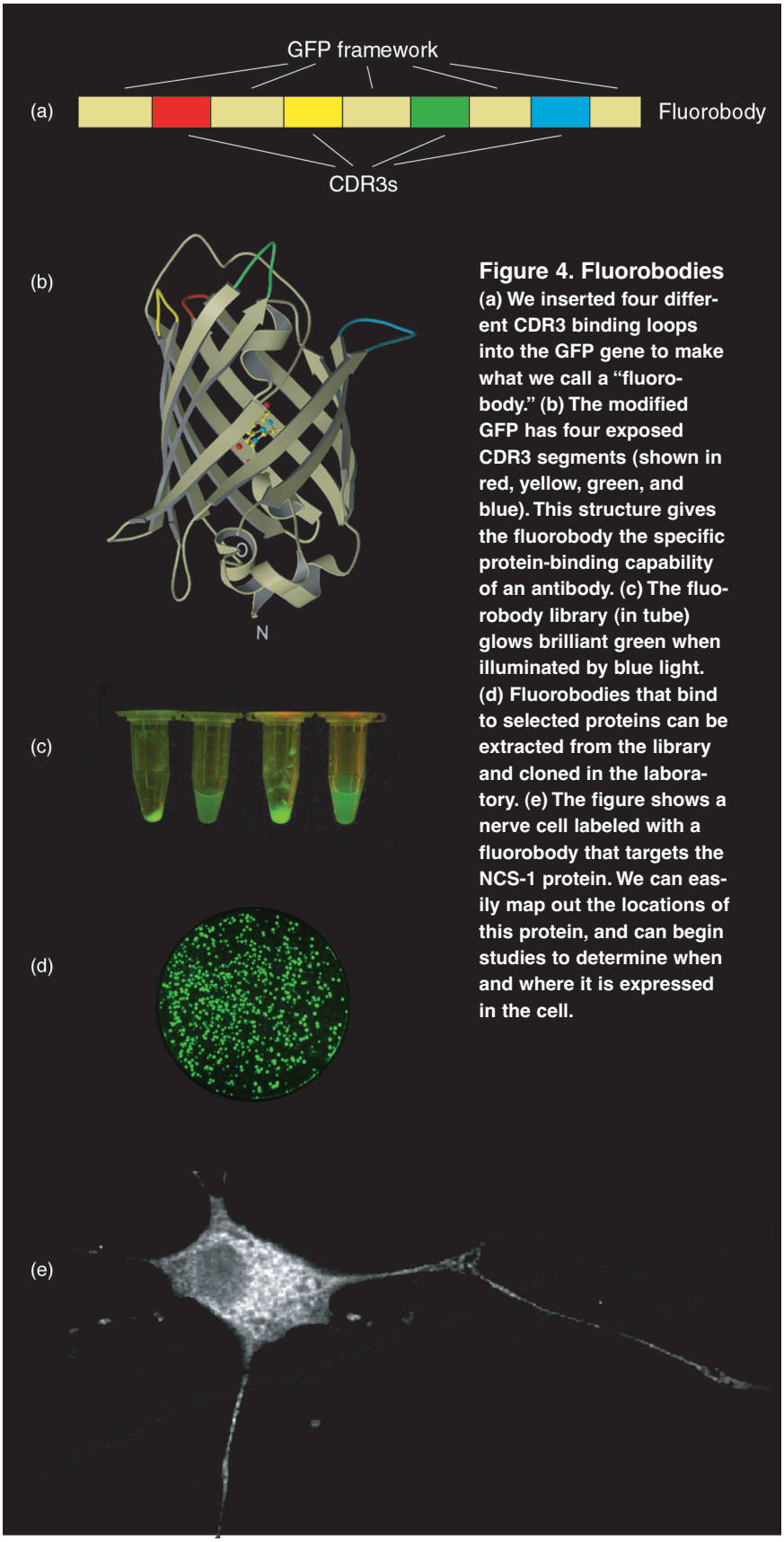
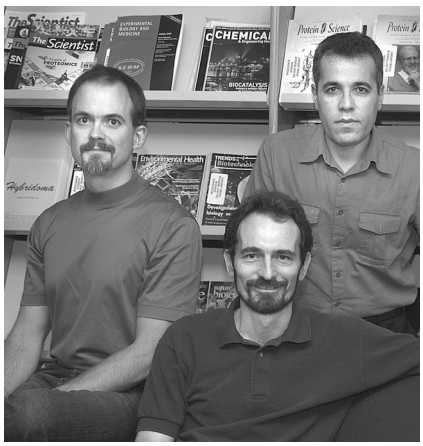


Figure 4. Fluorobodies
(a) We inserted four different CDR3 binding loops into the GFP gene to make what we call a "fluorobody." (b) The modified GFP has four exposed CDR3 segments (shown in red, yellow, green, and blue). This structure gives the fluorobody the specific protein-binding capability of an antibody. (c) The fluorobody library (in tube) glows brilliant green when illuminated by blue light. (d) Fluorobodies that bind to selected proteins can be extracted from the library and cloned in the laboratory. (e) The figure shows a nerve cell labeled with a fluorobody that targets the NCS-1 protein. We can easily map out the locations of this protein, and can begin studies to determine when and where it is expressed in the cell.



Geoffrey Waldo received a bachelor of science degree in chemistry from Wright State University, Ohio, and a master of science degree and a Ph.D. in physical chemistry from the University of Michigan at Ann Arbor. Since 1993, he has been at Los Alamos, where he has applied his expertise to the study of protein evolution and folding.

Andrew Bradbury was born in England and received his bachelor of arts from the University of Oxford, and bachelor of medicine and bachelor of surgery degrees from the University of London. He then received a Ph.D. in immunology from the University of Cambridge. Subsequently, Andrew spent 10 years in Italy developing biomolecular diversity technology. In 1999, he joined the Laboratory, where he continues his interest in the field.

Ahmet Zeytun was born in Turkey and received a bachelor of science degree in plant genetics from Ondokuzmayis University, a master of science degree in molecular genetics from Ohio State, and a Ph.D. in molecular immunology from Virginia Tech University. He joined Los Alamos in 2000 to work on the development of fluorobodies.