

SPOTLIGHT

# Open-source recombinant monoclonal secondary nanobodies

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**Secondary antibodies are everyday reagents in biomedical research that are generated in animals. In this issue, Pleiner et al. (2018. *J. Cell Biol.* <https://doi.org/10.1083/jcb.201709115>) describe several single domain antibody fragments against antibodies from mouse and rabbit, so-called nanobodies that are easily produced recombinantly, and characterize their use in Western blotting, enzyme-linked immunosorbent assay, and immunofluorescence assays.**

Antibodies are ubiquitous reagents in research and diagnostic laboratories that allow the detection of protein, lipid, or carbohydrate molecules. Usually, in assays such as ELISA, Western blotting, or immunofluorescence, the so-called primary antibody that binds to the target molecule is then detected by a so-called secondary antibody. This secondary antibody has specificity against the species-specific immunoglobulin isotype of the primary antibodies and carries a label that allows for a read-out based on fluorescence, enzymatic chemoluminescence, or chromogenic detection. Antibodies are products of the immune system of jawed vertebrates and are generated by the adaptive immune system in response to the detection of nonself antigen. This process is used biotechnologically by injecting target molecules as antigen into animals. The immune system of the animal will then develop a response against the injected antigen and release antigen-targeting antibodies into blood plasma that are harvested later by bleeding and/or killing the animal. Often, but not always, the generated antibody will then be useful to detect the antigen in experimental assays. Most antibodies used are Y-shaped, ~10-nm-long protein structures of ~150 kD in size, called IgG (Fig. 1). Unfortunately, the epitope-binding domain in IgG-type antibodies consists of both a heavy and a light chain polypeptide linked by disulfide bonds and is thus difficult to prepare recombinantly. The use of animals, mostly mammals, to generate IgG antibodies is cost and labor intensive and presents concerns regarding animal welfare. It was thus immediately clear that the discovery of heavy chain only antibodies in camelids (Hamers-Casterman et al., 1993) would have significant impact on antibody technology and production. By injection of antigen into camelids it is now possible to generate antibodies consisting only of heavy chains with an epitope-binding domain merely 1.5 × 2.5 nm and 13 kD in size. By truncating this domain from the remainder of the heavy chain, the nanobody can be

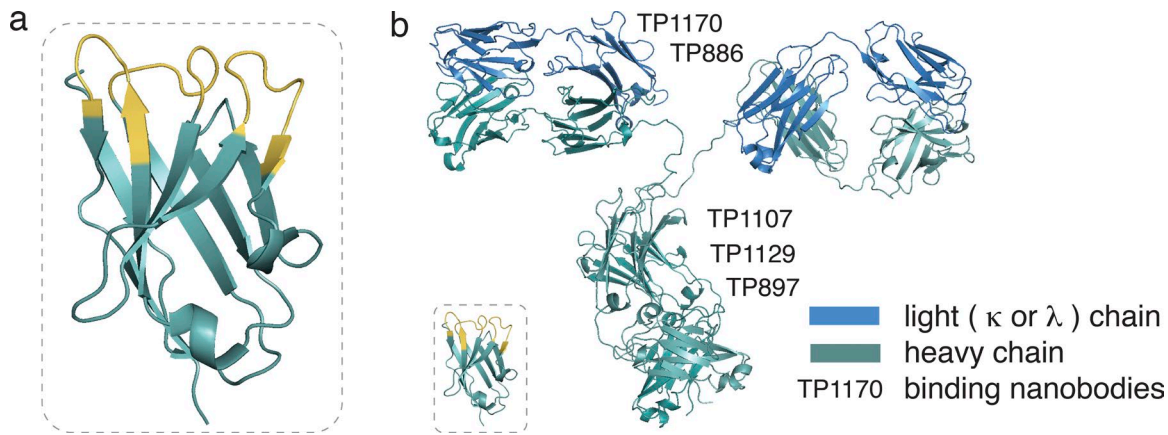
recombinantly expressed as a single polypeptide chain in bacterial culture (Fig. 1 a). This method of production is much more efficient than harvesting antibody by bleeding of live animals. It is even more productive than the generation of monoclonal antibodies from hybridoma cells, immortalized antibody-producing cells (mostly from mice) that secrete antibodies into the culture medium. Nanobodies are thus extremely cost efficient, highly specific, highly soluble, and stable, very small binders that can be easily modified at the genetic level for labeling purposes in many ways (Schumacher et al., 2017). Because of the relatively limited size of the three conserved hypervariable loops of the nanobodies, the generation of complete libraries is possible (Moutel et al., 2016) and by now many nanobodies against different targets are available. One of the most widely used nanobody is one against GFP (Rothbauer et al., 2006) and by now many nanobodies against various fluorescent proteins exist together with nanobodies against very common cellular molecules, such as tubulin (Mikhaylova et al., 2015), and against small protein tags (Braun et al., 2016). Recently, nanobodies have received increased attention because of the realization that their small size increases the effective resolution of cellular structures when used in superresolution methods that allow a resolution in the size range of large proteins (Ries et al., 2012).

In this issue, Pleiner et al. present several nanobodies raised against rabbit and mouse IgG antibodies. They characterize their capabilities as replacements for secondary antibodies in antibody-based assays such as Western blotting, ELISA, and immunofluorescence. A concern taken into account by the authors is that the use of nanobodies as secondary detection reagents is by no means a fail-safe strategy. The traditional IgG sandwich-labeling strategy with primary and secondary antibodies is in itself a signal amplification strategy. In a polyclonal secondary antibody mixture, several labeled secondary antibodies may bind

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**Figure 1. Nanobodies as secondary antibodies.** (a) Structure of the variable domain of an alpaca heavy chain antibody ( $V_{HH}$ ). The variable loops are emphasized in yellow (PDB accession number 30GO). (b) Structure of an IgG antibody with main nanobodies binding to  $F_{ab}$  or  $F_c$  domains to rabbit or mouse antibodies described in the manuscript. Inset shows comparative size of nanobody (PDB accession number 1IGT).

to a primary antibody and as a result of their comparably large size these may carry several labeling agents each. The necessarily monoclonal nanobody does not in itself provide such amplification and thus might not be competitive in some of these assays.

Pleiner et al. (2018) map a large number of nanobody binders generated after immunization of alpacas with rabbit and mouse antibodies and map them to the crystallizable fragment ( $F_c$ ) and antigen binding fragment ( $F_{ab}$ ) domains of the single rabbit IgG and the four different mouse IgG isotypes (IgG1, IgG2a, IgG2b, and IgG3). For some of them, even the nature of the light chain ( $\kappa$  or  $\lambda$ ) they bind to could be determined. Although several nanobodies cross react to different species, after affinity maturation of some binders, the authors identify a set of nanobodies against the  $F_{ab}$  and  $F_c$  domains of rabbit and mouse IgG, respectively, and against mouse IgG  $F_c$  isotypes (Fig. 1 b). They then go on to test these nanobodies as secondary binders in chemiluminescent and fluorescent detection methods for Western blotting and find them to be equally effective as traditional sandwich labeling. The use of a combination of nanobodies against light and heavy chains proves at times to be even more potent than IgG-mediated labeling.

In another series of experiments, Pleiner et al. (2018) test fluorescence-labeled nanobodies as labeling agents in immunofluorescence microscopy. Wisely, they chose a set of different targets to control for detection efficiency by the primary antibody to compare staining intensity and found that secondary nanobodies can be useful detection reagents in this important assay.

An appealing approach proposed here is the precoupling of nanobodies to specific primary antibodies that allows for one-step immunolabeling to speed up experiments or to perform multi-color immunolabeling with antibodies of the same species and isotype. This will allow for assays that are impossible in traditional experiments and prove to be important where only antibodies from a single species are available for a range of targets. Collectively, immunofluorescence staining with single nanobodies is generally weaker than with secondary antibodies and only a cocktail of  $F_c$  domain- and Fab domain-directed nanobodies matches the signal strength of traditional secondary antibodies. Matching the qualities of secondary antibodies using the smaller nanobodies

is considered to be a great success. The authors go on to show that when anti-light chain nanobodies are used, resolution is improved in single molecule localization microscopy in comparison to traditional sandwich labeling, although labeling density, a critical parameter in superresolution microscopy, is not very high.

Overall, although the nanobodies presented do not necessarily prove to be superior reagents to antibodies when used in Western blotting or immunofluorescence, Pleiner et al. (2018) present convincing data that they can work as well as traditional secondary antibodies. This is very important, as it allows for the use of recombinant reagents that can easily and relatively cheaply be generated with techniques available in most laboratories. This will especially help laboratories that are limited in resources worldwide. The future will show whether these reagents will indeed be used widely or, like other secondary antibody-detecting reagents based on the bacterial IgG-binding proteins protein A or protein G, be a rather seldom used alternative, especially because secondary nanobodies are already commercially available. By making the sequences public, providing detailed methods, and making expression plasmids available on Addgene, the authors have certainly provided a set of excellent tools to the community.

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