TECHNOLOGY FEATURE

ANTIBODY ANARCHY: A CALL TO ORDER

Antibodies used in research often give murky results. Broader awareness and advanced technologies promise clarity.

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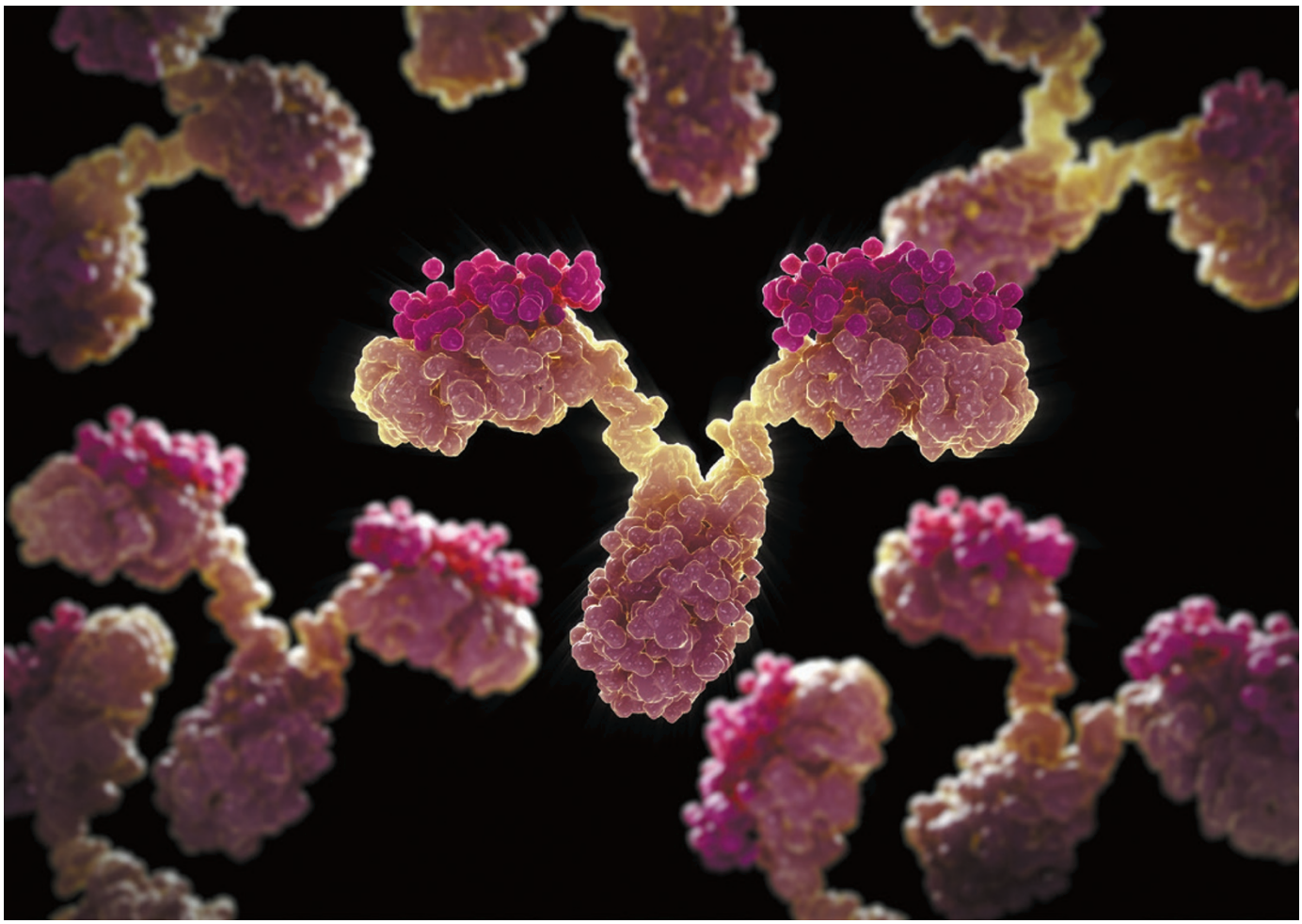


Antibodies, with their distinctive Y-shape, are among the most widely used — and most vexing — reagents in biology.

BY MONYA BAKER

A mouse first alerted Clifford Saper to the fact that antibodies were misleading the scientific community. As editor-in-chief of the *Journal of Comparative Neurology* between 1994 and 2011, he handled scores of papers in which scientists relied on antibodies to flag the locations of neurotransmitters and their receptors. Around the turn of the century, related investigations began to roll in from researchers using knockout mice, animals genetically engineered to not express a target gene. The results were unsettling. Antibody staining in knockout animals should have shown radically different patterns from those in unmodified animals. But all too often the images were identical. "As we saw more and more retractions due to this, I began to realize that we had no systematic way to evaluate papers that used antibodies," recalls Saper, now chair of neurology at Beth Israel Deaconess Medical Center in Boston, Massachusetts.

Thus began a one-journal revolution. Saper and his editorial colleagues set up a policy of requiring extensive validation data on each antibody[1]. The policy was good for rigour, but not submissions, he recalls. "Many authors were caught in the middle, and found it easier to publish their papers elsewhere." But Saper persisted. His efforts eventually culminated in the JCN Antibody Database, an inventory of a few thousand antibodies that can be ▶

trusted for neuroanatomy.

Today, biomedical researchers still collect tales of antibody woe faster than country-music labels spin out sad songs. The most common grumble is the cheating reagent: the antibody purchased to detect protein X surreptitiously binds protein Y (and perhaps ignores X altogether). Another complaint is 'lost treasure': a run of promising experiments that stalls when a new batch of antibodies fails to reproduce previous findings (see 'A market in a bind').

But technological advances and shifts in the scientific community now promise to cut through this antibody quagmire.

Antibodies are ubiquitous tools in the life sciences. Perhaps their most popular use is in western blotting to reveal the presence of a particular protein in cells or tissue samples, but they are also used to visualize proteins under the microscope by immunohistochemistry and immunofluorescence, as well as in many other applications that stem from an antibody's presumed ability to bind specific biomolecules. A 2015 report from online purchasing portal Biocompare puts the market for research antibodies at US$2.5 billion a year and growing. The choice is dazzling: there are hundreds of vendors supplying products.

It is alarming, then, to discover that antibodies can be unreliable reagents. Insufficient specificity, sensitivity and lot-to-lot consistency have resulted in false findings and wasted efforts. Antibody unreliability has taken its toll across studies in cancer, metabolism, ageing, immunology and cell signalling, and in any field concerned with researching complex biomolecules. The waste, in terms of time and resources, is colossal. Losses from purchasing poorly characterized antibodies have been estimated at $800 million per year, not counting the impact of false conclusions, uninterpretable (or misinterpreted) experiments, wasted patient samples and fruitless research time[2].

Mathias Uhlén, a protein researcher at the Royal Institute of Technology in Stockholm, says that frustration with research antibodies has been building for years[3] and that the time is finally ripe for improvements. "There is a big interest in the community to clean this up."

SPURRED TO ACT

Discontent has spurred action along various fronts. In September, Uhlén chaired the inaugural meeting for a working group on antibody validation hosted by the Human Proteome Organization, an international consortium based in Vancouver, Canada, that supports large-scale projects for understanding proteins. That same month, the Federation of American Societies for Experimental Biology hosted roundtables to explore problems with antibodies. It expects to issue recommendations early next year. The US National Institutes of Health (NIH) is also on the case. Starting in January next year, grant applications must include a new section describing efforts to authenticate antibodies and other key resources required for experiments. Far-reaching solutions are likely to be hammered out at a meeting hosted by the Global Biological Standards Institute next September. The gathering will be held in Asilomar, California, where scientists gathered 40 years ago to set cautionary approaches for using recombinant genetic technology to manipulate DNA.

"We're hoping that the community will come up with consensus guidelines," says Jon Lorsch, director of the US National Institute of General Medical Sciences in Bethesda, Maryland. That way, both grant applicants and reviewers will have resources to turn to when describing how they will authenticate their materials.

Such resources could take the form of a menu of broad-strokes criteria. "We are not talking about good and bad antibodies but antibodies that work in specific assays and specific context," says Uhlén. Evaluation categories might include knockdown and knockout approaches to reveal whether an antibody still binds even in the absence of the target protein. Another approach would be to tag a target protein with a fluorescent marker to reveal whether the antibody also binds untagged proteins. A third category could compare a new antibody with a well-characterized one. Finally, researchers could run the antibody and whatever it binds through a mass spectrometer to analyse bound molecules for the expected protein fragments.

Several vendors have announced their own characterization efforts, and new technologies are helping. Alan Hirzel, chief executive officer of Abcam, a life-sciences reagents provider in Cambridge, UK, says that to verify that its commercial antibodies perform as expected, the

A market in a bind

An antibody that performs differently across experiments can cause calamity. But the performance of these reagents is linked to how they are manufactured.

Polyclonal antibodies are made by collecting the blood of an animal immunized with the target antigen. Any particular lot will therefore only be available as long as the animal lives. To produce monoclonal antibodies, a host animal is immunized with the target protein or relevant portion of it, then the B lymphocytes that recognize and respond to that antigen are fused to a myeloma cell line that can be cultured indefinitely to produce the desired antibody.

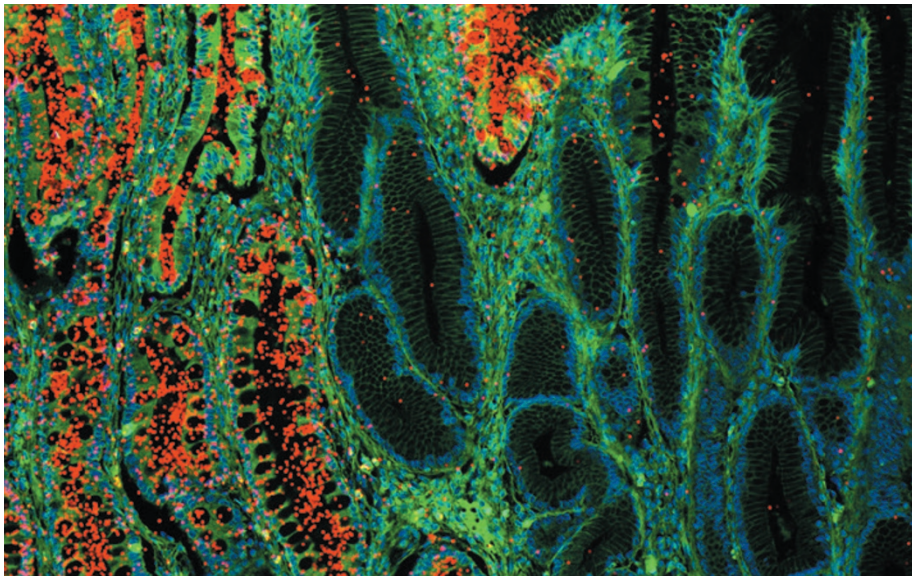
Recombinant antibodies are unlike traditional monoclonals because they can be manufactured without animals. Instead, these antibodies are made by identifying an exact gene sequence for an antibody — either by sequencing an animal's immune cells to find those that produce antibodies with highest affinity for the target, or sequentially shuffling gene sequences and testing the resultant proteins. That gene can then be introduced into an appropriate cell line to produce antibodies. Because the identity of the antibody is precisely defined, the cell line can be regenerated if the original colony dies or mutates.

The pursuit of antibody quality has inspired two publicly funded initiatives aimed at generating collections of validated antibodies and other protein-binding reagents. These produced thousands of new binders, but the Protein Capture Reagents programme, which launched in 2010, is already winding down, as is the European Union-funded Affinomics consortium, which launched in 2007 (ref. 8). Advocates say that the chosen targets, such as transcription factors, were particularly problematic and that further investments in such reagents would yield larger pay-offs.

Meanwhile, polyclonals command a large swathe of the market. A project that profiled reagents used across 10,000 biomedical papers published since 2006 found references to 1,293 polyclonals, 755 monoclonals and only 1 recombinant. Some researchers think that polyclonal antibodies, which can target a protein in multiple ways, are not only easy to manufacture but also particularly good at recognizing proteins in diverse contexts.

Eric McIntush is chief scientific officer of Bethyl Laboratories in Montgomery, Texas, which has been selling polyclonal antibodies for over 40 years and plans to start selling recombinants in 2016. The research world needs both, he says. Companies simply cannot afford to sink funds into products that they may never sell. The widespread availability of polyclonals, which are currently the least expensive antibody to develop, may encourage experiments on under-investigated proteins. As targets become more defined and are needed for translational applications, he says, there will be a market for recombinant products.

But researchers such as Andreas Plückthun, a protein engineer at the University of Zurich in Switzerland, think that polyclonals and monoclonals should be eliminated entirely in favour of defined binders. He agrees that many proteins are not addressed by existing reagents but does not see the point in making undefined products such as polyclonals. "Why not use something where the genes can be identified or kept?" he asks. M.B.



Pairs of antibodies can be designed to signal (red) only when both detect the same target protein[9].

company is using a genome editing method called CRISPR–Cas9, which makes precise changes in DNA. The company is testing antibodies on human cell lines in which target genes have been disrupted by CRISPR–Cas9 and then posting results for each reagent tested.

"We now really have the technologies we need that allow us to carry out those characterizations, whereas 5 or 10 years ago, we simply didn't," says Klaus Lindpaintner, chief scientific officer at Thermo Fisher Scientific, a life-sciences tools provider in Waltham, Massachusetts. Those companies with characterization data are starting to view this as a competitive advantage. In June this year, life-sciences company Bio-Rad in Hercules, California, launched a line of antibodies that have been tested for off-target activity in western blots against 12 different cell lines. Since mid-2014, Proteintech, an antibody manufacturer in Chicago, Illinois, has been using small interfering RNA to knock down gene expression in each new antibody product — assessing whether the signal subsides with the expression of the target gene. Such efforts are nascent, however, with only a tiny fraction of companies' catalogues being subjected to validation.

"Providers cannot guarantee that a given antibody will work for every tissue type."

And not all companies disclose the specific conditions of testing, or whether an antibody has performed poorly under those conditions, says Gordon Whiteley, lab director at the NIH's Antibody Characterization Program, which aims to create reliable antibodies for use in cancer biology. The example his programme sets in terms of supplying testing protocols and resulting data could be just as important as the reagents themselves, he says.

There will be no single best way to test antibodies, says Roberto Polakiewicz, chief scientific officer of Cell Signaling Technology, an antibody manufacturer in Danvers, Massachusetts. "Developing an antibody is a scientific endeavour. You need people who know what experiments to do to validate an antibody." If customers cannot see the data and make their own judgements, they need to look for a new antibody, he says.

But researchers sometimes take only a cursory look at data, and many do not realize that antibodies' performance in a given tissue or application, such as western blotting, says little about whether it will work in other sorts of experiments.

And commercial providers cannot guarantee that a given antibody will work for every tissue type and experimental condition, warns Paul Sawchenko, a neuroscientist at the Salk Institute in San Diego, California. "Unless one is so fortunate as to have had someone else demonstrate specificity in the same tissue from the same species under the same experimental conditions, you should be obliged to do this yourself."

VITAL INFORMATION

It would be more efficient to learn from other researchers' work, but fewer than half of the publications that describe antibody experiments report which specific reagent was actually used[4]. Even when authors do include a catalogue number, companies may discontinue products and sell off lines, making them hard to track, says Anita Bandrowski, an information scientist at the University of California, San Diego. Bandrowski is group leader at the Resource Identification Initiative, an NIH-backed programme involving a diverse group of academic collaborators. The initiative has been instrumental in establishing unique identifiers for antibodies and persuading dozens of journals to ask authors to specifically name which antibodies they are using.

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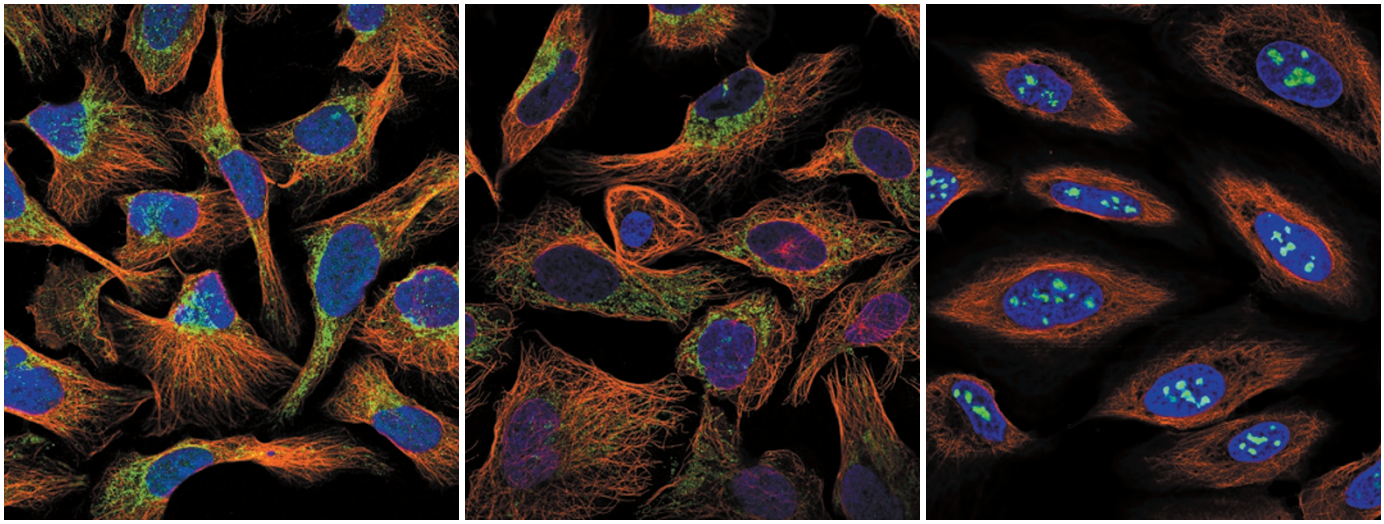

Three antibodies (green) against the same mitochondrial protein. The unexpected pattern on the right shows the third antibody binds an unintended protein.

Information is beginning to accumulate. More than two dozen web portals have sprung up to help researchers select antibodies. Some collect user reviews on antibody performance and offer comparison tools. The Antibody Validation Channel, a project of the scientific publisher F1000, allows researchers to post their accounts and even request peer review. Biocompare has hired a content editor whose sole focus is to reach out to the research community and get them to write reviews.

Some antibody suppliers, such as St John's Laboratory in London, offer researchers free products in exchange for testing and sharing the results. Antibodies-online, a market place for antibodies, arranges for an independent third party to perform validation. At Antibodypedia's knockdown initiative, launched in September, life scientists can earn hundreds of dollars in free reagents if they submit data showing that gene-silencing reagents such as small interfering RNA or CRISPR–Cas9 eliminate an antibody signal for a given target.

But many scientists are wary of information from anonymous reviews. Data supplied by both users and companies can be sparse, and some projects share data only if they confirm that an antibody works as expected. "Sometimes it seems easier to hire a detective than to order a specific antibody," concludes an overview of antibody portals[5].

FUTURE ASSESSMENTS

Some researchers are developing mechanisms to compare antibodies directly. Aled Edwards at the University of Toronto, Canada, is director of the international Structural Genomics Consortium (SGC). He and his SGC colleagues used mass spectrometry to detect and compare the sets of proteins pulled down by immunoprecipitation with more than 1,000 antibodies[6]. The collaboration ran across 5 reference laboratories, took 4 years and cost US$3 million, not counting in-kind donations. Ultimately, it established a procedure to score antibody quality and share quantitative information about its performance, specifically for 'pull-down experiments', in which proteins are pulled out of solution using antibodies.

Fridtjof Lund-Johansen, a proteomics researcher at Oslo University Hospital in Norway, is developing an ambitious bead assay that tests thousands of antibodies at once[7]. The plan is to separate cellular proteins into many different fractions, then profile the proteins in each fraction using two different methods. One is mass spectrometry and the other is a bead-based array with thousands of antibodies. The mass spectrometry data serve as a reference for the results obtained with antibodies. Turning the idea into a refined assay will take considerable work, Lund-Johansen admits. "It is extremely ambitious. It is totally crazy, but it is the only way to go." Other scientists are intrigued at the approach but wonder if it will predict antibody performance in common techniques.

Blanket assessments of antibodies can be overinterpreted, says Ulf Landegren, a proteomics technology developer at Uppsala University in Sweden. "It is far more meaningful to discuss the ability of assays to detect the correct protein, rather than whether antibodies or other binders bind the right protein." A case in point is cross-reactivity, when an antibody binds proteins other than its specified target. Cross-reactivity depends not just on a particular antibody, but also on the complexity of a sample, the concentration of the antibody and the rarity of the target protein. He recommends that rather than relying on a single antibody, researchers should instead test antibodies in pairs that are designed to bind to different parts of a target protein. Parts of a sample labelled with both reagents are less likely to represent off-target binding.

One problem with this approach is that it is hard for scientists to know if they are purchasing different antibodies. Vendors often obtain products from different sources and are not required to disclose the original manufacturer. As a result, researchers who want to compare several antibodies may end up comparing identical products sold by several vendors. A handful of companies, including Genlogica and One World Labs, both in San Diego, California, only sell products labelled by the original manufacturer and offer 'trial size' antibody batches so that researchers can test products side by side in their labs.

The toughest challenge is not so much in antibody characterization but in persuading cell biologists to hold back on using antibodies until these are thoroughly evaluated, says Edwards, although he doubts that scientists will become savvier unless funders and publishers force the issue. "Right now we have an unregulated market, where you don't have to have any quality to sell your product." In other words, he says, guidelines, characterization data and conscientious vendors only matter if researchers invest effort into selecting reagents. ■

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1. Saper, C. B. & Sawchenko, P. E. *J. Comp. Neurol.* **465,** 161–163 (2003).
2. Bradbury, A. *et al. Nature* **518,** 27–29 (2015).
3. Bordeaux, J. *et al. BioTechniques* **48,** 197–209 (2010).
4. Vasilevsky, N. A. *et al. PeerJ* **1,** e148 (2013).
5. Pauly, D. & Hanack, K. *F1000Research* http://dx.doi.org/10.12688/f1000research.6894.1 (2015).
6. Marcon, E. *et al. Nature Methods* **12,** 725–731 (2015).
7. Wu, W. *et al. Mol. Cell. Proteomics* **8,** 245–257 (2009).
8. *Nature Methods* **12,** 373 (2015).
9. Conze, T. *et al. Glycobiology* **20,** 199–206 (2010).

CORRECTION

The Technology Feature 'Connectomes make the map' (*Nature* **526,** 147–149; 2015) misnamed the MultiSEM model and gave the wrong citation in reference 3. MultiSEM 505 should have been Zeiss MultiSEM, and ref. 3 should have referred to Zingg, B. *et al. Cell* **156,** 1096–1111 (2014).