Special Issue on

Industrial Biotechnology-Made in Germany:
The path from policies to sustainable energy, commodity and specialty products

Edited by:
Dr. Thomas Brück
Professor of Industrial Biocatalysis, Dept. of Chemistry, Technische Universität München (TUM), Germany
Novel Antibody Format Provides Efficient Tools for Research and Drug Discovery

Yurlova, L.1, Buchfellner, A.1,2, Zolghadr, K.*

1 ChromoTek GmbH, IZB, Germany
2 Natural and Medical Sciences Institute (NMI) at the University of Tuebingen, Germany

Keywords
Heavy-chain antibody; Single domain antibody; VHH; Nano-Trap; Nano-Booster; Chromobody

Abstract
Antibody-based reagents are indispensable for a broad range of biomedical sciences as well as for bioproduction. Here we highlight novel derivatives of heavy-chain antibodies and elaborate upon their beneficial application in proteomics, cell biological research and drug discovery. This special class of antibodies from Camelidae provides distinct advantages over conventional antibodies due to the naturally originating single-chain structure of their antigen-binding domains. At ChromoTek we have now developed new immunization and screening strategies to convert these advantages into superior tools for a broad range of applications.

First, these extremely stable antigen-binding fragments of heavy chain antibodies (V\textsubscript{H}s, single domain antibodies, sdABs) can be easily produced as recombinant proteins in bacteria with constant quality criteria. When coupled to solid matrices, the highly specific V\textsubscript{H}s can function as Nano-Traps®, facilitating efficient affinity purification of proteins for proteomic analyses.

Second, the tenfold smaller size of V\textsubscript{H}s than that of the commonly used immunoglobulins offers a versatile substitute to traditional antibody staining as V\textsubscript{H}s can also be fluorescently labeled. TheseNano-Booster termed reagents are therefore especially useful for super-resolution microscopy and possess higher tissue penetrating ability.

The third exciting application of V\textsubscript{H}s as live cell biomarkers opens new possibilities for cell biological and pharma research. When V\textsubscript{H}s are genetically fused to fluorescent proteins and expressed inside cells, these so-called Chromobodies® enable unique intracellular live cell antibody staining, unthinkable with conventional antibodies. These fluorescent nanoprobes are of special interest for high content analysis (HCA) in drug discovery, since they permit real-time analyses of the effects of drug candidates at endogenous targets. Here we show examples for Chromobodies® highlighting important cellular biomarkers. Beyond visualization, Chromobodies® can be designed to specifically modulate (e.g. inhibit) their intracellular targets.

Further possible implementations of these extremely small, stable and soluble single-chain camelid antibody fragments vary from co-crystallization assistance and neutralization of toxins, to crop protection and eventual therapeutics.

Abbreviations
- BrdU: Bromodeoxyuridine
- CCC: Cell Cycle Chromobody
- ChIP: Chromatin immunoprecipitation
- Co-IP: Co-immunoprecipitation
- DNMT1: DNA (cytosine-5)-methyltransferase 1
- ELISA: Enzyme-linked immunosorbent assay
- FISH: Fluorescence in situ hybridization
- GFP: Green fluorescent protein
- GST: Glutathione S-transferase
- HCA: High content analysis
- IF: Immunofluorescence
- IgG: Immunoglobulin G
- IHC: Immunohistochemistry
- IP: Immuno precipitation
- MAPKAPK2, MK2: MAP kinase-activated protein kinase 2
- NHS: N-hydroxysuccinimide
- PALM: Photoactivated localization microscopy
- PARP: Poly (ADP-ribose) polymerase
- PBS: Phosphate buffered saline
- PCNA: Proliferating cell nuclear antigen
- PFA: Paraformaldehyde
- RFP: Red fluorescent protein
- RIP: RNA immunoprecipitation
- SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- STED: Stimulated emission depletion (microscopy)
- STORM: Stochastic optical reconstruction microscopy
- U2OS: Human osteosarcoma cell line
- V\textsubscript{H}s: Variable domain of heavy chain of heavy chain antibody
- 3D-SIM: Structured illumination microscopy

Introduction
The natural role of antibodies as blood plasma proteins is to identify and bind other biomolecules, viruses and microbial toxins in this way defending us against infection [1]. This ability of antibodies to recognize their particular targets (termed antigens) is broadly employed in biomedical research, enabling specific and quantitative detection of target molecules among a myriad of others. Antibody-based tools are indispensable in basic experimental science, as well as in medicine for diagnostics purposes and as of late even in therapeutics [2].

The drawback of these powerful molecules is that conventional antibodies are large proteins consisting of several polypeptide chains which complicates their production and some of the applications. However, a couple of decades ago, the accidental finding of some oddly small antibodies in camel blood serum triggered the uncovering of a whole new class of antibodies. These camelid antibodies (present in camels, dromedaries, llamas, guanacos, vicunas and alpacas) were named...
heavy chain antibodies, as they are composed of heavy chains only and are devoid of light chains ([3,4], recent review in [5]). In the absence of light chains, the antigen binding part of these antibodies is reduced to a single domain (single domain antibody, sbAb), the so-called V_H domain (variable domain of heavy chain of heavy chain antibody) or nanobody (Figure 1). These V_H domains are just 12-15 kDa in size, which is one-tenth the size of a conventional IgG antibody. V_Hs are highly affine, extremely soluble and stable to temperature and chemicals [6-8].

These exceptional properties of V_Hs offer tangible advantages for various applications and even enable them to perform other functions for which no true alternative is available. At ChromoTek GmbH (Planegg, Germany), we put these advantages on service to basic science by converting V_Hs into superior research tools. Furthermore, V_Hs’ unique properties enable targeting and tracing of antigens in living cells [9]. This patented Chromobody® Technology is exclusively available to ChromoTek. The basic V_H patents have been developed at the Vlaams Instituut voor Biotechnologie (VIB, Belgium) and the Vrije Universiteit Brussel (VUB, Belgium). Dependent on the application field, ChromoTek and several other licensors have access to the V_H technology. Other key patent holders are Ablynx, Belgium (healthcare application of V_Hs) and BAC BV, Netherlands (now part of Life Technologies recently integrated into Thermo Fisher Scientific, USA; application of V_Hs in biopharmaceutical purification).

Since its launch in 2008, ChromoTek has developed new immunization and screening strategies to convert V_Hs into the first-rate products for biomedical science, appreciated by ~ 4500 customers all over the world. Below we discuss several most important research applications of V_Hs: efficient immunoprecipitation with Nano-Traps, super-resolution microscopy with Nano-Boosters and visualization of endogenous proteins in live cells with Chromobodies®. An overview comparison of these applications can be found in (Table 1).

V_H-based reagents: from immunization to screening and production

To develop a V_H-based reagent, first a specific V_H which is highly affine for a selected target must be obtained. For this, we follow an optimized process consisting of alpaca immunization, generation of a V_H-library, selection by immunopanning and verification by ELISA. In brief, alpacas (Vicugna pacos) are immunized with a purified target protein in a suitable adjuvant (e.g. from Gerbu Biotechnik GmbH, Germany) for a 3 month period including a 1 week “boost”. Lymphocytes are isolated from a blood sample and further used for mRNA-extraction and cDNA synthesis. These are then used as a template for amplification of V_H sequences with specific primers resulting in construction of a V_H library of ~10^7 individual DNA sequences. Here we employ one of the most widely used library methodologies, which is based on the use of filamentous phage [10], a virus that lives on Escherichia coli. Phage display technology (reviewed in [11,12]) has proven to be a powerful technique for presentation and selection of antibodies in general and single-chain V_Hs in particular [13].

Depending on the downstream application of a V_H (i.e. live cell or biochemical application) we subject a phage displayed library to one of the two different panning methods: an adapted immunopanning method, known as a solid phase panning [14], or, alternatively, a “native” panning technique [15]. These two methods differ by antigen presentation. In solid phase panning, the antigen is a purified protein or peptide, which is passively adsorbed on a solid support, e.g. on a microplate. Passive adsorption, however, has been shown to be able to induce conformational changes in proteins, potentially rendering a large proportion of nonfunctional proteins [16]. In contrast to this, in “native” panning the antigen is presented as a tagged fusion protein enriched from a mammalian cell lysate [15]. In comparison to the solid phase method, with the “native panning” we aim at presentation of the antigen in its intracellular conformation with posttranslational modifications in order to thereby increase the chance of finding antibodies for cell based applications. Three consecutive panning rounds are performed to enrich phages, carrying an antibody fragment, specific for the presented antigen [9].

V_Hs which were selected by immunopanning are further tested for antigen recognition in ELISA [17], [18]. ELISA-positive binders are then characterized for their expression level, solubility, affinity and specificity. For the bacterial production, selected V_Hs are cloned in appropriate bacterial expression vectors. Different expression methods have to be tested since V_Hs can be either produced in the bacterial cytoplasm [19] or in the periplasm, where the oxidising environment supports disulfide bond formation [13]. Soluble antibody fragments produced in E. coli are purified by affinity chromatography via the hexahistidine-peptide genetically fused to the C-terminus of a V_H [20] followed by gel filtration chromatography [21]. In comparison to conventional production of monoclonal or polyclonal antibodies (e.g. isolation from serum, or purification from culture media of hybridoma cells), bacterial production of recombinant antibody fragments like V_Hs is economic and sustainable, providing a protein of consistent quality with negligible batch-to-batch variations [18]. Purified V_Hs are then modified for appropriate functionality by coupling to solid matrices or fluorescent dyes to create our biochemical reagents Nano-Traps and Nano-Boosters.

Figure 1 Comparison of conventional antibodies, consisting of four polypeptide chains (e.g. mouse IgG, ~150 kDa) and much simpler camelid heavy-chain antibodies. A binding domain of a heavy-chain antibody (in blue, VHH fragment or sdAb) is formed by a single polypeptide chain and is just ~15 kDa in size.
Table 1: Overview of application areas for VₙH-based research tools.

<table>
<thead>
<tr>
<th>Application</th>
<th>Nano-Trap</th>
<th>Nano-Booster</th>
<th>Chromobody*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Format</td>
<td>VₙH-solid support</td>
<td>VₙH-chemical dye</td>
<td>VₙH-GFP/RFP (DNA)</td>
</tr>
<tr>
<td>Alternative Technologies</td>
<td>Conventional IP (e.g. IgG with Protein-A/G beads)</td>
<td>Conventional IF/IHC (primary + secondary antibody)</td>
<td>Overexpression of genetic fusions of GFP/RFP with the target protein</td>
</tr>
<tr>
<td>Advantages of VₙH Technology</td>
<td>2X faster IP, robust against t°C and chemicals (e.g. for mass-spec), no interference from immunoglobulin chains</td>
<td>2X faster IF, close proximity of signal to the target (for super-resolution microscopy)</td>
<td>Detection of endogenous (not overexpressed) proteins in live cells, endogenous target inhibition</td>
</tr>
<tr>
<td>References</td>
<td>[22,25-34]</td>
<td>[35-41]</td>
<td>[9,42-45]</td>
</tr>
</tbody>
</table>

* - in development

Abbreviations: please refer to the full list of abbreviations at the beginning of the manuscript.

Product Line: Nano-Traps®

Nano-Trap reagents enable highly efficient one-step isolation of the target proteins and their interaction partners from cell lysates or tissues [22]. These immunoprecipitation reagents consist of target-specific VₙHs covalently coupled to agarose or magnetic particles (beads). Nano-Trap technology provides notable improvements in speed, robustness and quality of immunoprecipitation in comparison to the conventional immunoprecipitation techniques employing IgGs coupled to Protein-A/G beads [23].

Firstly, to manufacture Nano-Traps, the NHS (N-hydroxysuccinimide) coupling is employed to immobilize VₙHs on the beads, resulting in formation of amide bonds between the matrix and the primary amino groups of the VₙH proteins. This covalent bonding allows Nano-Traps to overcome the commonly encountered problem of interference from immunoglobulin bands with analysis of immunoprecipitates (e.g. on SDS-PAGE) [24]. Thus, in case of conventional immunoprecipitation with IgGs immobilized on Protein-A/G beads, elution in presence of detergents and reducing agents (e.g. boiling in SDS-sample buffer, containing SDS and β-mercaptoethanol), results in contamination of the eluate (“bound” fraction) with heavy and light chains of conventional antibodies. In contrast, even under these conditions, Nano-Traps ensure that the eluted protein is not contaminated with the antibodies, since VₙHs stay “on the bead” during elution due to both covalent binding of VₙHs to the matrices and monomeric nature of VₙHs (Figure 2A).

Secondly, extreme stability of VₙHs themselves ensures their robust binding performance under a broad range of conditions (up to 2M NaCl or 1% SDS or 8M Urea), which can be required for some high-end downstream applications, such as ubiquitination assay [25].

Next, immunoprecipitation with Nano-Traps is very efficient, which stems from the high affinity of the VₙH binding its target (dissociation constant for our GFP-Trap® lies in the picomolar range) [22]. Additionally, a higher density can be expected from the coupling of VₙHs to the beads, than from coupling of the full IgG molecules, due to the 10-times smaller size of the VₙH fragments.

Finally, since Nano-Traps are manufactured with the binders already immobilized on the beads, the pull-down procedure is significantly shorter than with conventional antibodies, which require additional time for coupling to Protein-A/G beads [23]. This results in an efficient immunoprecipitation with Nano-Traps within 30 minutes (Figure 2B).

One example for an extremely popular Nano-Trap is our GFP-Trap® (over 400 references on PubMed, listed also at www.chromotek.com/references/publications/) which connects cell biology with biochemistry. Fluorescent proteins are the commonly used tags in cell biology, and GFP-Trap enables easy immunoprecipitation of fluorescent fusion proteins and their interacting factors from transfected cells or stable cell lines (Figure 2A, 2B).

GFP-Trap has been employed for a multitude of biochemical analyses of the GFP-fusion proteins by means of immunoprecipitation (IP) / Co-IP followed by Western Blot detection [26, 27], mass spectroscopy [28-30], enzyme activity measurements (can be done directly on-beads) [31, 32], ChIP [33] or RIP analyses [34]. For example, GFP-Trap was one of the tools the authors used in a recent study of the role of the post-translational modification citrullination in regulation of pluripotency and histone H1 binding to chromatin [26]. For this, GFP-tagged histone H1.2 (wild-type vs. its mutant form) was expressed in embryonic stem (ES) cells, pulled-down with GFP-Trap and then subjected to citrullination immunoblot. In another publication, the authors analyzed an interaction between the two disease- and RNA-associated proteins, FMRP (fragile X protein) and Ataxin-2 in transgenic Drosophila using GFP-Trap in...
conjunction with Western blotting as one of the approaches [27]. In the most recent work, GFP-Trap was used to conduct in vivo high-throughput RNA-binding assays [34].

Besides GFP-Trap, we provide the following highly effective Nano-Trap reagents: RFP-Trap, GST-Trap, DNMT1-Trap. A number of other Nano-Traps specific against p53, Mdm4, MAPKAPK2, PARP and β-Arrestin is our pipeline and will become available within the next months.

Product Line: Nano-Booster

Nano-Booster reagents enable reactivation, enhancement and stabilization of the signals of fluorescent proteins for microscopy applications (Figure 3 and [35]).

As mentioned in the previous chapter, fluorescent proteins are commonly used in cell biology to facilitate studying protein localization and dynamics in living cells. However, fixation or other treatments (FISH, Click chemistry or BrdU detection) can dramatically decrease signal intensities. Our GFP- and RFP-Boosters are immuno- and histochemical reagents, consisting of small, highly affine V₃Hs coupled to bright fluorescent ATTO dyes from ATTO-TEC GmbH, Germany (www.atto-tec.com). ATTO NHS-esters react with amino groups of V₃Hs typically resulting in 1:1 labeling efficiency (one dye molecule per one V₃H molecule). Nano-Boosters can be easily applied in a one-step protocol: no incubation with secondary antibodies is required here. Already after a short 30-minute immunostaining of PFA-fixed cells with GFP-Booster, the fluorescent signal intensities increase ~2.5 times (data not shown) and become significantly more stable against bleaching (Figure 3, bottom). Enhancement of the fluorescent protein signals with our Nano-Boosters for conventional or confocal fluorescence microscopy can be exemplified with recent publications on spindle assembly checkpoint [36], on membrane remodeling in Dictyostelium discoideum [37] and on trafficking in mammalian ciliogenesis [38].

Another drawback of typical fluorescent proteins is that their photostability and quantum efficiency are not sufficient for super-resolution microscopy applications, such as 3D-SIM, STED or STORM/PALM. Although the signal properties of the fluorescent proteins can be improved through immunostaining, the large size of conventional antibodies displaces the dye from the target in a “linkage error” of ~ 10 nm [35], which is undesirable for single-molecule imaging. In contrast, close proximity of a dye to the target structure can be easily achieved with GFP- and RFP-Boosters due to the small size (1.5 nm X 2.5 nm) of the alpaca antibody fragments [35], [39]. Thus, Nano-Boosters combine molecular specificity of genetic tagging with the minimal linkage error and a high photon yield of organic dyes. This labeling scheme has been successfully applied for single-molecule nanoscopy of GFP-tagged proteins in mammalian cell lines, in cultured hippocampal neurons, and in budding yeast Saccharomyces cerevisiae [35]. Also, in a recent study of Drosophila patterning, the authors examined differential association of mRNAs with processing bodies (P bodies) in fly oocytes using structured illumination microscopy. To determine co-localization of bicoid (bcd) and gurken (gurk) ribonucleoprotein complexes with a P body marker protein, they detected the protein counterparts through genetic fusions to fluorescent proteins, which were immunostained with Nano-Booster and imaged with 3D-SIM [40]. The group of A. Furstenberg also used Nano-Boosters in super-resolution microscopy to quantitatively analyze dynamics of arrestin2 clustering in cultured mammalian cells upon G protein-coupled receptor stimulation and its dependence on cytoskeletal components [41].

Product Line: Chromobodies®

Chromobodies are unique molecular probes developed by
Central shortcomings by creating V\textsubscript{HH}-based nanoprobes termed proteins. Localization of endogenous proteins can be examined about distribution or dynamics of the endogenous cellular these exogenously expressed chimeras do not give information fusion of a target protein with a fluorescent protein. However, exogenously introduced chimeric proteins generated by a genetic real-time imaging applications [9].

ChromoTek to enable immunotracing of antigens in living cells in Chromobodies [42]. A hallmark advantage of V\textsubscript{HH}s over conventional antibodies lies in their ability to fold in the reducing environment of cytoplasm of eukaryotic cells, making V\textsubscript{HH}s suitable for a role as intracellular antibodies. Chromobodies\textsuperscript{*} are generated by genetic fusion of a target-specific V\textsubscript{HH} with a fluorescent protein. To develop a Chromobody against a target of interest, specific V\textsubscript{HH}s which were selected in a "native" panning, are cloned in-frame with fluorescent proteins, such as TagRFP or TagGFP2 under control of immediate early promoter of cytomegalovirus (P\textsubscript{CMV}) in mammalian expression vectors from Evrogen, Russia (www.evrogen.com/products/TagFPs.shtml). These Chromobody plasmids can be introduced into living cells (celllines, primary or stem cells) by means of transient transfection (e.g. with Lipofectamine\textsuperscript{®}, Thermo Fisher Scientific, MA, USA). Alternatively, Chromobodies are also available as stable cell lines for high-content analysis (HCA) and screening. In brief, stable HeLa and U2OS cell lines are generated by random integration of a Chromobody into cellular genome upon transfection followed by culturing under selection pressure (puromycin or G418) and single-cell sorting to gain individual clones. Transgene expression is validated by fluorescence microscopy and Western blotting. Stability of the genetic modification is confirmed after 1, 2 and 3 months in culture (respective paper describing the method is currently in preparation).

At ChromoTek we developed Chromobodies highlighting important cellular biomarker processes such as cell cycle (PCNA, Cell Cycle Chromobody), DNA methylation (DNMT1 Chromobody), PARYlation (PARP Chromobody), nuclear and cytoskeletal rearrangements (Lamin and Actin Chromobodies) (PCNA, Cell Cycle Chromobody), DNA methylation (DNMT1 Chromobody), PARYlation (PARP Chromobody), nuclear and cytoskeletal rearrangements (Lamin and Actin Chromobodies) (see Figure 4). With the help of Chromobodies these processes can for the first time be resolved based on the signals from the endogenous proteins, which is essential for understanding fundamental biological processes as well as for accurate distinguishing of health and disease at a cellular level. For example, DNA replication could recently be for the first time observed in high detail at endogenous level by subjecting our Cell Cycle Chromobody cell line that specifically labels endogenous PCNA to high-resolution confocal time-lapse microscopy [43].

Since Chromobodies\textsuperscript{*} permit real-time analyses of the effects of drug candidates at endogenous targets, they are of special interest for high content analysis in drug discovery. Several in-house and external studies demonstrated suitability of Chromobodies\textsuperscript{*} for automated HCA. For example, our Cell Cycle Chromobody (CCC) HeLa and U2OS stable cell lines enable screening of compounds such as cancer drugs for influencing cell cycle and cell viability in one experiment. (Figure 5) shows the results of an automated evaluation of the effect of anticancer drugs such as aphidicolin, nocodazole or staurosporine on the cell cycle, analyzed with CCC cell line. In another case study we demonstrate a straightforward visualization of apoptosis in real-time imaging using Lamin Chromobody cell line for high-content analysis [44].

Beyond visualization, Chromobodies can be designed to specifically modulate (e.g. inhibit) their intracellular targets [39], [45]. Inhibitory Chromobodies provide a useful tool for basic research and for target validation in drug discovery. Chromobodies could even serve as a lead molecule for investigation of targets considered undruggable.

![Figure 3](Image)

**Figure 3** RFP-Booster enables specific labeling of RFP-fusion proteins and increases signal stability.

Top: Confocal images of HeLa cells expressing mRFP-PCNA and stained with RFP-Booster. The cells were transiently transfected with mRFP-PCNA mammalian expression construct, fixed in 4% PFA, permeabilized with 0.5% triton-PBS, incubated with RFP-Booster_ATTOS94 for 30 min and stained with DAPI to highlight nuclei. Imaging was performed with UltraVIEW VoX (PerkinElmer, MA, USA) spinning disc microscope. Left to right: DAPI signal is in blue, PCNA/ RFP-Booster signal is in red, overlay image is on the right. Note that the red signal is strong and specific and clearly resolves the replication foci. Scale bar, 10 μm.

Bottom: Quantification of signal stability upon photobleaching. RFP-expressing cells were fixed and stained with RFP-Booster and compared with unstained cells in bleaching experiments. Bleaching and imaging was performed with Leica SP5 confocal microscope (Leica Microsystems GmbH, Germany) equipped with HeNe 2 mW 594 nm laser (60% laser intensity for bleaching, 15% for image acquisition). Signal intensities were quantified, starting values normalized to 100% and plotted against the bleaching time, s. Bar chart shows that RFP fluorescence bleaches very quickly upon irradiation with high laser intensity. In contrast, fluorescent signals remain stable when enhanced with RFP-Booster_ATTOS94 (in-house comparison tests, www.chromotek.com).
PERSPECTIVES AND VISIONS

Being extremely small, stable and soluble, the single-chain camelid antibody fragments, V_{HH}s, offer special advantages to biomedical sciences. At ChromoTek GmbH we develop superior V_{HH}-based tools for a broad range of applications in basic academic research: from immunoprecipitation to super-resolution microscopy. However, V_{HH}s can serve basic science also in a number of other ways. For example, V_{HH}s proved to assist crystallization process and structural determination of difficult-to-crystallize proteins and complexes, such as Ribonuclease A, components of bacterial secretion system, or archael mechanosensitive channel [46]. These single-domain binders restrain highly dynamic proteins, stabilize intrinsic flexible protein regions and mask counterproductive protein surfaces to facilitate efficient crystal formation. Furthermore, V_{HH}s can be engineered to assist specific depletion of their target by targeting it to the ubiquitin-proteasome pathway, as

Figure 4 Confocal images of human cells expressing Chromobodies that highlight important cellular markers. Image acquisition was performed with Leica SPS confocal microscope (Leica Microsystems GmbH, Germany) either live or after fixation. Left to right:

HeLa cells stably expressing Cell Cycle Chromobody (in green, different cell cycle stages are visualized: G phase, early, mid and late S phases),

HeLa cells transiently transfected with DNMT1 Chromobody (in red),

HeLa cells stably expressing Lamin Chromobody (green, visualizes nuclear morphology),

HeLa cells transiently transfected with Actin Chromobody (in red). Cells were fixed and counterstained for DNA (in blue). Scale bar, 10 μm. (Unpublished in-house experiments).

Figure 5 Top: A time-series was acquired by live-cell confocal imaging of a stable U2OS cell line expressing Cell Cycle Chromobody (U2OS-CCC). Cells were imaged live for 10 h with UltraView VoX (PerkinElmer, MA, USA) spinning disc microscope. A typical cell progressing through different stages of cell cycle is shown. Left to right: G2 phase, mitosis, G1 phase, early and mid S phases. Scale bar, 10 μm.

Bottom: Results of automated cell cycle analysis of Cell Cycle Chromobody cells treated with three different cell cycle inhibitors. CCC cells were seeded into 96-well plates (Greiner μ-clear, Greiner bio one, Germany), incubated for 24 hours with several concentrations of reference substances, fixed and counterstained for DNA. Two independent experiments were performed in duplicates. Automated image acquisition and analyses were carried out with INCell Analyzer 1000 and INCell Workstation software (GE Healthcare, Germany) respectively. For cell classification, a decision tree was generated based on nuclear size, shape and intensity and on the amount and size of nuclear granules. Note increased number of cells arrested in early S phase upon aphidicolin treatment, mitosis arrest upon treatment with higher concentrations of nocodazole and increased number of dead cells when subjected to increasing concentrations of staurosporine.
elegantly shown in [47]. Therefore, to assist scientists in their specific needs of single-domain affinity reagents, in addition to our portfolio of V₃H₉-products outlined above we offer isolation of V₃H₉s for specific customer targets.

Further implementations of single-chain antibody fragments outside of basic science include healthcare and diagnostics (in vivo imaging, neutralization of toxins and eventual therapeutics), biotechnological purification (former BAC BV, Netherlands), crop protection (AgroSave, Belgium) and environmental/food safety (AbCelex, Canada).

REFERENCES


