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Supra-blot: an accurate and reliable assay for detecting target proteins with a synthetic host molecule-enzyme hybrid

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In accordance with the rapid increase in demand for selective and spatial chemical tagging, and accurate detection of proteins of interest, we develop a sensitive protein detection method, termed "Supra-blot" capitalizing on high-affinity host-guest interaction between cucurbit[7]uril (CB[7]) and adamantylammonium (AdA). The method can directly detect chemically tagged proteins without false-positive signals caused by endogenous biomolecules. Not only a single specific protein, but also spatially localized proteins in cells and animal tissues were labeled with AdA, and selectively detected by a host molecule-enzyme hybrid, CB[7]-conjugated horseradish peroxidase (CB[7]-HRP) generating amplified chemiluminescence signals. This study shows the great potential of Supra-blot for accurate and reliable detection of proteins of interest in cells.

In proteomics, chemical biology has facilitated the selective modification of proteins of interest with chemical tags that can be imaged, isolated, and quantified via their non-covalent and covalent binding partners.[1, 2] For example, biotin has been utilized extensively as a chemical tag, since it can be conjugated to target proteins using various chemical and enzymatic tagging methods and binds with high selectivity and specificity to its binding partners, such as streptavidin (SA), for which the binding affinity (K_a) is ~10^{13} M^{-1}.[3, 4] Although this ligand-protein binding pair is useful in proteomics, it suffers from intrinsic drawbacks, including the large size of the components involved, and undesired enzymatic degradation of SA under biological conditions.[5, 6] Moreover, the selective binding of SA to biotin-tagged proteins suffers interference from endogenously biotinylated proteins, thereby leading to false-positive protein detection.[7] It is even more serious when used for animal tissues.[8-10] Some of these issues have been resolved with bio-orthogonal covalent approaches using click coupling reactions.[11, 12] However, this covalent coupling system presents another challenge: the use of a relatively high concentration (a few 10 μM) of coupling partners.[13] When necessary, metal ions (*e.g.*, Cu(I)) are utilized for efficient covalent coupling owing to slow reaction kinetics, which often causes unwanted aggregation of proteins.[14, 15] Thus, to overcome these barriers, novel chemical tags that can be selectively conjugated to proteins using readily accessible chemicals and enzymatic methods and be rapidly recognized by small, stable and bio-orthogonal binding partners are required for accurate and reliable protein detection.

Recently, adamantyl-(AdA) and ferrocenyl-ammonium (FcA) derivatives have emerged as new chemical tags for protein labeling.[16-18] Like biotin, they are small and can be selectively conjugated to any proteins of interest in cells and animals via chemical and enzymatic methods. In addition, they have an extremely high binding affinity (K_a >10^{13} M^{-1}) with selectivity for their host molecule, cucurbit[7]uril (CB[7]).[19-23] Additionally, this synthetic system possess beneficial features including a smaller size (~1 kDa) than biotin-SA (~ 53 kDa), negligible interference of the binding properties by endogenous biomolecules, no decomposition under biological conditions, and no enzymatic degradation. However, due to the lack of a direct sensitive detection method, AdA- and FcA-labelled proteins have not been directly detected but indirectly through protein staining using primary and secondary antibodies (Abs) conjugated with an enzyme for typical western blotting, which necessitates tedious and laborious antibody treatments and washing steps.[18] In general, horseradish peroxidase (HRP), a radical-generating enzyme, amplifies chemiluminescent signals that enable detection of pico- to femto-gram quantities of proteins of interest by an immunochemical technique.[24]

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Combining the ultrahigh binding affinity of CB[7] to AdA and the signal amplification ability of HRP, we herein demonstrate a new supramolecular chemistry-based protein detection assay, termed 'Supra-blot', for the accurate, reliable and direct detection of AdA-labelled proteins using HRP conjugated with CB[7] (CB[7]-HRP as a host molecule-enzyme hybrid; Fig. 1e). Specific cellular proteins of interest labelled with AdA on a genetically designated site, such as AdA-labelled histone H3 in mammalian cells, was successfully detected by treatment of CB[7]-HRP with luminol and H_2O_2 on a blotted nitrocellulose (NC) membrane. Furthermore, spatially localized proteins labelled with AdA in cells and animal tissues by peroxidases, such as an engineered ascorbate enzyme (APEX) and antibody-conjugated HRP, were successfully detected by Supra-blot as distinct protein bands on a NC membrane. These demonstrate the great potential of Supra-blot as a new chemical tool for accurate and reliable detection of both specific proteins and proteins spatially localized in regions of interest in cells.

CB[7]-HRP was prepared by conjugation of monoamine-functionalized CB[7] (MA-CB[7])[25-27] to aldehyde-functionalized HRP (Act-HRP; EZ-Link Plus Activated Peroxidase, Thermo Scientific) to form imine between CB[7] and Act-HRP prior to reductive amination by treatment with $NaBH_3CN$ (Fig. S1, See ESI for the synthetic detail). The conjugation of CB[7] to HRP was confirmed by SDS-PAGE experiments. Unlike native HRP, the reacted HRP treated with AdA-conjugated fluorescein isothiocyanate (AdA-FITC,[26] a strong guest of CB[7]) exhibited a fluorescent band (Fig. S2c) at the same position as the CBB-stained band matched that of native HRP (Fig. S2b). It suggests that MA-CB[7] was successfully conjugated to HRP, and the portal of the conjugated CB[7] was open and thus able to form a stable complex with AdA-conjugated molecules such as AdA-FITC. In addition, matrix-assisted laser desorption/ionization time of flight mass spectrometry of CB[7]-HRP (Fig. S3) revealed conjugation of *ca.* two MA-CB[7] to Act-HRP. The enzyme activity of CB[7]-HRP measured by the *O*-dianisidine assay[28] was found to be 10.4 ± 0.3 unit mg^{-1}, indicating that HRP retained its enzymatic activity after conjugation to MA-CB[7].

Next, we examined the ability of CB[7]-HRP for selective detection of a specific protein labeled with AdA in a cell lysate. AdA-conjugated BSA (see ESI) was used as a model protein. A number of different protein bands from a mixture of AdA-BSA and cell lysate were well-resolved on the Ponceau S-stained NC membrane, depending on molecular weight (lanes 3, 4 in Fig. S5b). After treating the membrane with CB[7]-HRP, luminol, and H_2O_2 sequentially, we observed a distinct protein band at ~70 kDa with a chemiluminescence signal (lane 4 in Fig. S5c), the position of which matched that of AdA-BSA (lane 2 in Fig. S5c) obtained in the same experiment with phosphate buffered saline (PBS) instead of cell lysate. This indicates that CB[7]-HRP selectively detected AdA-BSA in a mixture of cellular proteins.

Encouraged by the blotting results of the model protein, we tested the Supra-blot method for selective detection of a specific cellular protein namely, histone H3 (H3) to verify its applicability. We utilized HEK293T cells expressing the H3-SNAP-FLAG fusion protein (Fig. 2) for selective labelling of the target protein with benzylguanine (BG)-conjugated AdA (Fig.

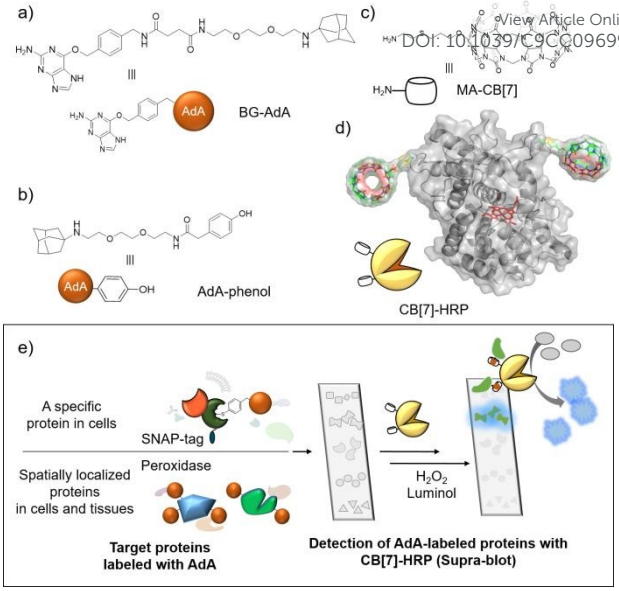

Fig. 1 Chemical structures a) BG-AdA and b) AdA-phenol and c) MA-CB[7], d) a hypothetical model of CB[7]-HRP and e) a scheme for detection of AdA-labeled proteins using Supra-blots.

1a). The protein consists of three different motifs: a target protein (H3), an expression verification tag (FLAG tag), and a self-labelling protein tag (SNAP-tag). The FLAG tag epitope (DYKDDDDK) can be selectively recognized by a corresponding anti-FLAG primary antibody.[29] The SNAP-tag is a mutant of *O*^{6}-alkylguanine-DNA alkyltransferase, which selectively labels chemical tags with BG moiety to itself.[30] Expression of the target protein in cells was confirmed by typical western blotting with anti-FLAG primary antibody and its corresponding HRP-conjugated secondary antibody (See ESI), showing a chemiluminescence band (~38 kDa) in the membrane from the cell lysate (Fig. S6). The cell lysate was treated with BG-AdA to label the H3-SNAP-FLAG with the AdA unit, and Supra-blot was performed on the membrane. The results showed that cellular proteins were well-resolved (Fig. 2b), with a single

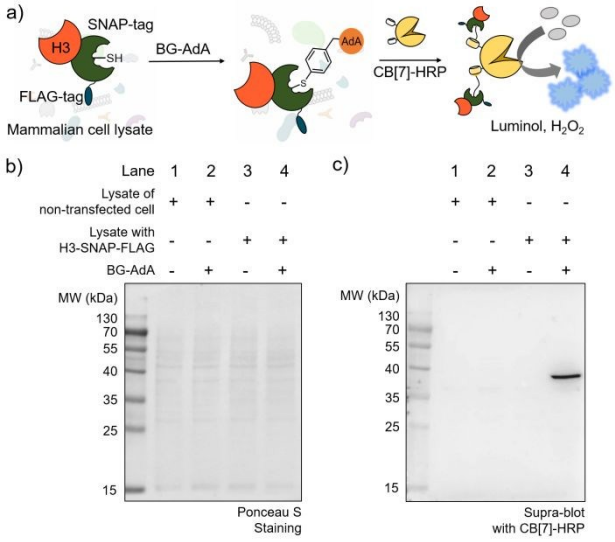

Fig. 2 a) Scheme for AdA labeling of the H3-SNAP-FLAG complex. b) Protein detection by Ponceau S and c) Supra-blotting

chemiluminescence band at the position of the target protein (~38 kDa) in the membrane (lane 4 in Fig. 2c). Additionally, no bands were detected in lysates of non-transfected cells (lanes 1, 2 in Fig. 2c) and cells not treated with BG-AdA (lane 3 in Fig. 2c), demonstrating that H3-SNAP-FLAG was successfully labelled with AdA, and only the AdA-labelled protein was detected by Supra-blot with CB[7]-HRP. Not limited to mammalian cells, a protein expressed in bacteria (*Escherichia coli*) labelled with a polyglycine-conjugaed AdA (GGG-AdA) using Sortase A enzyme was also selectively detected by Supra-blot (Fig. S7, See ESI for the detail). Interestingly, in the results of the model study with AdA-BSA (Fig. S5) and the studies on cells and bacteria (Fig. 2, S6, S7), bands for the target proteins were detected without the false-positives that are unavoidable in typical western blots with streptavidin-conjugated HRP (SA-HRP) when detecting proteins tagged with biotin.[7, 31] This indicates that Supra-blot not only can detect specific proteins, but is also a more accurate method than typical western blotting with SA-HRP.

To explore capabilities beyond detection of a single specific protein, Supra-blot was tested on a set of proteins spatially localized in a region of interest in a cell. For this investigation, an enzymatic proximity labelling method was employed using an engineered ascorbate peroxidase (APEX2) that generates radicals in situ upon H_2O_2 treatment for tagging AdA-phenol to neighbouring proteins close to APEX2.[3, 4] The enzyme can be targeted to different cellular locations via typical genetic engineering with designed plasmid DNA constructs. In this study, HEK293T cells were transfected with constructs harbouring V5 epitope (IPNPLLGLD)-tagged mitochondrial matrix-APEX2 (Matrix-V5-APEX2) or the APEX2 nuclear export signal (APEX2-V5-NES) to target APEX2 to the mitochondrial matrix or cytosol, respectively (Fig. 3).[32] Successful expression of Matrix-V5-APEX2 and APEX2-V5-NES was confirmed by detecting the target proteins via typical western blotting (Fig. S10) with anti-V5 antibody following the same experimental procedure used for H3-SNAP-FLAG (See ESI). For APEX proximity labelling of proteins with AdA-phenol, cells were incubated with AdA-phenol followed by H_2O_2 treatment for 1 min (See ESI). Supra-blot analysis of lysates of the cells clearly revealed different patterns of protein bands detected with CB[7]-HRP (Fig. 4a). These results reflected the ability of Supra-blot to recognize unique sets of proteins localized in subcellular regions of interest as distinct protein patterns. To verify the reliability of Supra-blot, we compared the Supra-blot results obtained from two types of cells transfected with different APEX-containing plasmid constructs, both of which targeted the same subcellular compartment (mitochondrial matrix). To validate this study, we additionally performed Supra-blot with cells expressing APEX2 conjugated to mitochondrial ribosomal protein L12 (MRPL12-V5-APEX2; MRPL12 is localized to the mitochondria matrix)[33] and compared the results with those obtained with cells expressing Matrix-V5-APEX2. As shown in lanes 2 and 3 of Fig. 4a, the protein band patterns were similar, demonstrating that Supra-blot can reliably detect a unique set of localized cellular proteins. Differences and similarities among Supra-blotted protein bands were more recognizable when the patterns were converted to a line scan analysis graph with molecular weight and staining intensity as x and y axes, respectively (Fig. 4b). The degree of overlap between graphs was represented as correlation values (0 to 1 for non-overlap and full overlap; Fig. 4c) using correlative functions (See ESI). For example, Matrix (Matrix-V5-APEX) vs. NES (NES-V5-APEX) and Matrix vs. MRPL12 (MRPL12-V5-APEX) were scored as 0.31 and 0.98, respectively, indicating that the Supra-blotted pattern of Matrix was more similar to that of MRPL12 than that of NES. Moreover, the correlation value between MRPL12 and Matrix was 0.98, indicating almost full overlap, confirming high similarity between the sets of detected proteins when APEX was located in the same region in cells. This suggests that Supra-blot constitutes a reliable technique for detection of spatially localized cellular proteins.

A typical western blot of SA-HRP-mediated detection of biotinylated proteins yielded unavoidable false-positive signals,

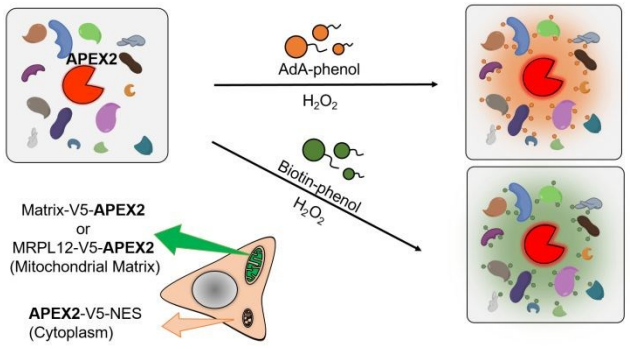

Fig. 3 Illustration of APEX2-mediated AdA (or biotin)-phenol labeling of spatially localized proteins in a cell.

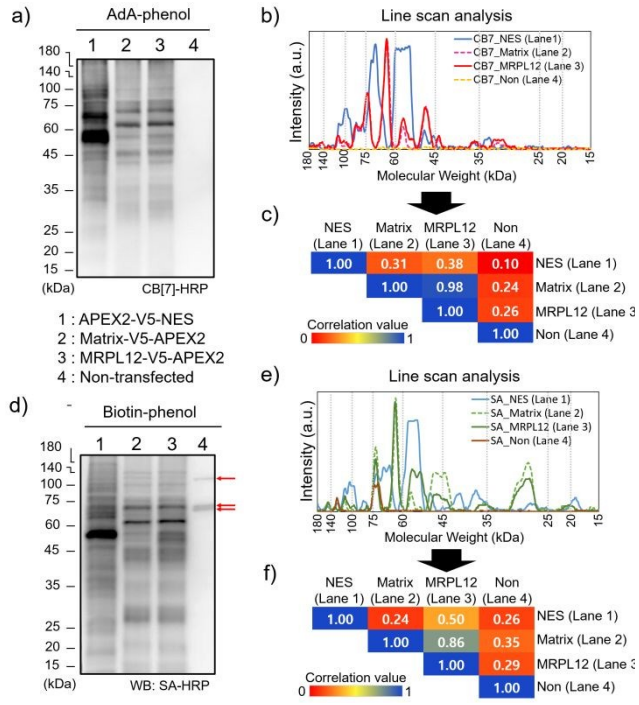

Fig. 4 a) Detection of AdA-phenol-labeled proteins by Supra-blot. b) Line scan analysis of proteins bands in a). c) Calculated correlation values for protein bands in b). d) Detection of biotin-phenol-labeled proteins by a western-blot with SA-HRP. e) Line scan analysis of proteins bands in d). f) Calculated correlation values for protein bands in e).

mostly due to endogenously biotinylated proteins (Fig. 4d-f),[5-10] as confirmed by the same experiments with biotin-phenol and SA-HRP instead of AdA-phenol and CB[7]-HRP. By contrast, Supra-blot did not suffer from false-positives, as verified using cells lacking APEX2 (lane 4 in Fig. 4a). Comparison of the correlation values between Supra-blot with CB[7]-HRP (Fig. 4c) and western blot with SA-HRP (Fig. 4f) revealed lower correlation for Supra-blot for different target sites (NES vs. non-transfected, and NES vs. MRPL12) and higher for the same target sites (MRPL12 vs. Matrix). This analysis strongly suggests that Supra-blot provides better accuracy and precision for detecting spatially localized cellular proteins with negligible false-positives. Similar results obtained with a different mammalian cell line, HeLa, (Fig. S11) confirmed the reliability of the Supra-blot for the detection of spatially localized proteins. In addition, we observed no false-positive protein bands when Supra-blot was used to analyse *Caenorhabditis elegans* (employed as a simple animal model) extracts, unlike the SA-HRP-based blot (Fig. S12). These results suggest that Supra-blot may be potentially applicable for the analysis of a wide variety of biological samples.

In summary, we reported a new protein detecting assay, termed Supra-blot using a host molecule-enzyme hybrid, CB[7]-HRP. Specific target proteins in cells and bacteria were labeled with AdA using SNAP-tag and Sortase A, respectively, and specifically detected in the form of a protein band on a NC membrane using CB[7]-based ultrastable and bio-orthogonal interaction. Furthermore, a set of proteins in regions of interest in cells and mouse brain tissues were also reliably detected after proximity labeling of proteins with AdA-phenol mediated by peroxidases such as APEX2 and antibody-conjugated HRP. Due to the use of synthetic high affinity host-guest interactions that are not interfered with biomolecules, Supra-blot provides accurate detection of target proteins without apparent false-positives. These findings demonstrate the great potential of Supra-blot as a reliable, precise and accurate tool for analysis of specific proteins and spatially localized proteins in cells.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

1 C. R. Jing and V. W. Cornish, *Acc. Chem. Res.*, 2011, **44**, 784-792.
2 Y. Kho, S. C. Kim, C. Jiang, D. Barma, S. W. Kwon, J. K. Cheng, J. Jaunbergs, C. Weinbaum, F. Tamanoi, J. Falck and Y. M. Zhao, *P. Natl. Acad. Sci. U.S.A.*, 2004, **101**, 12479-12484.
3 H. W. Rhee, P. Zou, N. D. Udeshi, J. D. Martell, V. K. Mootha, S. A. Carr and A. Y. Ting, *Science*, 2013, **339**, 1328-1331.
4 S. S. Lam, J. D. Martell, K. J. Kamer, T. J. Deerinck, M. H. Ellisman, V. K. Mootha and A. Y. Ting, *Nat. Methods*, 2015, **12**, 51-54.
5 G. Berntzen, O. H. Brekke, S. A. Mousavi, J. T. Andersen, T. E. Michaelsen, T. Berg, I. Sandlie and V. Lauvrak, *Protein Eng. Des. Sel.*, 2006, **19**, 121-128.
6 R. Jungmann, M. S. Avendano, J. B. Woehrstein, M. J. Dai, W. M. Shih and P. Yin, *Nat. Methods*, 2014, **11**, 313-318.
7 H. L. P. Tytgat, G. Schoofs, M. Driesen, P. Proost, E. J. M. Van Damme, J. Vanderleyden and S. Lebeer, *Microb. Biotechnol.*, 2015, **8**, 164-168.
8 M. Rusckowski, M. Fogarasi, B. Fritz and D. J. Hnatowich, *Nucl. Med. Biol.*, 1997, **24**, 263-268.
9 L. Horling, W. L. Neuhuber and M. Raab, *J. Neurosci. Methods*, 2012, **204**, 124-132.
10 G. Bussolati, P. Gugliotta, M. Volante, M. Pace and M. Papotti, *Histopathology*, 1997, **31**, 400-407.
11 V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem. Int. Ed.*, 2002, **41**, 2596-2599.
12 J. C. Jewett and C. R. Bertozzi, *Chem. Soc. Rev.*, 2010, **39**, 1272-1279.
13 C. Uttamapinant, A. Tangpeerachaikul, S. Grecian, S. Clarke, U. Singh, P. Slade, K. R. Gee and A. Y. Ting, *Angew. Chem. Int. Ed.*, 2012, **51**, 5852-5856.
14 K. Lang, L. Davis, J. Torres-Kolbus, C. J. Chou, A. Deiters and J. W. Chin, *Nat. Chem.*, 2012, **4**, 298-304.
15 V. Hong, N. F. Steinmetz, M. Manchester and M. G. Finn, *Bioconjugate Chem.*, 2010, **21**, 1912-1916.
16 D. A. Uhlenheuer, D. Wasserberg, C. Haase, H. D. Nguyen, J. H. Schenkel, J. Huskens, B. J. Ravoo, P. Jonkheijm and L. Brunsveld, *Chem. Eur. J.*, 2012, **18**, 6788-6794.
17 D. Wasserberg, D. A. Uhlenheuer, P. Neirynck, J. Cabanas-Danes, J. H. Schenkel, B. J. Ravoo, Q. An, J. Huskens, L. G. Milroy, L. Brunsveld and P. Jonkheijm, *Int. J. Mol. Sci.*, 2013, **14**, 4066-4080.
18 D. W. Lee, K. M. Park, M. Banerjee, S. H. Ha, T. Lee, K. Suh, S. Paul, H. Jung, J. Kim, N. Selvapalam, S. H. Ryu and K. Kim, *Nat. Chem.*, 2011, **3**, 154-159.
19 K. L. Kim, G. Sung, J. Sim, J. Murray, M. Li, A. Lee, A. Shrinidhi, K. M. Park and K. Kim, *Nat. Commun.*, 2018, **9**, 1712.
20 L. Isaacs, *Acc. Chem. Res.*, 2014, **47**, 2052-2062.
21 K. I. Assaf and W. M. Nau, *Chem. Soc. Rev.*, 2015, **44**, 394-418.
22 D. Shetty, J. K. Khedkar, K. M. Park and K. Kim, *Chem. Soc. Rev.*, 2015, **44**, 8747-8761.
23 S. J. Barrow, S. Kasera, M. J. Rowland, J. del Barrio and O. A. Scherman, *Chem. Rev.*, 2015, **115**, 12320-12406.
24 N. C. Veitch, *Phytochemistry*, 2004, **65**, 249-259.
25 Y. Ahn, Y. Jang, N. Selvapalam, G. Yun and K. Kim, *Angew. Chem. Int. Ed.*, 2013, **52**, 3140-3144.
26 K. M. Park, K. Baek, Y. H. Ko, A. Shrinidhi, J. Murray, W. H. Jang, K. H. Kim, J. S. Lee, J. Yoo, S. Kim and K. Kim, *Angew. Chem. Int. Ed.*, 2018, **57**, 3132-3136.
27 M. Li, A. Lee, K. L. Kim, J. Murray, A. Shrinidhi, G. Sung, K. M. Park and K. Kim, *Angew. Chem. Int. Ed.*, 2018, **57**, 2120-2125.
28 R. P. Gregory, *Biochem. J.*, 1966, **101**, 582-583.
29 A. Einhauer and A. Jungbauer, *J. Biochem. Bioph. Meth.*, 2001, **49**, 455-465.
30 K. Kolberg, C. Puettmann, A. Pardo, J. Fitting and S. Barth, *Curr. Pharm. Design*, 2013, **19**, 5406-5413.
31 T. C. Branon, J. A. Bosch, A. D. Sanchez, N. D. Udeshi, T. Svinkina, S. A. Carr, J. L. Feldman, N. Perrimon and A. Y. Ting, *Nat. Biotechnol.*, 2018, **36**, 880-887.
32 S. Y. Lee, M. G. Kang, J. S. Park, G. Lee, A. Y. Ting and H. W. Rhee, *Cell Rep.*, 2016, **15**, 1837-1847.
33 Y. V. Surovtseva, T. E. Shutt, J. Cotney, H. Cimen, S. Y. Chen, E. C. Koc and G. S. Shadel, *Proc. Natl. Acad. Sci. U.S.A.*, 2011, **108**, 17921-17926.

