

1 **Fluorescent amino acids as versatile building blocks for chemical biology**

2
3 Zhiming Cheng¹, Erkin Kuru², Amit Sachdeva³, Marc Vendrell^{1†}

4
5 ¹Centre for Inflammation Research, University of Edinburgh, Edinburgh, UK

6 ²Department of Genetics, Harvard Medical School, Boston, USA

7 ³School of Chemistry, University of East Anglia, Norwich, UK

8
9 †email: marc.vendrell@ed.ac.uk

10
11 **Abstract** | Fluorophores have transformed the way we study biological systems, enabling non-
12 invasive studies in cells and intact organisms, which increase our understanding of complex
13 processes at the molecular level. Fluorescent amino acids (FIAAs) have become an essential
14 chemical tool because they can be used to construct fluorescent macromolecules, such as
15 peptides and proteins, without disrupting their native biomolecular properties. Fluorescent and
16 fluorogenic amino acids with unique photophysical properties have been designed for tracking
17 protein–protein interactions *in situ* or imaging nanoscopic events in real-time with high spatial
18 resolution. In this Review, we discuss advances in the design and synthesis of FIAAs and how
19 they have contributed to the field of chemical biology in the past 10 years. Important areas of
20 research that we review include novel methodologies to synthesize building blocks with
21 tunable spectral properties, their integration into peptide and protein scaffolds using site-
22 specific genetic encoding and bio-orthogonal approaches, and their application to design novel
23 artificial proteins as well as to investigate biological processes in cells by means of optical
24 imaging.

25 26 **[H1] Introduction**

27
28 Fluorescence-based techniques have revolutionized our understanding of biological
29 processes, as they allow researchers to examine the localization, trafficking and activity of
30 biomolecules in cells, tissues and even whole intact organisms in a non-invasive manner.¹⁻⁴
31 Many natural peptides and proteins have key roles in various biological processes; however,
32 directly visualizing these molecules is hampered by the fact they generally do not exhibit strong
33 fluorescence emission. Whereas this low ‘background’ fluorescence boosts the sensitivity of
34 spectroscopy and imaging experiments, its weak levels highlight the need for labelling

35 strategies that can facilitate the detection of these macromolecules. Fluorescent labelling of
36 peptides and proteins has been successfully used in numerous ways, from the widely used
37 expression of fluorescent protein fusions (such as green fluorescent protein⁵ (GFP) and
38 subsequent derivatives^{6,7}) to the fusion of proteins with self-labelling tags (such as SNAP,
39 CLIP and HALO tags⁸⁻¹⁰) or modification with specific peptide sequences that can bind to
40 small molecules with high affinity (such as FLASH and ReASH^{11,12}). All of these approaches
41 have led to remarkable biological discoveries, but they typically involve a large structural
42 modification of the peptides or proteins of interest, which can alter their biomolecular
43 properties.

44 Non-natural fluorescent amino acids (FIAAs) have been developed as chemical
45 alternatives for the derivatization of peptides and proteins in a less disruptive manner. Owing
46 to their small size and similarity to the natural residues found in polypeptides, they can be used
47 to fluorescently label macromolecules while retaining their overall function with minimal
48 perturbation of the native protein structure. The toolbox of FIAAs expanded considerably in
49 the early 2000s, partly due to the development of numerous FIAAs¹³ with unique optical
50 properties, including environmental sensitivity¹⁴⁻¹⁶, responsive to metal chelation¹⁷, tunable
51 fluorescence emission and prolonged lifetime¹⁸). Importantly, these modular building blocks
52 can be introduced at specific sites in small peptides using solid-phase peptide synthesis¹⁹
53 (SPPS) or into larger proteins by genetic encoding²⁰ to generate fluorescent, native-like
54 biomolecules, enabling biological experiments that would be intractable by other means,
55 including analysis of protein conformational changes, monitoring of protein–protein
56 interactions and activity studies in live cells.

57 Over the past 10 years, a substantial increase in the number of versatile synthetic
58 strategies (such as multicomponent reactions²¹, metal-catalysed reactions²², light-induced
59 transformations²³ and bioorthogonal chemistry²⁴) to fine-tune the chemical structure of
60 fluorophores²⁵ and natural building blocks has accelerated the design and preparation of new
61 FIAAs. In this Review, we provide an overview of different chemical transformations that have
62 been used to generate novel building blocks with bespoke fluorescent properties and to
63 facilitate their site-specific integration into peptides and proteins. We also discuss how the
64 range of applications of FIAAs has extended to include many biological assays *in vitro*, in cells
65 and *in vivo*, which has been facilitated by a rapid expansion in the use of spectroscopic and
66 imaging-based techniques. Finally, we review the versatility of FIAAs from the point of view
67 of an end-user and present several examples of the application of FIAAs in chemical biology,

68 including molecular recognition studies, live-cell imaging and applications in synthetic biology
69 and microbiology (**Figure 1**).
70

71 [H1] Synthesis of FIAAs

72

73 [H2] Fluorescent analogues of natural amino acids

74 Three canonical amino acids, (tryptophan, tyrosine and phenylalanine, are fluorescent
75 and thus might have potential as naturally-occurring fluorophores²⁶; however, their optical
76 properties (such as excitation and emission wavelengths, brightness and photostability) are
77 suboptimal for most biological assays. Tryptophan, the most commonly used fluorophore
78 among natural amino acids, absorbs and emits in the ultraviolet range (absorption wavelength
79 (λ_{abs}) = 280 nm; emission wavelength (λ_{em}) = 346 nm) and its fluorescence quantum yield is
80 ~20%²⁷.

81 Initial attempts to improve the fluorescent properties of tryptophan yielded
82 azatryptophans as potential isosteric substitutes in proteins²⁸. Although 4-azatryptophans have
83 a longer emission wavelength (λ_{em} = 425 nm) than tryptophan and a large Stokes shift
84 (>130 nm), they are typically less bright than tryptophan. Alternative approaches have involved
85 the preparation of cyanotryptophans, which have improved fluorescence quantum yields
86 (approaching ~50%²⁹) owing to the incorporation of the strong electron withdrawing cyano
87 group. Cyanotryptophans can be synthesized from commercially available cyanoindoles, and
88 the red-shifted emission wavelength of 6-cyanotryptophan (**1a**) (λ_{em} = 370 nm) enabled its use
89 for Förster resonance energy transfer (FRET) experiments to study protein–DNA interactions.
90 Subsequent studies identified 4-cyanotryptophan (**1b**) as an amino acid with improved optical
91 properties³⁰. A key step in the synthesis of 4-cyanotryptophan is the palladium (Pd)-catalysed
92 incorporation of the cyano group at position 4 of L-tryptophan. Remarkably, the emission
93 maximum of 4-cyanotryptophan is in the blue visible region (λ_{em} = 405 nm) and it has a high
94 quantum yield (~80%), good photostability and a long fluorescence lifetime (~13.7 ns), which
95 makes it an interesting building block for spectroscopic and microscopic measurements of
96 proteins, such as the study of peptide–membrane interactions³¹. The structural similarity of
97 cyanotryptophans to tryptophan also makes them an attractive platform to explore enzyme-
98 based reactions, facilitating the preparation of enantiopure tryptophan analogues by
99 derivatization at multiple aromatic positions, using, for example, tryptophan synthase S
100 (TrpS)³² or variants of its β -subunit (TrpB)³³.

101

102 Extended tryptophan analogues have been also designed by conjugation of homocyclic
103 and heterocyclic systems to the indole moiety, and include tricyclic tryptophan analogues (**2**)

104 derived from pyrrolo(iso)quinolines³⁴, tryptophan derivatives obtained by hydrogen-mediated
105 coupling of 1,2,3-triazoles to indoles³⁵ (**3a**) and a series of 2-arylated tryptophans (**3b**) obtained
106 by Pd-mediated C–H functionalization³⁶. Cross-coupling reactions (such as Suzuki–Miyaura
107 couplings) have also been effective for preparing C4-substituted tryptophans³⁷, although the
108 effect of these substitutions on the optical properties of tryptophan has not been reported. Of
109 note, extended tryptophans have a red-shifted emission wavelength ($\lambda_{em.} = 420\text{--}480\text{ nm}$)³⁴⁻³⁶
110 and some have been incorporated in *Escherichia coli* dihydrofolate reductase (DHFR) using *in*
111 *vitro* translation, resulting in minimal disruption of DHFR function³⁸ and thus demonstrating
112 the potential of these molecules for monitoring conformational changes in macromolecular
113 structures.

114

115 **[H2] Non-natural FIAAs**

116 Despite the remarkable progress in the synthesis of fluorescent analogues of natural
117 amino acids, numerous non-natural FIAAs with different optical properties have been
118 developed (**Figure 2**). The synthetic approaches to generate non-natural FIAAs can be broadly
119 categorized into those that involve appending fluorescent moieties to amino acids and those
120 that involve *de novo* construction of amino acids with integrated chromophores (**Figure 2**).

121 **[H3] Palladium catalysis.** Pd-catalysed reactions are one of the most commonly used
122 approaches for the synthesis of non-natural FIAAs. Biphenyl derivatives of phenylalanine (**4a**)
123 have been prepared by Suzuki coupling of biphenyl boronic acids with iodophenylalanine^{39,40}.
124 Their emission maximum is red-shifted relative to that of phenylalanine (~340 nm versus 282
125 nm, respectively) and they have high quantum yields (~70%). Suzuki couplings have also been
126 used in the synthesis of fluorescent D-amino acids (FDAAs), including 4-acetamidobiphenyl
127 groups (**4b**), which have a slightly longer emission wavelength (~380 nm)⁴¹. Heck reactions
128 have been employed to extend the π -conjugation of tyrosine⁴², yielding styryl-containing
129 tyrosine analogues (**5**) with tunable fluorescence quantum yields (from 10% to 90%) and
130 emission maxima that cover the entire visible spectrum ($\lambda_{em.} = 400\text{--}800\text{ nm}$). Sonogashira
131 coupling reactions have been used to append aryl-containing acetylene derivatives to
132 benzoxazol-5-yl-alanine to produce FIAAs (**6**) with a high quantum yield and emission maxima
133 in ultraviolet and blue wavelengths ($\lambda_{em.} = 340\text{--}450\text{ nm}$)⁴³. Similarly, xanthone structures ($\lambda_{em.} = 380\text{ nm}$)
134 have been incorporated into the alanine core by Negishi couplings⁴⁴ (**7a**). The
135 high efficiency of Pd-catalysed couplings (such as the Buchwald–Hartwig reaction) has also

136 facilitated the synthetic optimization of widely used non-natural FIAAs, such as acridon-2-
137 ylalanine (ACD) (**7b**), which is one of the brightest blue-emitting FIAAs reported to date⁴⁵.

138

139 **[H3] C–H activation.** C–H activation is another synthetic methodology that has contributed
140 substantially to the development of non-natural FIAAs, particularly for the construction of new
141 tryptophan-based amino acids. Trp-BODIPY (**8a**) was the first BODIPY-containing tryptophan
142 analogue, which has excellent photophysical properties ($\lambda_{\text{abs.}} = 500 \text{ nm}$; $\lambda_{\text{em.}} = 530 \text{ nm}$) and is
143 compatible with SPPS⁴⁶. A similar approach was used to prepare Trp(redBODIPY) (**8b**), which
144 has a red-shifted maximum excitation and emission wavelength ($\lambda_{\text{abs.}} = 560 \text{ nm}$, $\lambda_{\text{em.}} =$
145 590 nm)⁴⁷, and also was extended for the synthesis of styryl-derivatized tryptophan analogues
146 by C–H olefination at the C2 position of the indole moiety⁴⁸. Given that the extension of π -
147 conjugation systems often leads to improved photophysical properties, as described above with
148 Heck reactions⁴², this strategy has the potential to yield FIAAs with novel optical properties.
149 Pd-catalysed sp^3 C–H activation has also been used to append green and red-fluorescent
150 BODIPY dyes to the side chains of alanine and phenylalanine (**9**) ($\lambda_{\text{em.}} = 510\text{--}625 \text{ nm}$)⁴⁹ and to
151 the C2 position of tryptophan by a novel ruthenium(II)-catalysed C–H alkylation⁵⁰.

152 **[H3] Copper and rhodium catalysis.** Further examples of metal-catalysed reactions include
153 the Ullmann-type reaction for coupling the fluorophore pyrene to the phenol group of tyrosine
154 (**10a**) using copper catalysis ($\lambda_{\text{em.}} = 390 \text{ nm}$; quantum yield $\sim 40\%$)⁵¹, or the widely used copper-
155 catalysed azide–alkyne cycloaddition (CuAAC) to conjugate standard fluorophores, such as
156 coumarins ($\lambda_{\text{em.}} = 380 \text{ nm}$), benzothiadiazoles ($\lambda_{\text{em.}} = 470 \text{ nm}$), fluorescein ($\lambda_{\text{em.}} = 510 \text{ nm}$),
157 dansyl ($\lambda_{\text{em.}} = 520 \text{ nm}$), nitrobenzodioxazoles (NBDs; $\lambda_{\text{em.}} = 530 \text{ nm}$), and naphthalimides ($\lambda_{\text{em.}} =$
158 550 nm), to the side chains of different amino acids^{52–54} (**11**). Fluorescent arylalanines (**12**)
159 ($\lambda_{\text{em.}} = 400 \text{ nm}$; quantum yield $\sim 60\%$) have been synthesized via the formation of C–C bonds
160 between several polyaromatic hydrocarbons and alanine using rhodium catalysis⁵⁵.

161 **[H3] Subheading.** Conventional reactions involving the formation of amides^{56–61} (**13**),
162 carbamates⁶² (**14**) or thioureas^{57,58,63} (**15**) have also been explored for modifying the side chains
163 of different amino acids with various fluorophores. Other reactions include Knoevenagel-like
164 condensation to derivatize aminodicarboxylate β -ketoesters with pyrene⁶⁴ (**16**) or synthesis of
165 a green-fluorescent FIAA (**17**) ($\lambda_{\text{em.}} = 540 \text{ nm}$) by coupling 4-dimethylamino-1-naphthalenes to
166 methyl (2*S*)-5-(dimethoxyphosphoryl)-4-oxo-2-(tritylamino)-pentanoate via a Horner–
167 Wadsworth–Emmons reaction⁶⁵. The simplicity of some reactions and the availability of
168 properly derivatized fluorescent building blocks has also facilitated the preparation of

169 collections of FIAAs. For example, using Michael addition, a small library of D-cysteine FIAAs
170 (18) with thiol-containing fluorophores was generated⁶⁶, and maleimide-4-aminophthalimide⁶⁷
171 (19) was coupled to L-cysteine. Furthermore, the Fukuyama–Mitsunobu reaction was used to
172 couple 6-acyl-2-naphthylamine to L-serine to produce 3-(6-acetylnaphthalen-2-ylamino)-2-
173 aminopropanoic acid (ANAP) (20), a building block with remarkable optical properties ($\lambda_{em.} =$
174 490 nm, quantum yield ~50%) that is widely used for fluorescent labelling of proteins⁶⁸. NBD-
175 like fluorophores have been conjugated by a nucleophilic aromatic substitution (S_{NAr}) reaction
176 to nucleophilic groups (21) in amino acid side chains (such as 3-amino-alanine)^{58,69}, whereas
177 phospholyl(borane) amino acids (22) have been produced by nucleophilic substitution of
178 fluorescent phospholide anions with iodo-derivatized amino acids⁷⁰, which enabled the
179 synthesis of a range of phospholyl derivatives with fluorescence properties covering a broad
180 spectrum of emission maxima wavelengths from ultraviolet to green ($\lambda_{em.} = 340\text{--}530$ nm).

181 **[H3] Flavone-based FIAAs.** Alternative synthetic approaches have involved the *de novo*
182 design of FIAAs by building fluorophores into an amino acid structure (Figure 3). For
183 example, flavone-based amino acids (23) (Figure 3a) are produced by constructing the
184 fluorophore 3-hydroxychromone from tyrosine by aldol condensation, followed by oxidative
185 cyclization (for example, the Algar–Flynn–Oyamada reaction)⁷¹⁻⁷³. Of note, flavone FIAAs
186 show excited-state intramolecular proton transfer (ESIPT) fluorescence, and they only
187 minimally perturb the structure of peptides owing to their small size. Another FIAA with a
188 built-in fluorophore, benzoacridone-modified alanine, was synthesized by an Ullmann-type
189 coupling between 3-chloro-2-naphthoic acid and aminophenylalanine, followed by cyclization
190 of 2-aminonaphthoic acid to yield green-fluorescent benzoacridone amino acid ($\lambda_{em.} = 500\text{--}550$
191 nm, quantum yield ~50%)⁷⁴. Furthermore, non-natural FIAAs have been synthesised by
192 building aryl-substituted pyridyl, pyrazole, benzotriazole and pyrazoloquinazoline (24)
193 heterocyclic chromophores on aspartic acid and asparagine structures ($\lambda_{em.} = 348\text{--}460$ nm)⁷⁵⁻⁷⁸
194 (Figure 3b).

195 **[H3] Coumarin based FIAAs.** The simplicity of the coumarin scaffold (that is, 1-benzopyran-
196 2-one) has facilitated the preparation of FIAAs integrating this fluorescent structure. Coumarin-
197 based FIAAs can be prepared by Pechmann condensation, either between an amino acid
198 containing a β -ketoester unit and a phenol derivative or between tyrosine and an ethyl
199 acetoacetate derivative^{79,80}. Alternatively, 7-(hydroxycoumarin-4-yl)ethylglycine is prepared
200 by Pd-catalysed cross coupling between 2,4-dimethoxyphenylboronic acid and amino acids
201 containing β -ketoester groups⁸¹. The brightness of coumarin-based FIAAs, with quantum

202 yields reaching 70%, together with their fairly small size, have made them excellent building
203 blocks for multiple applications in chemical biology.

204

205 **[H1] Bioactive fluorescent peptides**

206

207 **[H2] Studying biomolecular interactions**

208 Peptides are excellent scaffolds for biological studies, as they can be used to monitor
209 highly specific molecular interactions with a broad range of biomolecules. Fluorescent peptides
210 have traditionally been synthesized by coupling fluorophores to reactive groups (initially to
211 amines, carboxylic acids and thiols⁸², but now also to imidazoles⁸³ or phenols⁸⁴) on the side
212 chains of amino acids or in conveniently placed spacers. However, in some cases, this synthetic
213 approach can alter the native conformation of the peptide or its biological properties (such as
214 functional activity, binding affinity and/or subcellular localization)⁸⁵. One synthetic alternative
215 to minimize the effect of fluorescent labelling is to embed FIAAs within the sequence of the
216 peptides (to avoid the modification of polar groups, such as amines, carboxylic acids, and
217 thiols, which may be crucial for its bioactivity) or to optimize suitable spacers⁸⁶. These
218 methodologies rely on the chemical robustness and flexibility of SPPS, which enables the
219 efficient preparation of highly diverse peptides, including those that incorporate non-natural
220 FIAAs⁸⁷.

221 **[H3] Monitoring protein–protein and protein–DNA interactions.** Most biological
222 processes rely on the interaction of proteins with their binding partners. In this context,
223 fluorescent peptides have been developed to better understand protein-based interactions at the
224 molecular level and to help in the discovery of new drugs to modulate them. For example,
225 flavone-based solvatochromic FIAAs were incorporated at specific sites in a peptide sequence
226 from the human immunodeficiency virus-1 (HIV-1) nucleocapsid protein during SPPS^{71,73}.
227 These FIAAs have hydration-sensitive dual emission maxima ($\lambda_{em.} = 430$ nm and 530 nm),
228 which allowed the interaction between the nucleocapsid protein and nucleic acids to be
229 monitored to gain insights into the HIV-1 viral life cycle (**Figure 4a**). Other fluorescent
230 structures have been explored as solvatochromic amino acids with high sensitivity to their
231 surrounding microenvironments. For example, phthalimide-based amino acids have been
232 integrated into peptide structures to study dynamic protein–protein interactions, as in the case
233 of the PDZ domain⁸⁸ (**Figure 4b**), one of the most abundant protein interaction domains in
234 eukaryotes, or calmodulin⁸⁹, a major calcium-signal transduction protein. Subsequently, novel

235 fluorogenic peptides have been used as conformational reporters of calmodulin activity^{90,91}. In
236 this case, calmodulin-binding peptides were first identified by *in vitro* selection using tRNA
237 carrying an NBD-based amino acid⁹⁰, which was later replaced with a 4-*N,N*-dimethylamino-
238 1,8-naphthalimide amino acid that has a similar emission wavelength ($\lambda_{em.}= 530$ nm) but a
239 remarkably enhanced fluorogenic behaviour (up to 100-fold fluorescence increase after binding
240 to calmodulin)⁹¹. Furthermore, a fluorogenic peptide containing the environmentally-sensitive
241 1,5-naphthyridin-2(1*H*)-one amino acid has been used to obtain ratiometric fluorescence
242 measurements at two emission wavelengths (370 nm and 480 nm) in response to
243 conformational changes in calmodulin⁶¹.

244 **[H3] Monitoring peptide-membrane interactions.** Fluorogenic peptides can also be designed
245 to study peptide–membrane interactions, which are crucial for peptide-based therapeutics and
246 transfection reagents. Cationic peptides, including antimicrobial peptides, are suitable for this
247 purpose, given their high membrane-binding activity. Fluorogenic analogues of melittin, an
248 active component of honey bee venom, have been used to study the orientation of melittin in
249 lipid-rich membranes, revealing details about the varying insertion depths of different residues
250 (that is, flavone FIAAs) and that melittin is oriented parallel to the surface of cell membranes⁷².
251 Similar approaches have been used to study the membrane binding of neuropeptides, which
252 function as messengers between neurons. Analysis of the fluorescence of two analogues of the
253 insect neuropeptide heliocinin I (containing the FIAAs β -(4'-hydroxy-'-benzoyl) (ALB; $\lambda_{em.}=$
254 360 nm) or 6-dimethyl-amino-2-acyl-naphthalene (ALD; $\lambda_{em.}= 530$ nm)) interacting with
255 dodecylphosphatidylcholine micelles was used to develop a model of heliocinin I binding to
256 membranes⁹². Furthermore, Fmoc-L-4-cyanotryptophan was incorporated in the membrane-
257 interacting pH-(low) insertion peptide (pHLIP) during SPPS to measure peptide–membrane
258 binding constants using FRET experiments with the universal membrane stain DiO (ref.³¹)
259 **(Figure 4c).**

260 **[H3] Analytical applications.** Fluorogenic peptides have also been used as analytical tools for
261 the fluorometric detection of specific biomolecules. For example, peptide aptamers containing
262 an environmentally-sensitive NBD FIAA were evolved *in vitro* using ribosome display to
263 identify those that detect verotoxin, a protein that is produced by *E. coli* and is associated with
264 haemolytic uraemic syndrome⁹³. Furthermore, a 7-azatryptophan-containing peptide has been
265 used for detection of haeme levels in cells⁹⁴ using FRET-based measurements based on the
266 overlap between the emission spectrum of 7-azatryptophan ($\lambda_{em.}= 400$ nm) and the absorbance
267 of the haeme group. Peptides containing other fluorophores have also been used in FRET

268 assays to measure protease activity⁶⁹, including benzoacridone amino acids to detect active
269 caspase 3 (ref.⁷⁴), a key enzyme in the apoptotic pathway.

270

271 [H2] Live-cell optical imaging

272

273 Optical microscopy has allowed researchers to examine the movement of biomolecules
274 inside the cell with high spatial and temporal resolution. Fluorescent peptides are perfectly
275 suited for optical imaging, as they can target specific proteins in cells and also contain optical
276 reporters (that is, FIAAs) that are easily detected using fluorescence microscopes⁹⁵. To speed
277 up the detection of pulmonary infections, fluorogenic Trp-BODIPY-containing antimicrobial
278 peptides were designed for visualization of the deadly fungal pathogen *Aspergillus fumigatus*
279 in *ex vivo* human lung tissue⁹⁶ (**Figure 4d**). This residue is an optimal reporter as it retains the
280 molecular recognition of the native tryptophan and also provides a fluorogenic readout on
281 specific binding to fungal cells. Other fluorogenic antimicrobial peptides have enabled *in situ*
282 detection of bacteria in explanted whole human lungs using real-time optical
283 endomicroscopy⁹⁷. The versatility of Trp-BODIPY has led to the preparation of peptide
284 imaging agents for additional targets. For example, a fluorogenic cyclic peptide that mimics
285 milk fat globule EGF factor 8 (MFGE8; also known as lactadherin), a protein with high binding
286 affinity for phosphatidylserine, has been used to image the release of apoptotic bodies into the
287 extracellular space during programmed cell death⁹⁸. A red analogue, Trp-redBODIPY, has
288 been used to prepare fluorogenic cyclic peptides that bind to keratin 1 (KRT1). These peptides
289 were synthesized using Pd-catalysed C–H activation during SPPS, a labelling approach that is
290 less disruptive than conventional lysine derivatization and enabled imaging studies to
291 understand the interaction between immune cells and cancer cells in aggressive breast cancer
292 tumours⁴⁷.

293 Imaging studies can be also performed to acquire functional readouts from cells. For
294 example, a fluorogenic coumarin-based amino acid ($\lambda_{em.} = 460$ nm), the first phosphotyrosine-
295 mimetic FIAA, was used to report the endogenous phosphatase activity of protein tyrosine
296 phosphatases in live cells⁹⁹ (**Figure 4e**). The wash-free imaging capabilities of fluorogenic
297 peptides makes them valuable tools for applications where samples must be rapidly analysed
298 with few processing steps, such as in clinical diagnostics or metabolic engineering. For
299 example, NBD-aminophenylalanine (NBD-amPhe) has been used as a building block for the
300 preparation of epithelial cell adhesion molecule (EpCAM)-binding peptides that can detect

301 circulating tumour cells in the blood¹⁰⁰. Furthermore, fluorogenic peptide aptamers have been
302 used to image in real time the production of paramylon (a carbohydrate granule similar to
303 starch) by the microalga *Euglena gracilis*, thereby opening new opportunities in metabolic
304 engineering¹⁰¹.

305 **[H1] Fluorescent D-amino acids in bacteria**

306 **[H3] Visualizing bacterial cell wall growth**

307 Bacterial cells contain two main types of macromolecules that are assembled from
308 amino acids — namely, proteins, which consist of L-amino acids, and peptidoglycans, which
309 contain both L-amino acids and D-amino acids. Peptidoglycans are complex polymers that form
310 the cell wall of bacteria and coordinate multiple important processes, including cell growth and
311 division¹⁰². Their biological importance has made peptidoglycans the target of many
312 antibiotics, and peptidoglycan biosynthesis has become an area of extensive research for the
313 discovery of new antimicrobial drugs¹⁰³. The discovery of fluorescent D-amino acids (FDAAs)
314 has provided researchers in this field with non-invasive probes to visualize key steps during
315 the peptidoglycan biosynthesis in bacterial cells^{104,105}.

316 Taking advantage of the inherent promiscuity of taxonomically-diverse bacterial to
317 incorporate D-amino acids as peptidoglycan metabolites¹⁰⁶⁻¹⁰⁸, modified D-amino acids have
318 been used to specifically label sites of new cell wall growth in real time^{109,110}. Structurally-
319 diverse FDAAs, including different reporters of varying size and optical properties, have been
320 synthesised¹¹¹ (**Figure 5a**). For example, ethynyl-D-alanine (EDA), azido-D-alanine (ADA)
321 and dipeptides, such as ethynyl-D-alanyl-D-alanine (EDA-DA), have been used to label
322 peptidoglycans in different species of bacteria by cycloadditions with fluorophores. These
323 bioorthogonal approaches require two reaction steps that can compromise cell viability;
324 however, these 'clickable' D-amino acids are small and thus are minimally disruptive and
325 generally compatible with multiple reporters. For example, the small size and biological
326 stability of AlexaFluor 488-conjugated EDA-DA enabled the demonstration that
327 peptidoglycans are present in the cell wall of the human pathogen *Chlamydia trachomatis*¹¹¹.
328 Alternatively, FDAAs have been employed for single-step labelling of peptidoglycans during
329 their biosynthesis in bacterial cells, for example, by the metabolic incorporation of FDAAs into
330 new peptidoglycans to label bacteria with minimal perturbation of the cells and simpler
331 protocols (that is, fewer washing steps). The good selectivity for bacteria and straightforward
332 application of FDAAs has opened multiple research avenues in different biological

333 applications, ranging from *in vivo* analysis of the gut microbiota in mice¹¹² and in human fecal
334 samples¹¹³ to light-induced strategies for killing pathogenic bacteria¹¹⁴.

335 [H3] Multi-colour and multiplexed imaging

336 The simple, modular design of FDAAs and their convenient synthesis by condensation
337 of standard fluorophores to D-amino acids (such as D-lysine and D-diaminopropionic acid), has
338 not only facilitated their diversification with a broad range of fluorophores but has also enabled
339 imaging experiments to answer questions about bacterial growth and division with high spatial
340 resolution. For example, live-cell imaging experiments in which three differently-coloured
341 FDAAs, NBD-3-amino-D-alanine (NADA; $\lambda_{em} = 538$ nm), tetramethylrhodamine-D-lysine
342 (TDL; $\lambda_{em} = 565$ nm) and 7-hydroxycoumarin-D-alanine (HADA; $\lambda_{em} = 450$ nm), were time-
343 pulsed to record the chronological steps of cell wall growth in *Streptomyces venezuelae* (**Figure**
344 **5b**). This approach has also been used to monitor the production of peptidoglycans in single
345 bacterial cells in different environments, (such as during antibiotic treatment¹¹⁵ or
346 transplantation¹⁰⁹, and to track dynamic interactions between different bacterial species. In a
347 notable example of the latter, the predation cycle of *Bdellovibrio bacteriovorus* (a small
348 bacterial species that preys on larger bacteria) and its prey *E. coli* was studied by super-
349 resolution imaging of the multiplexed FDAAs HADA, BODIPY-3-amino-D-alanine (BADA;
350 $\lambda_{em} = 512$ nm) and tetramethylrhodamine-3-amino-D-alanine (TADA; $\lambda_{em} = 565$ nm)¹¹⁶.

351 Numerous studies have investigated the mechanism by which small and large FDAAs
352 can be incorporated into peptidoglycans of multiple bacterial species. Most FDAAs behave as
353 substrate analogues of solvent-accessible, periplasmic peptidoglycan transpeptidases, which
354 are responsible for the assembly of the cell wall material during growth and division^{117,118}. For
355 example, HADA was used as an activity-based probe of peptidoglycan transpeptidases to
356 monitor the spatial distribution of new cell wall versus old cell wall in live *Bacillus subtilis*¹¹⁷.
357 Owing to their multiplexing capabilities, FDAAs can also be combined with fluorescently-
358 tagged peptidoglycan biosynthetic proteins for multi-colour imaging studies. For example,
359 metabolic incorporation of HADA was combined with the localization of key proteins in cell
360 division (for example, FtsZ) in evolutionarily distinct rod-shaped bacterial species (such as *B.*
361 *subtilis*¹¹⁹ and *E. coli*¹²⁰), and MreB was colocalized with an EDA-DA-labelled peptidoglycan
362 ring during cell division in *Chlamydia trachomatis*¹²¹. Of note, these studies support a new
363 paradigm in which bacterial division progresses directionally around the cell, in contrast to the
364 previously described model of uniform cell division.

365 [H3] Tools for high-throughput screenings

366 However, macromolecular labelling of peptidoglycan structures with conventional
367 FDAAs has constraints on temporal resolution due to the washing steps that are needed to
368 reduce the background fluorescence from unincorporated free amino acids. These technical
369 limitations prompted the design of a series of FDAAs that are non-fluorescent in low viscosity
370 environments (that is, when the FDAA is moving freely in aqueous media) and fluoresce
371 strongly when their intramolecular conformation is constrained (for example, after
372 incorporation into peptidoglycans)¹²² (**Figure 5c**). These so-called rotor-FDAAs (RfDAAs)
373 consist of an electron-donating tetrahydroquinoline core structure coupled to electron-
374 withdrawing carboxy-2-cyanovinyl groups to create efficient push-pull systems (**Figure 3c**).
375 The resulting water-soluble amino acids Rf420DL ($\lambda_{em} = 420$ nm), Rf470DL ($\lambda_{em} = 470$ nm)
376 and Rf490DL ($\lambda_{em} = 490$ nm) have enabled wash-free and real-time imaging of peptidoglycan
377 biosynthesis, making possible the first high-throughput *in vitro* assay to probe the activity of
378 peptidoglycan transpeptidases. The imaging capabilities of these FDAAs will facilitate their
379 application in biological studies in which monitoring biological events in real time is of
380 extreme importance, including morphogenesis and drug screening studies.

381

382 **[H1] Genetically-encoded FIAAs**

383 **[H3] Encoding FIAAs into proteins**

384 Proteins can be endowed with fluorescence properties by numerous methods, including
385 by enzymatic and chemical site-specific labelling with optical reporters and by *in vitro*
386 translation of proteins with fluorescent tags¹²³⁻¹²⁷. These methods have undoubtedly aided the
387 study of complex biological processes but they do suffer from some limitations, including
388 potential off-target fluorescence and restriction to *in vitro* assays. Bearing this in mind, the
389 genetic encoding of FIAAs has rapidly evolved to enable the generation of artificial proteins
390 that might overcome some of these challenges¹²⁸.

391 Site-specific incorporation of non-natural amino acids by genetic encoding in cells is generally
392 achieved by assigning an amber stop codon to the non-natural amino acid and supplying the
393 cells with an exogenous aminoacyl-tRNA synthetase (aaRS) and its corresponding transfer
394 ribonucleic acid (tRNA). Importantly, the exogenous aaRS and tRNA must be orthogonal and
395 not cross react with the endogenous aaRSs and tRNAs of the host cell. Then, the orthogonal
396 aaRS specifically couples the orthogonal tRNA to the non-natural amino acid so that the
397 ‘charged’ tRNA is used by the ribosome for site-specific incorporation of the non-natural
398 amino acid (**Figure 6a**). Rapid advances in this technology have led to the efficient synthesis

399 of artificial proteins containing non-natural amino acids in live cells and animals¹²⁹⁻¹³¹. To date,
400 more than 150 non-natural amino acids, including bioorthogonal, photoreactive and
401 photocaged amino acids, as well as amino acids with electron paramagnetic resonance (EPR),
402 infrared (IR) and nuclear magnetic resonance (NMR) labels, have been genetically encoded in
403 different organisms¹³². The development of organisms with synthetic genomes¹³³ and the
404 evolution of quadruplet-codon-decoding ribosomes^{134,135} will expand the applicability of new
405 genetically-encoded FIAAs with different chemical structures and/or additional photophysical
406 properties to label proteins.

407 [H3] Genetically-encoded FIAAs in prokaryotes

408 The FIAAs that have been genetically incorporated into proteins (**Figure 6b**) can be
409 broadly classified by whether they are expressed in prokaryotic or in eukaryotic cells.
410 Following the discovery and application of L-(7-hydroxycoumarin-4-yl)ethylglycine (CouA;
411 $\lambda_{em.} = 380$ nm in neutral form; $\lambda_{em.} = 450$ nm in anionic form), acridon-2-ylalanine (Acid; $\lambda_{em.} =$
412 446 nm) and 4-biphenyl-l-phenylalanine (terphenylA; $\lambda_{em.} = 342$ nm) were developed as
413 additional FIAAs for site-specific incorporation into proteins in *E. coli*^{20,45,136}. In these cases,
414 evolved mutant tyrosyl-aaRSs from the archaeon *Methanococcus jannaschii* (*MjTyrRS*) were
415 used to generate ‘charged’ tRNAs inside the cell, which resulted in successful ribosomal
416 incorporation. Of note, the *MjTyrRS*–*MjtRNA* pair is orthogonal in prokaryotes and has been
417 evolved for incorporation of several other non-natural amino acids.

418 CouA is synthesized by incorporating a β -keto ester in the amino acid side chain and then
419 reacting it with resorcinol by Pechmann condensation²⁰. The ease of synthesis and efficient
420 incorporation of CouA into proteins has facilitated the widespread application of CouA in
421 biological studies. As the emission spectrum of CouA overlaps with the excitation spectrum of
422 cyan fluorescent protein (CFP), CouA was used to engineer a new CFP with a long Stokes shift
423 of ~ 110 nm (ref.¹³⁷). In this case, the precise incorporation of CouA at 20 Å from the CFP
424 fluorophore results in efficient FRET so that CFP fluorescence (at 426 nm) is detected when
425 CouA is excited (at 365 nm) (**Figure 6c**). Furthermore, the sensitivity of CouA to pH and
426 polarity changes has also been exploited to produce artificial proteins with readouts that are
427 sensitive to environmental conditions¹³⁸. For example, this approach was used to investigate
428 the difference in substrate specificity between the haloalkane dehalogenases DhaA and
429 DbjA¹³⁹. Steady-state and time-resolved fluorescence measurements of dehalogenase mutants
430 containing CouA at specific sites revealed greater hydration in DbjA than in DhaA, which
431 correlates with the substrate specificity of the two proteins. CouA has also been genetically

432 encoded into the transient receptor potential cation channel subfamily V member 1 (TRPV1)
433 to study the dynamics of receptor activation, with changes in the fluorescence being correlated
434 with the opening and closing of the channel on binding of capsaicin¹⁴⁰.

435 [H3] Genetically-encoded FIAAs in eukaryotes

436 Lysine derivatives of 7-hydroxycoumarin (Lys-Cou) have been genetically encoded
437 into proteins using evolved mutants of *Methanosarcina barkeri* pyrrolysyl RS (PylRS)–
438 tRNA_{CUA}¹⁴¹. The PylRS–tRNA pair^{142,143} has become an extremely useful tool for genetic
439 encoding of non-natural amino acids because it is orthogonal to prokaryotes and eukaryotes
440 and therefore can be used in cells of evolutionarily diverse origins, including *E. coli*,
441 *Saccharomyces cerevisiae*, mammalian cells and even whole intact organisms, such as the
442 nematode worm *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*¹⁴⁴. ACD
443 has also been encoded in fluorescent proteins for expression in prokaryotes. The high quantum
444 yield (~95%, compared with 63% for CouA), efficient synthesis and high photostability of
445 ACD are some of its key advantages.

446 Among the FIAAs that have been genetically encoded in eukaryotes, the most notable
447 are dansyl and prodan derivatives, such as dansylalanine ($\lambda_{em.} = 540$ nm) and ANAP ($\lambda_{em.} = 490$
448 nm)¹⁴⁵⁻¹⁴⁷ (**Figure 6b**). These FIAAs have been incorporated into proteins expressed in *S.*
449 *cerevisiae* using evolved mutants of the *E. coli*-derived leucyl-RS–leucyl-tRNA_{CUA} pair, which
450 is orthogonal to those found in eukaryotic cells. A remarkable feature of these amino acids is
451 that their emission maximum and quantum yield can vary drastically depending on the polarity
452 of the medium; for example, the quantum yield of dansyl-based amino acids vary from 50%
453 ($\lambda_{em.} = 510$ nm) in ethyl acetate to <10% ($\lambda_{em.} = 578$ nm) in water¹⁴⁸; the emission maxima for
454 ANAP are 490 nm in water and 420 nm in ethyl acetate. This sensitivity to the dielectric
455 constant of the surrounding environment has been exploited to investigate the dynamics of
456 different protein structures. For example, ANAP was used to map the regions of the voltage-
457 gated potassium ion channel ion channel that are inaccessible to chemical labelling¹⁴⁹ (**Figure**
458 **6d**). First, the FIAA was genetically encoded into different positions in the ion channel
459 sequence, either the extracellular and intracellular regions, and then electrophysiology and
460 fluorescence measurements were performed concurrently to identify the regions of the protein
461 that cooperatively lead to opening of the channel. Similarly, ANAP has been also used to
462 examine the catalytic activity of a voltage-sensing phosphatase¹⁵⁰ and to gain insights into
463 protein–protein interactions using FRET measurements, as in the case of the pro-apoptotic
464 protein BAX and the anti-apoptotic protein HSP70 (ref.¹⁵¹). Different mutants of BAX with

465 ANAP encoded at three different positions in the protein were tested for binding to HSP70
466 fused to yellow fluorescent protein (YFP), so that FRET signals were detected only when the
467 two proteins were in close proximity. This FRET signal was used as a reporter of the interaction
468 between BAX and HSP70 and helped to discern the mechanism of small molecule-induced
469 inhibition of this interaction. These examples highlight how genetically-encoded FIAAs can be
470 used to gain insights into protein dynamics and folding, as well as the interactions between
471 proteins and other biomolecules.

472

473 [H1] Conclusions and outlook

474 Advances in the chemical derivatization of heterocyclic building blocks and
475 fluorophores have accelerated the design and synthesis of FIAAs with improved properties for
476 chemical biology studies. To date, the unique reactivity of the indole moiety^{152,153} has resulted
477 in the synthesis of multiple tryptophan analogues by different transformations (such as Pd-
478 catalysed couplings and C–H activation). Site-specific modification of other amino acids,
479 including, amongst others, histidine^{154,155}, phenylalanine⁴⁹ and tyrosine^{156,157}, will help to
480 enable other residues with complementary functionalities to be fluorescently labelled and,
481 ultimately, lead to broader chemical diversity. Similarly, the emergence of new chemistry in
482 the field of synthetic fluorophores will enable the construction of FIAAs with additional
483 properties. These properties include not only physicochemical properties, such as small
484 size^{158,159} to reduce the effect of labelling in peptides and proteins, but also optical features for
485 spectroscopy and imaging experiments, including near-infrared emission^{160,161}, super-
486 resolution capabilities^{162,163}, photoactivatable behaviour^{164,165} or suitability for multimodal
487 imaging (for example, optoacoustics and positron emission tomography)^{166,167}. A greater
488 number and variety of FIAAs will create avenues to identify new roles for peptides and proteins
489 in biological systems. FIAAs with longer emission wavelengths will improve the penetration
490 depth that is required for *in vivo* imaging studies, and photoswitching FIAAs may allow
491 researchers to image the localization and trafficking of proteins using super-resolution
492 microscopy. New FIAAs might also find important applications in other areas of chemistry in
493 which they may have been underexplored to date, such as the material sciences, where
494 fluorescent building blocks embedded in supramolecular structures could provide dynamic
495 readouts to characterize the formation and properties of new materials in real time, including
496 nanofibres and peptide hydrogels^{168,169}.

497 For biological applications, the design of new methodologies in synthetic biology will
498 facilitate the integration of new modular blocks into different macromolecular structures. An

499 area that will benefit substantially from these developments is genetically-encoded FIAAs.
500 Although currently available genetically-encoded FIAAs hold huge potential for studying the
501 dynamics, folding and biomolecular interactions of proteins, their number and types are
502 limited. Most fluorophores absorb at very short wavelengths that require high energy
503 excitation and lead to limited tissue penetration and poor signal-to-background ratios, [which
504 precludes their use in whole organisms. Non-natural amino acids showing good spectral
505 overlap with existing fluorophores or fluorescent proteins would be also extremely useful to
506 build FRET pairs within proteins and thereby study dynamic conformational changes with
507 increased resolution. The UV excitation wavelengths of current FIAAs also reduce their
508 compatibility with photocaging groups, which are typically cleaved when illuminated at those
509 wavelengths (for example, 365 nm)^{170,171}. Therefore, synthetic routes that are able to red-
510 shift the excitation and emission wavelengths¹⁷² of non-natural FIAAs would have a
511 remarkable impact on the design of new artificial proteins. However, this synthetic effort will
512 need to be matched with advances in the biological counterpart, where the identification of
513 new aaRS–tRNA pairs that can recognize and incorporate such molecules will be essential
514 for the successful encoding of novel FIAAs into protein structures.

515

516 **References**

517

- 518 1 Chan, J., Dodani, S. C. & Chang, C. J. Reaction-based small-molecule fluorescent probes
519 for chemoselective bioimaging. *Nat. Chem.* **4**, 973-984, (2012).
- 520 2 Lavis, L. D. & Raines, R. T. Bright building blocks for chemical biology. *ACS Chem.*
521 *Biol.* **9**, 855-866 (2014).
- 522 3 Klymchenko, A. S. Solvatochromic and fluorogenic dyes as environment-sensitive
523 probes: design and biological applications. *Acc. Chem. Res.* **50**, 366-375 (2017).
- 524 4 Park, S. J. et al. Mechanistic elements and critical factors of cellular reprogramming
525 revealed by stepwise global gene expression analyses. *Stem Cell Res.* **12**, 730-741 (2014).
- 526 5 Shimomura, O., Johnson, F. H. & Saiga, Y. Extraction, purification and properties of
527 aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *J. Cell.*
528 *Comparative Physiol.* **59**, 223-239 (1962).
- 529 6 Tsien, R. Y. The green fluorescent protein. *Ann. Rev. Biochem.* **67**, 509-544 (1998).
- 530 7 Heim, R., Cubitt, A. B. & Tsien, R. Y. Improved green fluorescence. *Nature* **373**, 663-
531 664 (1995).

- 532 8 Keppeler, A. et al. A general method for the covalent labeling of fusion proteins with small
533 molecules in vivo. *Nat. Biotechnol.* **21**, 86-89 (2003).
- 534 9 Gautier, A. et al. An engineered protein tag for multiprotein labeling in living cells.
535 *Chem. Biol.* **15**, 128-136 (2008).
- 536 10 Los, G. V. et al. HaloTag: A novel protein labeling technology for cell imaging and
537 protein analysis. *ACS Chem. Biol.* **3**, 373-382 (2008).
- 538 11 Griffin, B. A., Adams, S. R. & Tsien, R. Y. Specific covalent labeling of recombinant
539 protein molecules inside live cells. *Science* **281**, 269 (1998).
- 540 12 Martin, B. R., Giepmans, B. N. G., Adams, S. R. & Tsien, R. Y. Mammalian cell-based
541 optimization of the biarsenical-binding tetracysteine motif for improved fluorescence and
542 affinity. *Nat. Biotechnol.* **23**, 1308-1314 (2005).
- 543 13 Krueger, A. T. & Imperiali, B. Fluorescent amino acids: modular building blocks for the
544 assembly of new tools for chemical biology. *ChemBioChem* **14**, 788-799 (2013).
- 545 14 Nitz, M., Mezo, A. R., Ali, M. H. & Imperiali, B. Enantioselective synthesis and
546 application of the highly fluorescent and environment-sensitive amino acid 6-(2-
547 dimethylaminonaphthoyl) alanine (DANA). *Chem. Commun.* 1912-1913 (2002).
- 548 15 Vázquez, M. E., Blanco, J. B. & Imperiali, B. Photophysics and biological applications
549 of the environment-sensitive fluorophore 6-n,n-dimethylamino-2,3-naphthalimide. *J.*
550 *Amer. Chem. Soc.* **127**, 1300-1306 (2005).
- 551 16 Socher, E. & Imperiali, B. FRET-Capture: a sensitive method for the detection of
552 dynamic protein interactions. *ChemBioChem* **14**, 53-57 (2013).
- 553 17 Shults, M. D. & Imperiali, B. Versatile fluorescence probes of protein kinase activity. *J.*
554 *Amer. Chem. Soc.* **125**, 14248-14249 (2003).
- 555 18 Venkatraman, P. et al. Fluorogenic probes for monitoring peptide binding to class II
556 MHC proteins in living cells. *Nat. Chem. Biol.* **3**, 222-228 (2007).
- 557 19 Vázquez, M. E., Rothman, D. M. & Imperiali, B. A new environment-sensitive
558 fluorescent amino acid for Fmoc-based solid phase peptide synthesis. *Org. Biomol.*
559 *Chem.* **2**, 1965-1966 (2004).
- 560 20 Wang, J., Xie, J. & Schultz, P. G. A genetically encoded fluorescent amino acid. *J. Amer.*
561 *Chem. Soc.* **128**, 8738-8739 (2006).
- 562 21 Kielland, N., Vendrell, M., Lavilla, R. & Chang, Y.-T. Imaging histamine in live
563 basophils and macrophages with a fluorescent mesoionic acid fluoride. *Chem. Commun.*
564 **48**, 7401-7403 (2012).

- 565 22 Weiss, J. T. et al. Extracellular palladium-catalysed dealkylation of 5-fluoro-1-propargyl-
566 uracil as a bioorthogonally activated prodrug approach. *Nat. Commun.* **5**, 3277-3285
567 (2014).
- 568 23 Ramil, C. P. & Lin, Q. Photoclick chemistry: a fluorogenic light-triggered in vivo ligation
569 reaction. *Curr. Opin. Chem. Biol.* **21**, 89-95 (2014).
- 570 24 Devaraj, N. K. The future of bioorthogonal chemistry. *ACS Cent. Sci.* **4**, 952-959 (2018).
- 571 25 de Moliner, F., Kielland, N., Lavilla, R. & Vendrell, M. Modern synthetic avenues for
572 the preparation of functional fluorophores. *Angew. Chem. Int. Ed.* **56**, 3758-3769 (2017).
- 573 26 Teale, F. W. & Weber, G. Ultraviolet fluorescence of the aromatic amino acids. *Biochem.*
574 *J.* **65**, 476-482 (1957).
- 575 27 Ghisaidoobe, A. B. T. & Chung, S. J. Intrinsic tryptophan fluorescence in the detection
576 and analysis of proteins: a focus on Förster resonance energy transfer techniques. *Int. J.*
577 *Mol. Sci.* **15**, 22518-22538 (2014).
- 578 28 Merkel, L., Hoesl, M. G., Albrecht, M., Schmidt, A. & Budisa, N. Blue fluorescent amino
579 acids as in vivo building blocks for proteins. *ChemBioChem* **11**, 305-314 (2010).
- 580 29 Talukder, P. et al. Cyanotryptophans as novel fluorescent probes for studying protein
581 conformational changes and DNA–protein interaction. *Biochemistry* **54**, 7457-7469
582 (2015).
- 583 30 Hilaire, M. R. et al. Blue fluorescent amino acid for biological spectroscopy and
584 microscopy. *Proc. Nat. Acad. Sci.* **114**, 6005-6009 (2017).
- 585 **Small analogue of a naturally-occurring amino acid that behaves as a non-**
586 **disruptive building block for the preparation of fluorescent peptides.**
- 587 31 Zhang, K. et al. Synthesis and application of the blue fluorescent amino acid l-4-
588 cyanotryptophan to assess peptide–membrane interactions. *Chem. Commun.* **55**, 5095-
589 5098 (2019).
- 590 32 Winn, M., Francis, D. & Micklefield, J. De novo biosynthesis of “non-natural” thaxtomin
591 phytotoxins. *Angew. Chem. Int. Ed.* **57**, 6830-6833 (2018).
- 592 33 Boville, C. E., Romney, D. K., Almhjell, P. J., Sieben, M. & Arnold, F. H. Improved
593 synthesis of 4-cyanotryptophan and other tryptophan analogues in aqueous solvent using
594 variants of TrpB from *Thermotoga maritima*. *J. Org. Chem.* **83**, 7447-7452 (2018).
- 595 34 Talukder, P., Chen, S., Arce, P. M. & Hecht, S. M. Efficient asymmetric synthesis of
596 tryptophan analogues having useful photophysical properties. *Org. Lett.* **16**, 556-559
597 (2014).

- 598 35 Wen, J. et al. Highly N₂-selective coupling of 1,2,3-triazoles with indole and pyrrole.
599 *Chem. Eur. J.* **20**, 974-978 (2014).
- 600 36 Williams, T. J., Reay, A. J., Whitwood, A. C. & Fairlamb, I. J. S. A mild and selective
601 Pd-mediated methodology for the synthesis of highly fluorescent 2-arylated tryptophans
602 and tryptophan-containing peptides: a catalytic role for Pd⁰ nanoparticles? *Chem.*
603 *Commun.* **50**, 3052-3054 (2014).
- 604 37 Bartoccini, F., Bartolucci, S., Mari, M. & Piersanti, G. A simple, modular synthesis of
605 C₄-substituted tryptophan derivatives. *Org. Biomol. Chem.* **14**, 10095-10100 (2016).
- 606 38 Talukder, P. et al. Tryptophan-based fluorophores for studying protein conformational
607 changes. *Bioorg. Med. Chem.* **22**, 5924-5934 (2014).
- 608 39 Chen, S. et al. Fluorescent biphenyl derivatives of phenylalanine suitable for protein
609 modification. *Biochemistry* **52**, 8580-8589 (2013).
- 610 40 Chen, S. et al. Detection of dihydrofolate reductase conformational change by FRET
611 using two fluorescent amino acids. *J. Amer. Chem. Soc.* **135**, 12924-12927 (2013).
- 612 41 Maity, J., Honcharenko, D. & Strömberg, R. Synthesis of fluorescent D-amino acids with
613 4-acetamidobiphenyl and 4-N,N-dimethylamino-1,8-naphthalimido containing side
614 chains. *Tet. Lett.* **56**, 4780-4783 (2015).
- 615 42 Cheruku, P. et al. Tyrosine-derived stimuli responsive, fluorescent amino acids. *Chem.*
616 *Sci.* **6**, 1150-1158 (2015).
- 617 **A toolbox of tyrosine-based FIAAs with tunable emission and reversible pH and**
618 **redox responses, showing potential for biosensing applications.**
- 619 43 Bylińska, I., Guzow, K., Wójcik, J. & Wiczak, W. New non-proteinogenic fluorescent
620 amino acids: benzoxazol-5-yl-alanine derivatives containing acetylene unit. Synthesis,
621 spectral and photophysical properties. *J. Photochem. Photobiol. A: Chem.* **364**, 679-685
622 (2018).
- 623 44 Hoppmann, C., Alexiev, U., Irran, E. & Rück-Braun, K. Synthesis and fluorescence of
624 xanthone amino acids. *Tet. Lett.* **54**, 4585-4587 (2013).
- 625 45 Speight, L. C. et al. Efficient synthesis and in vivo incorporation of acridon-2-ylalanine,
626 a fluorescent amino acid for lifetime and Förster resonance energy transfer/luminescence
627 resonance energy transfer studies. *J. Amer. Chem. Soc.* **135**, 18806-18814 (2013).
- 628 46 Mendive-Tapia, L. et al. Preparation of a Trp-BODIPY fluorogenic amino acid to label
629 peptides for enhanced live-cell fluorescence imaging. *Nat. Protoc.* **12**, 1588-1619 (2017).
- 630 47 Subiros-Funosas, R. et al. Fluorogenic Trp(redBODIPY) cyclopeptide targeting keratin
631 1 for imaging of aggressive carcinomas. *Chem. Sci.* **11**, 1368-1374 (2020).

632 **Development of an optically enhanced Trp-redBODIPY and validation in cyclic**
633 **peptides for imaging aggressive carcinomas.**

- 634 48 Terrey, M. J., Holmes, A., Perry, C. C. & Cross, W. B. C–H Olefination of tryptophan
635 residues in peptides: control of residue selectivity and peptide–amino acid cross-linking.
636 *Org. Lett.* **21**, 7902-7907 (2019).
- 637 49 Wang, W., Lorion, M. M., Martinazzoli, O. & Ackermann, L. BODIPY peptide labeling
638 by late-stage C(sp³)–H activation. *Angew. Chem. Int. Ed.* **57**, 10554-10558 (2018).
- 639 50 Schischko, A. et al. Late-stage peptide C–H alkylation for bioorthogonal C–H activation
640 featuring solid phase peptide synthesis. *Nat. Commun.* **10**, 3553-3561 (2019).
- 641 51 Pereira, G., Vilaça, H. & Ferreira, P. M. T. Synthesis of new β -
642 amidodehydroaminobutyric acid derivatives and of new tyrosine derivatives using
643 copper catalyzed C–N and C–O coupling reactions. *Amino Acids* **44**, 335-344 (2013).
- 644 52 Bag, S. S., Jana, S. & Pradhan, M. K. Synthesis, photophysical properties of triazolyl-
645 donor/acceptor chromophores decorated unnatural amino acids: Incorporation of a pair
646 into Leu-enkephalin peptide and application of triazolylperylene amino acid in sensing
647 BSA. *Bioorg. Med. Chem.* **24**, 3579-3595 (2016).
- 648 53 Benedetti, E., Veliz, A. B. E., Charpenay, M., Kocsis, L. S. & Brummond, K. M.
649 Attachable solvatochromic fluorophores and bioconjugation studies. *Org. Lett.* **15**, 2578-
650 2581 (2013).
- 651 54 Li, C. et al. Click chemistry to fluorescent amino esters: synthesis and spectroscopic
652 studies. *Eur. J. Org. Chem.* **2010**, 2395-2405 (2010).
- 653 55 Ferreira, P. M. T., Monteiro, L. S., Pereira, G., Castanheira, E. M. S. & Frost, C. G.
654 Synthesis of fluorescent alanines by a rhodium-catalysed conjugate addition of
655 arylboronic acids to dehydroalanine derivatives. *Eur. J. Org. Chem.* **2013**, 550-556
656 (2013).
- 657 56 Hsu, Y.-P. et al. Full color palette of fluorescent D-amino acids for in situ labeling of
658 bacterial cell walls. *Chem. Sci.* **8**, 6313-6321 (2017).
- 659 57 Gütschow, M. & Häußler, D. Fluorescently labeled amino acids as building blocks for
660 bioactive molecules. *Synthesis* **48**, 245-255 (2015).
- 661 58 Kuru, E., Tekkam, S., Hall, E., Brun, Y. V. & Van Nieuwenhze, M. S. Synthesis of
662 fluorescent D-amino acids and their use for probing peptidoglycan synthesis and bacterial
663 growth in situ. *Nat. Protoc.* **10**, 33-52 (2014).

664 59 Katritzky, A. R., Ozcan, S. & Todadze, E. Synthesis and fluorescence of the new
665 environment-sensitive fluorophore 6-chloro-2,3-naphthalimide derivative. *Org. Biomol.*
666 *Chem.* **8**, 1296-1300 (2010).

667 60 Esteves, C. I. C., Silva, A. M. F., Raposo, M. M. M. & Costa, S. P. G. Unnatural benz-
668 X-azolyl asparagine derivatives as novel fluorescent amino acids: synthesis and
669 photophysical characterization. *Tetrahedron* **65**, 9373-9377 (2009).

670 61 Yokoo, H., Kagechika, H, Ohsaki, A. & Hirano, T. A polarity-sensitive fluorescent
671 amino acid and its incorporation into peptides for the ratiometric detection of
672 biomolecular interactions. *ChemPlusChem* **84**, 1716-1719 (2019).

673 62 Shukla, L., Moodie, L. W. K., Kindahl, T. & Hedberg, C. Synthesis and spectroscopic
674 properties of fluorinated coumarin lysine derivatives. *J. Org. Chem.* **83**, 4792-4799
675 (2018).

676 63 Bag, S. S. & De, S. Isothiocyanyl alanine as a synthetic intermediate for the synthesis of
677 thioureayl alanines and subsequent aminotetrazolyl alanines. *J. Org. Chem.* **82**, 12276-
678 12285 (2017).

679 64 Mohite, A. R. & Bhat, R. G. Enantiopure synthesis of side chain-modified α -amino acids
680 and 5-cis-alkylprolines. *J. Org. Chem.* **77**, 5423-5428 (2012).

681 65 Fowler, L. S., Ellis, D. & Sutherland, A. Synthesis of fluorescent enone derived α -amino
682 acids. *Org. Biomol. Chem.* **7**, 4309-4316 (2009).

683 66 Navo, C. D. et al. Cell-penetrating peptides containing fluorescent d-cysteines. *Chem.*
684 *Eur. J.* **24**, 7991-8000 (2018).

685 67 Wörner, S., Röncke, F., Ulrich, A. S. & Wagenknecht, H-A. 4-Aminophthalimide acids
686 as small and environment-sensitive fluorescent probes for transmembrane peptides.
687 *ChemBioChem* **21**, 618-622 (2020).

688 68 Xiang, Z. & Wang, L. Enantiospecific synthesis of genetically encodable fluorescent
689 unnatural amino acid 1-3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid. *J.*
690 *Org. Chem.* **76**, 6367-6371 (2011).

691 69 Zhao, Y., Pirrung, M. C. & Liao, J. A fluorescent amino acid probe to monitor efficiency
692 of peptide conjugation to glass surfaces for high density microarrays. *Mol. BioSyst.* **8**,
693 879-887 (2012).

694 70 Arribat, M., Rémond, E., Clément, S., Lee, A. V. D. & Cavelier, F. Phospholyl(borane)
695 amino acids and peptides: stereoselective synthesis and fluorescent properties with large
696 stokes shift. *J. Am. Chem. Soc.* **140**, 1028-1034 (2018).

- 697 71 Strizhak, A. V. et al. Two-color fluorescent L-amino acid mimic of tryptophan for
698 probing peptide–nucleic acid complexes. *Bioconjugate Chem.* **23**, 2434-2443 (2012).
- 699 **First report of a tyrosine-based FIAA exhibiting excited-state intramolecular proton**
700 **transfer and hydration sensitive dual emission.**72 Postupalenko, V. Y. et al. Dual-
701 fluorescence L-amino acid reports insertion and orientation of melittin peptide in cell
702 membranes. *Bioconjugate Chem.* **24**, 1998-2007 (2013).
- 703 73 Sholokh, M. et al. Fluorescent amino acid undergoing excited state intramolecular proton
704 transfer for site-specific probing and imaging of peptide interactions. *J. Phys. Chem. B*
705 **119**, 2585-2595 (2015).
- 706 74 Taki, M., Yamazaki, Y., Suzuki, Y. & Sisido, M. Introduction of a highly photodurable
707 and common-laser excitable fluorescent amino acid into a peptide as a FRET acceptor
708 for protease cleavage detection. *Chem. Lett.* **39**, 818-819 (2010).
- 709 75 Bell, J. D. et al. Synthesis and photophysical properties of benzotriazole-derived
710 unnatural α -amino acids. *J. Org. Chem.* **84**, 10436-10448 (2019).
- 711 76 Gilfillan, L., Artschwager, R., Harkiss, A. H., Liskamp, R. M. J. & Sutherland, A.
712 Synthesis of pyrazole containing α -amino acids via a highly regioselective
713 condensation/aza-Michael reaction of β -aryl α,β -unsaturated ketones. *Org. Biomol.*
714 *Chem.* **13**, 4514-4523 (2015).
- 715 77 Harkiss, A. H., Bell, J. D., Knuhtsen, A., Jamieson, A. G. & Sutherland, A. Synthesis
716 and fluorescent properties of β -pyridyl α -amino acids. *J. Org. Chem.* **84**, 2879-2890
717 (2019).
- 718 78 Bell, J. D. et al. Conformationally rigid pyrazoloquinazoline α -amino acids: one- and
719 two-photon induced fluorescence. *Chem. Commun.* **56**, 1887-1890 (2020).
- 720 79 Häußler, D. & Gütschow, M. Synthesis of a fluorescent-labeled bisbenzamidine
721 containing the central (6,7-dimethoxy-4-coumaryl)alanine building block. *Heteroatom*
722 *Chem.* **26**, 367-373 (2015).
- 723 80 Koopmans, T., van Haren, M., van Ufford, L. Q., Beekman, J. M. & Martin, N. I. A
724 concise preparation of the fluorescent amino acid 1-(7-hydroxycoumarin-4-yl)
725 ethylglycine and extension of its utility in solid phase peptide synthesis. *Bioorg. Med.*
726 *Chem.* **21**, 553-559 (2013).
- 727 81 Moodie, L. W. K., Chammaa, S., Kindahl, T. & Hedberg, C. Palladium-mediated
728 approach to coumarin-functionalized amino acids. *Org. Lett.* **19**, 2797-2800 (2017).

- 729 82 Fernandez, A., Thompson, E. J., Pollard, J. W., Kitamura, T. & Vendrell, M. A
730 fluorescent activatable AND-gate chemokine CCL2 enables in vivo detection of
731 metastasis-associated macrophages. *Angew. Chem. Int. Ed.* **58**, 16894-16898 (2019).
- 732 83 Joshi, P. N. & Rai, V. Single-site labeling of histidine in proteins, on-demand
733 reversibility, and traceless metal-free protein purification. *Chem. Commun.* **55**, 1100-
734 1103 (2019).
- 735 84 Cheng, M. H. Y., Savoie, H., Bryden, F. & Boyle, R. W. A convenient method for
736 multicolour labelling of proteins with BODIPY fluorophores via tyrosine residues.
737 *Photochem. Photobiol. Sci.* **16**, 1260-1267 (2017).
- 738 85 Zhao, C. et al. Searching for the optimal fluorophore to label antimicrobial peptides. *ACS*
739 *Comb. Sci.* **18**, 689-696 (2016).
- 740 86 Vendrell, M. et al. Biotin ergopeptide probes for dopamine receptors. *J. Med. Chem.* **54**,
741 1080-1090 (2011).
- 742 87 Palomo, J. M. Solid-phase peptide synthesis: an overview focused on the preparation of
743 biologically relevant peptides. *RSC Adv.* **4**, 32658-32672 (2014).
- 744 88 Sainlos, M., Iskenderian, W. S. & Imperiali, B. A general screening strategy for peptide-
745 based fluorogenic ligands: probes for dynamic studies of PDZ domain-mediated
746 interactions. *J. Am. Chem. Soc.* **131**, 6680-6682 (2009).
- 747 **Pioneering work on solvatochromic phthalimide amino acids and their integration**
748 **into peptide structures to study dynamic protein-protein interactions.**
- 749 89 Loving, G. & Imperiali, B. A versatile amino acid analogue of the solvatochromic
750 fluorophore 4-n,n-dimethylamino-1,8-naphthalimide: a powerful tool for the study of
751 dynamic protein interactions. *J. Am. Chem. Soc.* **130**, 13630-13638 (2008).
- 752 90 Wang, W. et al. A fluorogenic peptide probe developed by in vitro selection using tRNA
753 carrying a fluorogenic amino acid. *Chem. Commun.* **50**, 2962-2964 (2014).
- 754 91 Wang, W. et al. Fluorogenic enhancement of an in vitro-selected peptide ligand by
755 replacement of a fluorescent group. *Anal. Chem.* **88**, 7991-7997 (2016).
- 756 92 Heru, C., Jurgen, S., Tino, Z. & Horst, A. Fluorescent analogues of the insect
757 neuropeptide helicokinin I: synthesis, photophysical characterization and biological
758 activity. *Prot. Pept. Lett.* **17**, 431-436 (2010).
- 759 93 Manandhar, Y. et al. In vitro selection of a peptide aptamer that changes fluorescence in
760 response to verotoxin. *Biotechnol. Lett.* **37**, 619-625 (2015).

- 761 94 Newton, L. D., Pascu, S. I., Tyrrell, R. M. & Eggleston, I. M. Development of a peptide-
762 based fluorescent probe for biological heme monitoring. *Org. Biomol. Chem.* **17**, 467-
763 471 (2019).
- 764 95 Zhao, C., Mendive-Tapia, L. & Vendrell, M. Fluorescent peptides for imaging of fungal
765 cells. *Arch. Biochem. Biophys.* **661**, 187-195 (2019).
- 766 96 Mendive-Tapia, L. et al. Spacer-free BODIPY fluorogens in antimicrobial peptides for
767 direct imaging of fungal infection in human tissue. *Nat. Commun.* **7**, 10940-10948
768 (2016).
- 769 **First report of Trp-BODIPY as a fluorogenic amino acid to non-invasively label**
770 **peptides for live-cell and *ex vivo* tissue imaging**
- 771 97 Akram, A. R. et al. Enhanced avidity from a multivalent fluorescent antimicrobial peptide
772 enables pathogen detection in a human lung model. *Sci. Rep.* **9**, 8422-8431 (2019).
- 773 98 Subiros-Funosas, R. et al. A Trp-BODIPY cyclic peptide for fluorescence labelling of
774 apoptotic bodies. *Chem. Commun.* **53**, 945-948 (2017).
- 775 99 Ge, J., Li, L. & Yao, S. Q. A self-immobilizing and fluorogenic unnatural amino acid
776 that mimics phosphotyrosine. *Chem. Commun.* **47**, 10939-10941 (2011).
- 777 100 K.C, T. B. et al. Wash-free and selective imaging of epithelial cell adhesion molecule
778 (EpCAM) expressing cells with fluorogenic peptide ligands. *Biochem. Biophys. Res.*
779 *Commun.* **500**, 283-287 (2018).
- 780 101 Maeno, T. et al. Targeted delivery of fluorogenic peptide aptamers into live microalgae
781 by femtosecond laser photoporation at single-cell resolution. *Sci. Rep.* **8**, 8271-8279
782 (2018).
- 783 102 Egan, A. J. F., Cleverley, R. M., Peters, K., Lewis, R. J. & Vollmer, W. Regulation of
784 bacterial cell wall growth. *FEBS J.* **284**, 851-867 (2017).
- 785 103 Dörr, T., Moynihan, P. J. & Mayer, C. Bacterial cell wall structure and dynamics. *Front.*
786 *Microbiol.* **10**, 2051 (2019).
- 787 104 Radkov, A. D., Hsu, Y.-P., Booher, G. & VanNieuwenhze, M. S. Imaging bacterial cell
788 wall biosynthesis. *Ann. Rev. Biochem.* **87**, 991-1014 (2018).
- 789 105 Hsu, Y.-P., Booher, G., Egan, A., Vollmer, W. & VanNieuwenhze, M. S. D-Amino acid
790 derivatives as in situ probes for visualizing bacterial peptidoglycan biosynthesis. *Acc.*
791 *Chem. Res.* **52**, 2713-2722 (2019).
- 792 106 de Pedro, M. A., Quintela, J. C., Höltje, J. V. & Schwarz, H. Murein segregation in
793 *Escherichia coli*. *J. Bacteriol.* **179**, 2823-2834 (1997).

- 794 107 Cava, F., de Pedro, M. A., Lam, H., Davis, B. M. & Waldor, M. K. Distinct pathways for
795 modification of the bacterial cell wall by non-canonical D-amino acids. *EMBO J.* **30**,
796 3442-3453 (2011).
- 797 108 Lupoli, T. J. et al. Transpeptidase-mediated incorporation of D-amino acids into bacterial
798 peptidoglycan. *J. Am. Chem. Soc.* **133**, 10748-10751 (2011).
- 799 109 Siegrist, M. S. et al. D-amino acid chemical reporters reveal peptidoglycan dynamics of
800 an intracellular pathogen. *ACS Chem. Biol.* **8**, 500-505 (2013).
- 801 110 Kuru, E. et al. In situ probing of newly synthesized peptidoglycan in live bacteria with
802 fluorescent D-amino acids. *Angew. Chem. Int. Ed.* **51**, 12519-12523 (2012).
- 803 **Development of a toolbox of fluorescent D-amino acids for labelling peptidoglycans**
804 **and monitor bacterial cell wall growth.**
- 805 111 Liechti, G. W. et al. A new metabolic cell-wall labelling method reveals peptidoglycan
806 in *Chlamydia trachomatis*. *Nature* **506**, 507-510 (2013).
- 807 112 Hudak, J. E., Alvarez, D., Skelly, A., von Andrian, U. H. & Kasper, D. L. Illuminating
808 vital surface molecules of symbionts in health and disease. *Nat. Microbiol.* **2**, 17099-
809 17106 (2017).
- 810 113 Wang, W. et al. Assessing the viability of transplanted gut microbiota by sequential
811 tagging with D-amino acid-based metabolic probes. *Nat. Commun.* **10**, 1317-1323
812 (2019).
- 813 114 Hu, F. et al. Visualization and in situ ablation of intracellular bacterial pathogens through
814 metabolic labeling. *Angew. Chem. Int. Ed.* doi:10.1002/anie.201910187 (2019).
- 815 115 Kuru, E. Mechanisms of incorporation for D-amino acid probes that target peptidoglycan
816 biosynthesis. *ACS Chem. Biol.* **14**, 2745-2756 (2019).
- 817 116 Kuru, E. et al. Fluorescent D-amino-acids reveal bi-cellular cell wall modifications
818 important for *Bdellovibrio bacteriovorus* predation. *Nat. Microbiol.* **2**, 1648-1657
819 (2017).
- 820 117 Morales Angeles, D. et al. Pentapeptide-rich peptidoglycan at the *Bacillus subtilis* cell-
821 division site. *Mol. Microbiol.* **104**, 319-333 (2017).
- 822 118 Baranowski, C. et al. Maturing *Mycobacterium smegmatis* peptidoglycan requires non-
823 canonical crosslinks to maintain shape. *eLife* **7**, e37516 (2018).
- 824 119 Bisson-Filho, A. W. et al. Treadmilling by FtsZ filaments drives peptidoglycan synthesis
825 and bacterial cell division. *Science* **355**, 739-743 (2017).

- 826 120 Yang, X. et al. GTPase activity–coupled treadmilling of the bacterial tubulin FtsZ
827 organizes septal cell wall synthesis. *Science* **355**, 744-747 (2017).
- 828 121 Liechti, G. et al. Pathogenic *Chlamydia* lack a classical sacculus but synthesize a narrow,
829 mid-cell peptidoglycan ring, regulated by MreB, for cell division. *PLOS Pathogens* **12**,
830 e1005590 (2016).
- 831 122 Hsu, Y.-P. et al. Fluorogenic D-amino acids enable real-time monitoring of peptidoglycan
832 biosynthesis and high-throughput transpeptidation assays. *Nat. Chem.* **11**, 335-341
833 (2019).
- 834 **Rotor fluorogenic D-amino acids for real-time visualization of transpeptidase reactions**
835 **and high-throughput screening of antibacterial drugs.**123 Rodriguez, E. A. et al. The
836 growing and glowing toolbox of fluorescent and photoactive proteins. *Trends Biochem.*
837 *Sci.* **42**, 111-129 (2017).
- 838 124 Jing, C. & Cornish, V. W. Chemical tags for labeling proteins inside living cells. *Acc.*
839 *Chem. Res.* **44**, 784-792 (2011).
- 840 125 Spicer, C. D. & Davis, B. G. Selective chemical protein modification. *Nat. Commun.* **5**,
841 4740-4753 (2014).
- 842 126 Kajihara, D. et al. FRET analysis of protein conformational change through position-
843 specific incorporation of fluorescent amino acids. *Nat. Methods* **3**, 923-929 (2006).
- 844 127 Goto, Y., Katoh, T. & Suga, H. Flexizymes for genetic code reprogramming. *Nat. Protoc.*
845 **6**, 779-790 (2011).
- 846 128 Lang, K. & Chin, J. W. Cellular incorporation of unnatural amino acids and
847 bioorthogonal labeling of proteins. *Chem. Rev.* **114**, 4764-4806 (2014).
- 848 129 Young, D. D. & Schultz, P. G. Playing with the molecules of life. *ACS Chem. Biol.* **13**,
849 854-870 (2018).
- 850 130 Chin, J. W. Expanding and reprogramming the genetic code of cells and animals. *Ann.*
851 *Rev. Biochem.* **83**, 379-408 (2014).
- 852 131 Chin, J. W. Expanding and reprogramming the genetic code. *Nature* **550**, 53-60 (2017).
- 853 132 Dumas, A., Lercher, L., Spicer, C. D. & Davis, B. G. Designing logical codon
854 reassignment – expanding the chemistry in biology. *Chem. Sci.* **6**, 50-69 (2015).
- 855 133 Fredens, J. et al. Total synthesis of *Escherichia coli* with a recoded genome. *Nature* **569**,
856 514-518 (2019).
- 857 134 Wang, K. et al. Optimized orthogonal translation of unnatural amino acids enables
858 spontaneous protein double-labelling and FRET. *Nat. Chem.* **6**, 393-403 (2014).

- 859 135 Sachdeva, A., Wang, K., Elliott, T. & Chin, J. W. Concerted, rapid, quantitative, and site-
860 specific dual labeling of proteins. *J. Am. Chem. Soc.* **136**, 7785-7788 (2014).
- 861 136 Lampkowski, J. S., Uthappa, D. M. & Young, D. D. Site-specific incorporation of a
862 fluorescent terphenyl unnatural amino acid. *Bioorg. Med. Chem. Lett.* **25**, 5277-5280
863 (2015).
- 864 137 Kuhn, S. M., Rubini, M., Müller, M. A. & Skerra, A. Biosynthesis of a fluorescent protein
865 with extreme pseudo-stokes shift by introducing a genetically encoded non-natural amino
866 acid outside the fluorophore. *J. Am. Chem. Soc.* **133**, 3708-3711 (2011).
- 867 138 Lacey, V. K. et al. A fluorescent reporter of the phosphorylation status of the substrate
868 protein STAT3. *Angew. Chem. Int. Ed.* **50**, 8692-8696 (2011).
- 869 139 Amaro, M. et al. Site-specific analysis of protein hydration based on unnatural amino
870 acid fluorescence. *J. Am. Chem. Soc.* **137**, 4988-4992 (2015).
- 871 140 Steinberg, X. et al. Conformational dynamics in TRPV1 channels reported by an encoded
872 coumarin amino acid. *eLife* **6**, e28626 (2017).
- 873 141 Luo, J. et al. Genetically encoded optochemical probes for simultaneous fluorescence
874 reporting and light activation of protein function with two-photon excitation. *J. Am.*
875 *Chem. Soc.* **136**, 15551-15558 (2014).
- 876 142 Wan, W., Tharp, J. M. & Liu, W. R. Pyrrolysyl-tRNA synthetase: an ordinary enzyme
877 but an outstanding genetic code expansion tool. *Biochim. Biophys. Acta – Prot. Proteom.*
878 **1844**, 1059-1070 (2014).
- 879 143 Srinivasan, G., James, C. M. & Krzycki, J. A. Pyrrolysine encoded by UAG in archaea:
880 charging of a UAG-decoding specialized tRNA. *Science* **296**, 1459-1462 (2002).
- 881 144 Elliott, T. S. et al. Proteome labeling and protein identification in specific tissues and at
882 specific developmental stages in an animal. *Nat. Biotechnol.* **32**, 465-472 (2014).
- 883 145 Summerer, D. et al. A genetically encoded fluorescent amino acid. *Proc. Nat. Acad. Sci.*
884 **103**, 9785-9789 (2006).
- 885 146 Lee, H. S., Guo, J., Lemke, E. A., Dimla, R. D. & Schultz, P. G. Genetic incorporation
886 of a small, environmentally sensitive, fluorescent probe into proteins in *Saccharomyces*
887 *cerevisiae*. *J. Am. Chem. Soc.* **131**, 12921-12923 (2009).
- 888 **Seminal work on the genetic encoding of the environmentally-sensitive amino acid**
889 **ANAP into protein structures.**
- 890 147 Chatterjee, A., Guo, J., Lee, H. S. & Schultz, P. G. A genetically encoded fluorescent
891 probe in mammalian cells. *J. Am. Chem. Soc.* **135**, 12540-12543 (2013).

- 892 148 Chen, R. F. Fluorescence of dansyl amino acids in organic solvents and protein solutions.
893 *Arch. Biochem. Biophys.* **120**, 609-620 (1967).
- 894 149 Kalstrup, T. & Blunck, R. Dynamics of internal pore opening in Kv channels probed by
895 a fluorescent unnatural amino acid. *Proc. Nat. Acad. Sci.* **110**, 8272-8277 (2013).
896 **The incorporation of ANAP into proteins enabled the investigation of the gating**
897 **mechanism of voltage-gated potassium channels.**
- 898 150 Sakata, S., Jinno, Y., Kawanabe, A. & Okamura, Y. Voltage-dependent motion of the
899 catalytic region of voltage-sensing phosphatase monitored by a fluorescent amino acid.
900 *Proc. Nat. Acad. Sci.* **113**, 7521-7526 (2016).
- 901 151 Park, S.-H., Ko, W., Lee, H. S. & Shin, I. Analysis of protein–protein interaction in a
902 single live cell by using a FRET system based on genetic code expansion technology. *J.*
903 *Am. Chem. Soc.* **141**, 4273-4281 (2019).
- 904 152 Preciado, S. et al. Synthesis and biological evaluation of a post-synthetically modified
905 Trp-based diketopiperazine. *MedChemComm* **4**, 1171-1174 (2013).
- 906 153 Mendive-Tapia, L. et al. New peptide architectures through C–H activation stapling
907 between tryptophan–phenylalanine/tyrosine residues. *Nat. Commun.* **6**, 7160-7168
908 (2015).
- 909 154 Chen, X. et al. Histidine-specific peptide modification via visible-light-promoted C–H
910 alkylation. *J. Am. Chem. Soc.* **141**, 18230-18237 (2019).
- 911 155 Peciak, K., Laurine, E., Tommasi, R., Choi, J.-W. & Brocchini, S. Site-selective protein
912 conjugation at histidine. *Chem. Sci.* **10**, 427-439 (2019).
- 913 156 Ban, H., Gavriluk, J. & Barbas, C. F. Tyrosine bioconjugation through aqueous ene-
914 type reactions: a click-like reaction for tyrosine. *J. Am. Chem. Soc.* **132**, 1523-1525
915 (2010).
- 916 157 Tilley, S. D. & Francis, M. B. Tyrosine-selective protein alkylation using π -
917 allylpalladium complexes. *J. Am. Chem. Soc.* **128**, 1080-1081 (2006).
- 918 158 Benson, S. et al. SCOTfluors: small, conjugatable, orthogonal, and tunable fluorophores
919 for in vivo imaging of cell metabolism. *Angew. Chem. Int. Ed.* **58**, 6911-6915 (2019).
- 920 159 Su, L. et al. Cu(I)-catalyzed 6-endo-dig cyclization of terminal alkynes, 2-bromoaryl
921 ketones, and amides toward 1-naphthylamines: applications and photophysical
922 properties. *J. Am. Chem. Soc.* **141**, 2535-2544 (2019).
- 923 160 Mellanby, R. J. et al. Tricarbocyanine *N*-triazoles: the scaffold-of-choice for long-term
924 near-infrared imaging of immune cells in vivo. *Chem. Sci.* **9**, 7261-7270 (2018).

- 925 161 Cosco, E. D. et al. Flavylium polymethine fluorophores for near- and shortwave infrared
926 imaging. *Angew. Chem. Int. Ed.* **56**, 13126-13129 (2017).
- 927 162 Tang, J. et al. Single-atom fluorescence switch: a general approach toward visible-light-
928 activated dyes for biological imaging. *J. Am. Chem. Soc.* **141**, 14699-14706 (2019).
- 929 163 Zheng, Q. et al. Rational design of fluorogenic and spontaneously blinking labels for
930 super-resolution imaging. *ACS Cent. Sci.* **5**, 1602-1613 (2019).
- 931 164 Raymo, F. M. Photoactivatable synthetic fluorophores. *Phys. Chem. Chem. Phys.* **15**,
932 14840-14850 (2013).
- 933 165 Zhang, Y., Tang, S., Sansalone, L., Baker, J. D. & Raymo, F. M. A photoswitchable
934 fluorophore for the real-time monitoring of dynamic events in living organisms. *Chem.*
935 *Eur. J.* **22**, 15027-15034 (2016).
- 936 166 Hendricks, J. A. et al. Synthesis of [¹⁸F]BODIPY: bifunctional reporter for hybrid
937 optical/positron emission tomography imaging. *Angew. Chem. Int. Ed.* **51**, 4603-4606
938 (2012).
- 939 167 Zhou, E. Y., Knox, H. J., Liu, C., Zhao, W. & Chan, J. A conformationally restricted aza-
940 BODIPY platform for stimulus-responsive probes with enhanced photoacoustic
941 properties. *J. Am. Chem. Soc.* **141**, 17601-17609 (2019).
- 942 168 Onogi, S. et al. In situ real-time imaging of self-sorted supramolecular nanofibres. *Nat.*
943 *Chem.* **8**, 743-752 (2016).
- 944 169 Beesley, J. L. & Woolfson, D. N. The de novo design of α -helical peptides for
945 supramolecular self-assembly. *Curr. Opin. Biotechnol.* **58**, 175-182 (2019).
- 946 170 Davis, L. & Greiss, S. Genetic encoding of unnatural amino acids in *C. elegans*. *Meth.*
947 *Mol. Biol.* **1728**, 389-408 (2018).
- 948 171 Bridge, T. et al. Site-specific encoding of photoactivity in antibodies enables light-
949 mediated antibody-antigen binding on live cells. *Angew. Chem. Int. Ed.* **58**, 17986-
950 17993. (2019).
- 951 172 Ren, T.-B. et al. A general method to increase stokes shift by introducing alternating
952 vibronic structures. *J. Am. Chem. Soc.* **140**, 7716-7722 (2018).

953
954
955

956 **Acknowledgements**

957 E. K. acknowledges funding of a Fellowship from the Life Sciences Research Foundation. A.S.
958 acknowledges funding from the Wellcome Trust (204593/Z/16/Z) and the Biotechnology and

959 Biological Research Sciences Council (BB/R004692/1). M.V. acknowledges funding from an
960 ERC Consolidator Grant (771443). The authors thank S. Shaikh for useful comments and
961 technical support with the graphical illustrations.

962

963 **Author contributions**

964 All authors contributed equally to the preparation of this manuscript.

965

966 **Competing interests**

967 The authors declare no competing interests.

968

969 **Peer review information**

970 *Nature Reviews Chemistry* thanks A. Klymchenko and the other, anonymous, reviewer(s) for
971 their contribution to the peer review of this work.

972

973 **Publisher's note**

974 Springer Nature remains neutral with regard to jurisdictional claims in published maps and
975 institutional affiliations.

976

977

978

979 **Figure legends**

980 **Figure 1. Applications of FIAAs in chemical biology.** The design and synthesis of new
981 fluorescent amino acids (FIAAs) has created multiple opportunities for *in vitro* biomolecular
982 recognition assays (part **a**), solid-phase peptide synthesis (SPPS) of fluorescent peptides for
983 optical imaging (part **b**), genetic encoding of fluorescent building blocks into proteins (part **c**)
984 and microbiology experiments to study bacterial growth (part **d**). Images in part **b** reproduced
985 with permission from ref. 96, Springer Nature Limited. Image in part **d** reproduced with
986 permission from ref. 112, Springer Nature Limited.

987 **Figure 2. Representative non-natural FIAAs developed in the past decade.** Chemical
988 structures and basic spectral properties of fluorescent analogues of tryptophan (part **a**) and
989 fluorescent amino acids (FIAAs) obtained by appending fluorophores to amino acids using a
990 broad range of chemical reactions (part **b**). ϕ_F , fluorescence quantum yield; Pd, palladium; Rh,
991 rhodium; CuAAC, copper-catalysed azide–alkyne cycloaddition.

992 **Figure 3. Synthetic schemes for the *de novo* preparation of non-natural FIAAs.** **a** | Design
993 of flavone-based fluorescent amino acids (FIAAs) building on tyrosine derivatization⁷¹⁻⁷³. **b** |
994 Pyrazoloquinazolines derived from L-aspartic acid as one-photon and two-photon
995 fluorophores⁷⁸. **c** | Synthesis of rotor fluorogenic D-amino acids (RfDAAs) for labelling
996 peptidoglycan structures in bacterial cells¹²². Boc, *tert*-butoxycarbonyl; DCM,
997 dichloromethane; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DIPEA, *N,N*-
998 diisopropylethylamine; DMF, *N,N*-dimethyl formamide; Fmoc-OSu, *N*-(9-
999 fluorenylmethyloxycarbonyl)succinimide; TFA, trifluoroacetic acid ; THF, tetrahydrofuran;
1000 Trt-Cl, triphenylmethyl chloride; TSTU, *N,N,N',N'*-tetramethyl-*O*-(*N*-succinimidyl)uronium
1001 tetrafluoroborate.

1002 **Figure 4. Bioactive fluorescent peptides for structural studies and optical imaging.** **a** |
1003 Schematic representation of the interaction between the HIV-1 nucleocapsid peptide W37
1004 (labelled with the flavone-based fluorescent amino acid (FIAA) M3HFaa) and
1005 oligonucleotides. The graph shows the fluorescence emission spectra of the W37–M3HFaa
1006 peptide on binding to different nucleic acids. **b** | Schematic illustration of the binding between
1007 a fluorescent peptide containing the FIAA 4-DMAP and a PDZ domain. The graph shows the
1008 fluorescence emission spectra of the 4-DMAP-containing peptide in the unbound state and
1009 bound to the PDZ domain. **c** | Illustration of the pH-(low) insertion peptide (pHLIP) containing

1010 4-cyanotryptophan (which acts as a FRET donor, and DiO-stained large unilamellar vesicles
1011 (LUVs), which act as FRET acceptors, for the study of peptide–membrane interactions. The
1012 graph depicts the FRET measurements taken at 512 nm with increasing concentrations of
1013 labelled pHLIP. **d** | Chemical structure of the Trp-BODIPY-labelled cyclic peptide PAF26 and
1014 live-cell confocal imaging of *Aspergillus fumigatus* over time. Scale bar: 2.5 μm . Multi-photon
1015 fluorescence microscopy images of *ex vivo* human lung tissue after incubation with RFP-
1016 expressing *A. fumigatus* (red) and the Trp-BODIPY-labelled PAF26 peptide (green). Second
1017 harmonic generation images are shown in cyan. Scale bar: 10 μm . Fluorescence lifetime images
1018 depicting autofluorescent tissue structures (white arrows) and a PAF26-stained *A. fumigatus*
1019 hypha (yellow arrows). Scale bar: 20 μm . **e** | pER peptide containing a self-immobilizing
1020 coumarin FIAA that mimics phosphotyrosine (pTyr) to report endogenous activity of protein
1021 tyrosine phosphatases (PTPs). The graph depicts time-dependent fluorescence measurements
1022 of the pER peptide before and after ultraviolet (UV) irradiation (365 nm) in HeLa cell lysates.
1023 Fluorescence microscopy images of HeLa cells after incubation with pER and UV irradiation
1024 to visualize intracellular PTP activity (green: pER peptide, red: commercial tracker). Part **a**
1025 reproduced with permission from ref. 73, American Chemical Society. Part **b** was reproduced
1026 with permission from ref. 88, American Chemical Society. Part **c** reproduced with permission
1027 from ref. 31, The Royal Society of Chemistry. Part **d** reproduced with permission from ref. 96,
1028 Springer Nature Limited. Part **e** reproduced with permission from ref. 99, The Royal Society
1029 of Chemistry.

1030 **Figure 5. Fluorescent D-amino acids for studying bacterial growth.** **a** | Peptidoglycans are
1031 polymers of L- and D-amino acids and sugars, and a major component of bacterial cell walls.
1032 The peptidoglycan layer is considerably thicker in Gram-positive than in Gram-negative
1033 bacteria. **b** | Chemical structures and optical properties of ‘clickable’ and fluorescent D-amino
1034 acids (FDAAs) used for labelling peptidoglycan structures in Gram-positive and Gram-
1035 negative bacteria. **c** | Time-lapse multicolour microscopy images of the bacterium *Streptomyces*
1036 *venezuelae* by pulsed-incubation with NBD-3-amino-D-alanine (NADA; green),
1037 tetramethylrhodamine-D-lysine (TDL; red), and 7-hydroxycoumarin-D-alanine (HADA; blue).
1038 **d** | Schematic representation of the fluorescence activation of the rotor FDAA Rf470DL on
1039 peptidoglycan labelling and fluorescence microscopy images of *Bacillus subtilis* depicting
1040 peptidoglycan labelling using Rf470DL (red) or HADA (cyan) before and after washing. Part
1041 **c** reproduced with permission from ref. 110, Wiley VCH. Part **d** reproduced with permission
1042 from ref. 122, Springer Nature Limited.

1043 **Figure 6. Genetically encoded site-specific incorporation of FIAAs in live cells.** **a** | Non-
1044 natural amino acids are site-specifically incorporated into proteins by assigning an amber stop
1045 codon (UAG) to non-natural amino acids and supplying the cells with an exogenous aminoacyl-
1046 tRNA synthetase (aaRS) and its corresponding tRNA, which charges the tRNA with the non-
1047 natural amino acid. **b** | Chemical structures of genetically-encoded fluorescent amino acids
1048 (FIAAs) and their optical properties. **c** | Adjusting the photophysical properties of a fluorescent
1049 protein. Site-specific incorporation of L-(7-hydroxycoumarin-4-yl)ethylglycine (CouA) at
1050 position 39 in cyan fluorescent protein (CFP), resulting in efficient FRET between CouA and
1051 CFP. When CouA (in blue) is excited at 360 nm, emission from the CFP fluorophore (in cyan)
1052 at 476 nm is observed. **d** | Mapping ion channel dynamics. 3-(6-acetylnaphthalen-2-ylamino)-
1053 2-aminopropanoic acid (ANAP) is site-specifically incorporated into both extracellular and
1054 intracellular positions (black circles) in a voltage-gated potassium ion channel. Voltage-
1055 dependent changes in the fluorescence emission of ANAP revealed information about the
1056 regions of the ion channel that move cooperatively on opening of the channel. Red spheres in
1057 the structure are potassium ions. Part **c** adapted with permission from ref. 137, American
1058 Chemical Society. Part **d** adapted with permission from ref. 149, National Academy of
1059 Sciences USA.

1060

1061 **ToC blurb**

1062 Fluorescent amino acids are widely used as building blocks for non-perturbative labelling of
1063 peptides and proteins. This Review covers recent advances in the design and synthesis of
1064 FIAAs with bespoke optical properties for different applications in biological studies.