

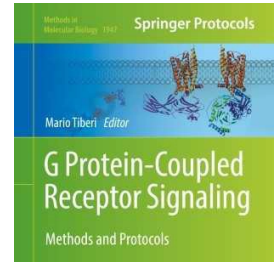
Sep 22, 2021

Cell-Free Protein Synthesis

In 1 collection

DOI

dx.doi.org/10.17504/protocols.io.bqnwmvfe



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DOI: dx.doi.org/10.17504/protocols.io.bqnwmvfe

External link: https://link.springer.com/protocol/10.1007/978-1-4939-9121-1_4

Protocol Citation: Anne Zemella, Theresa Richter, Lena Thoring, Stefan Kubick 2021. Cell-Free Protein Synthesis. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.bqnwmvfe>

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Protocol status: Working

Created: December 13, 2020

Last Modified: September 22, 2021

Protocol Integer ID: 45494

Keywords: Cell-free protein synthesis, G protein-coupled receptor, Protein modification, Non-canonical amino acids, Amber suppression, Confocal laser scanning microscopy,



Abstract

This is part 3.3 of the "A Combined Cell-Free Protein Synthesis and Fluorescence-Based Approach to Investigate GPCR Binding Properties" collection of protocols: <https://www.protocols.io/view/a-combined-cell-free-protein-synthesis-and-fluores-bqntmven>

Collection Abstract: Fluorescent labeling of de novo synthesized proteins is in particular a valuable tool for functional and structural studies of membrane proteins. In this context, we present two methods for the site-specific fluorescent labeling of difficult-to-express membrane proteins in combination with cell-free protein synthesis. The cell-free protein synthesis system is based on Chinese Hamster Ovary Cells (CHO) since this system contains endogenous membrane structures derived from the endoplasmic reticulum. These so-called microsomes enable a direct integration of membrane proteins into a biological membrane. In this protocol the first part describes the fluorescent labeling by using a precharged tRNA, loaded with a fluorescent amino acid. The second part describes the preparation of a modified aminoacyl-tRNA-synthetase and a suppressor tRNA that are applied to the CHO cell-free system to enable the incorporation of a non-canonical amino acid. The reactive group of the non-canonical amino acid is further coupled to a fluorescent dye. Both methods utilize the amber stop codon suppression technology. The successful fluorescent labeling of the model G protein-coupled receptor adenosine A2A (Adora2a) is analyzed by in-gel-fluorescence, a reporter protein assay, and confocal laser scanning microscopy (CLSM). Moreover, a ligand-dependent conformational change of the fluorescently labeled Adora2a was analyzed by bioluminescence resonance energy transfer (BRET).

For **Introduction and Notes**, please see: <https://www.protocols.io/view/a-combined-cell-free-protein-synthesis-and-fluores-bqntmven/guidelines>

Materials

2.3 Materials for Cell-Free Protein Synthesis and Fluorescent Labeling of Modified Adora2a

1. Coding sequence for the modified adenosine A_{2A} receptor (Uniprot: P29274, amino acids 1-340, with and without amber stop codon at nucleotide sequence coding for P215) with a receptor C-terminally fused Nanoluciferase (Fig. 1). Flanking sequences containing 5' regulatory sequences (T7 RNA polymerase promotor, Cricket paralysis virus (CRPV) IGR IRES sequence (Genbank accession no. AF218039, nucleotides 6025-6216)) and 3' regulatory sequences (T7 terminator), cloned with the coding sequence into a plasmid (BioCat or Thermo Fisher Scientific).
2. 1.5 ml reaction tubes.
3. CHO lysate prepared as described [19, 20] (*see Note 1*).
4. 10× translation mix: 300 mM HEPES-KOH (pH 7.6), 2250 mM KOAc, 2.5 mM spermidine, 1 mM of each canonical amino acid (Merck) and 39 mM Mg(OAc)₂.
5. 5× energy: 100 mM creatine phosphate, 1.5 mM GTP, 1.5 mM CTP, 1.5 mM UTP, 8.75 mM ATP and 0.5 mM m⁷G(ppp)G cap analogue.
6. 100 μM Polyguanylic acid (polyG, IBA).
7. T7 RNA polymerase (f.c. 1 U/μl).
8. ¹⁴C-leucine.
9. 100 μM Bodipy-TMR-lysine-tRNACUA (BP-CUA, biotechrabbit).
10. 100 μM Bodipy-TMR-lysine-tRNAGAA (BP-GAA, biotechrabbit).
11. 100 μM eAzFRS.
12. 100 μM tRNATyrCUA.
13. 100 mM p-propargyloxy-L-phenylalanine (pPa, Iris Biotech).
14. 5 mM Copper(II) sulfate (CuSO₄).
15. 2.5 mM Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, Iris Biotech).
16. 80 mM Sodium ascorbate (NaAsc).
17. Phosphate-buffered saline (PBS).
18. 100 μM Sulfo-Cy5-azide (Lumiprobe).

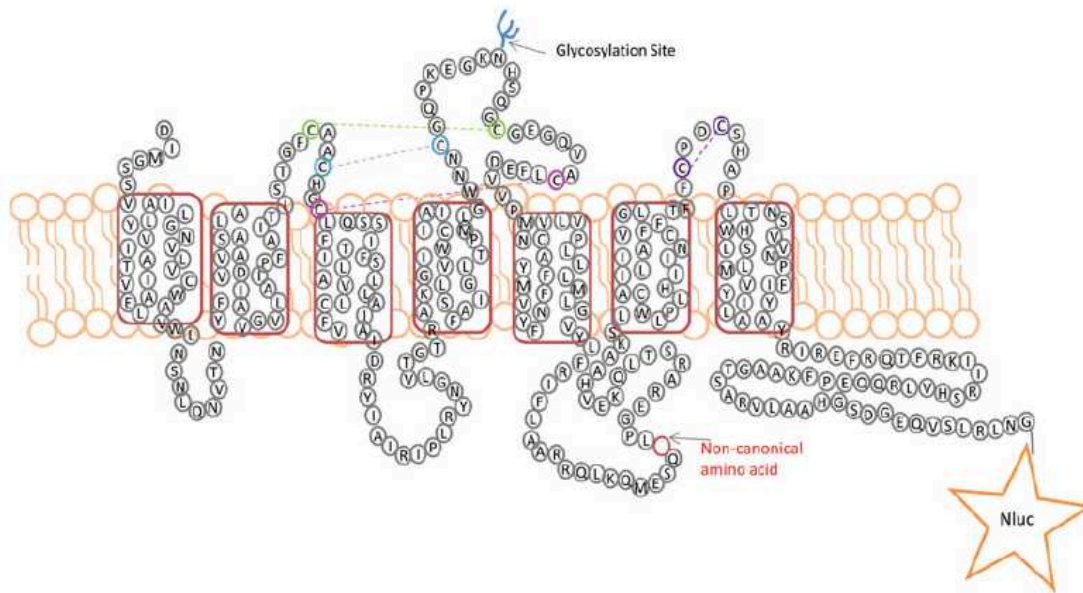










Fig. 1 Schematic illustration of the Adora2a receptor with a C-terminally fused Nluc. Arrows indicate the glycosylation site and the position of the non-canonical amino acid. Disulfide bridges are indicated by the colors green, blue, pink, and purple

Safety warnings









! For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

3.3 Cell-Free Protein Synthesis

- 1 Thaw all required components for cell-free protein synthesis  On ice (see **Note 8**).
- 2 The cell-free protein synthesis is performed in a coupled mode where transcription and translation reaction take place in one vessel. A standard reaction is composed of  40 % CHO lysate ,  10 micromolar (μM) polyG ,  1 x translation mix , and  1 x energy mix . Add plasmid at a concentration of  40 nanomolar (nM) (see **Note 9**). Add  1 U/ μL T7 RNA polymerase for the transcription reaction and ^{14}C -leucine (specific radioactivity of 66.67 dpm/pmol) for further analysis of de novo synthesized proteins. Fill the reaction with water to the final volume of  50 μL .



3.3.1 Fluorescent Labeling with Bodipy-TMR-Lysine

- 3 Thaw the Bodipy-TMR-lysine-tRNACUA (BP-CUA) and Bodipy-TMR-lysine-tRNAGAA (BP-GAA)  On ice and keep it dark (see **Note 10**).
- 4 Pipette  2 micromolar (μM) precharged tRNA to the cell-free reaction and incubate the prepared cell-free protein synthesis reaction by  27 $^{\circ}\text{C}$ for  03:00:00 and shaking at  600 rpm (see **Note 11**). Cover the thermomixer with a lid or aluminum foil to prevent the reaction of any light.
- 5 Take a  5 μL aliquot of the translation mixture for SDS-PAGE. Centrifuge the translation reaction at  16000 x g, 4 $^{\circ}\text{C}$, 00:15:00 . Take  5 μL of the supernatant and resuspend the pellet (microsomal fraction) in an equal volume PBS in comparison to the volume of the translation reaction. This step is required for the analysis of the localization of the synthesized protein. The expectations of the incorporation of the different Bodipy-tRNAs are described in Table 1 (see **Note 12**).

3h



15m



A	B	C	D
Construct	Precharged tRNA	Expectation	
		Synthesis	Fluorescence
Adora2a	BP-GAA	Full-length protein	Highly fluorescently labeled protein with statistical incorporation of ncAA
Adora2a	BP-CUA	Full-length protein	The full-length protein is not labeled with the

A	B	C	D
			fluorescent dye and the ncAA is not incorporated.
Adora2a_amb	BP-CUA	Full-length protein and partial termination product	The full-length protein is fluorescently labeled. The fluorescence signal has a lower intensity in comparison to the statistically labeled Adora2a due to the site-specific incorporation of the ncAA at one defined position. Termination product is not labeled.
Adora2a_amb	BP-GAA	Termination product	The termination product is highly fluorescently labeled due to the statistical incorporation of the ncAA. No full-length product is visible.

Table 1

Expectations of cell-free protein synthesis and fluorescent labeling of different Adora2a constructs with Bodipy-tRNA directed to phenylalanine codons (GAA) or directed to the amber stop codon (CUA)

- An example of the performance of the fluorescent labeling of Adora2a with or without incorporated non-canonical amino acid determined by in-gel-fluorescence and autoradiography, and the measurement of the reporter protein are shown in Fig. 2. In-gel-fluorescence and autoradiography (Fig. 2a, c) showed the expected results as described in Table 1. The reporter protein assay (Fig. 2b) is based on the activity of a receptor C-terminally fused Nanoluciferase (Nluc). Nluc activity can only be measured after the translation of the full-length fusion protein. The Adora2a synthesized by using the DNA construct without an amber stop codon (Adora2a + BP-GAA/ BP-CUA) resulted in a fusion protein with a detectable Nluc activity. The fusion protein translated from the amber stop codon DNA construct (Adora2a_amb + BP-CUA) showed a fivefold reduced Nluc activity. This result implicates a rather low incorporation efficiency of the precharged tRNA addressing the amber stop codon. The combination of Adora2a_amb with BP-GAA showed no luciferase activity since only the termination product was translated. Protein yields were calculated by scintillation counting and resulted in approximately [IM] 20 µg/ml in translation mixture. In the microsomal fraction and the supernatant, approximately [IM] 6 µg/ml and [IM] 14 µg/ml were detected, respectively. A similar Adora2a distribution was obtained by in-gel-fluorescence, luciferase activity, and autoradiography. Previously reported distributions of membrane proteins are comparable to the here described distribution [7]. A comparable protein yield was calculated for the termination product.

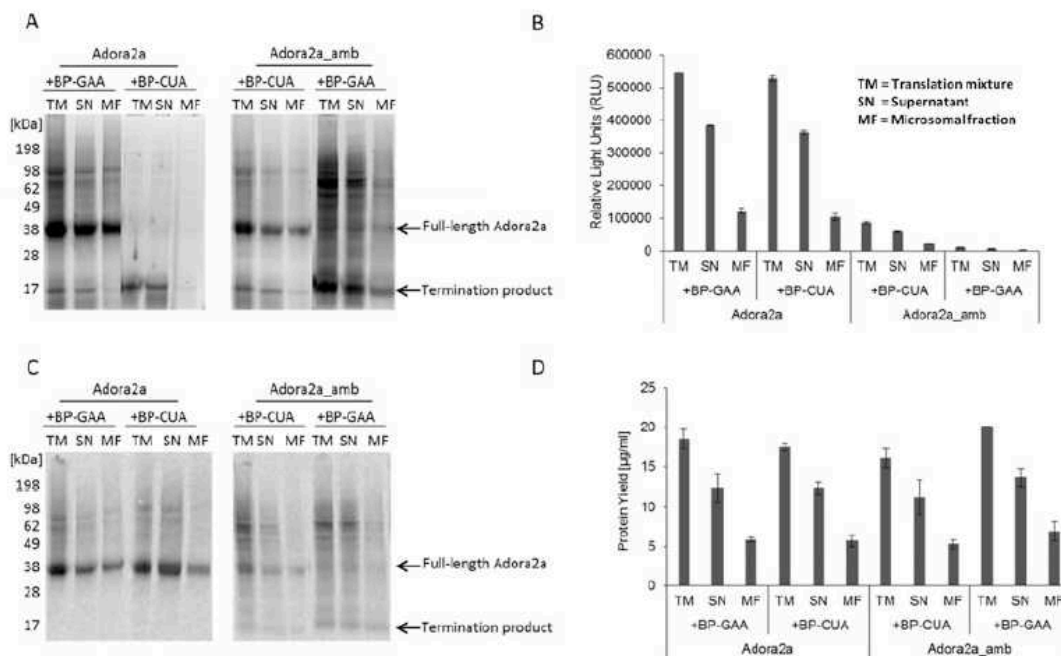


Fig. 2 Analysis of fluorescent labeling by using precharged tRNAs. **(a)** In-gel-analysis of Adora2a by statistical and site-specific incorporation of a fluorescently labeled amino acid. The translation mixture (TM) was fractionated into supernatant (SN) and microsomal fraction (MF). **(b)** Analysis of the translation of a reporter protein (Nluc) fused to the C-terminus of Adora2a by measuring the luminescence of the Nluc. **(c)** Autoradiographic analysis of de novo synthesized Adora2a by incorporation of ^{14}C -leucine during the cell-free synthesis reaction. **(d)** Determination of protein yield of de novo synthesized Adora2a by scintillation counting

3.3.2 Analysis of Ligand- Dependent Conformational Change Using a BRET Assay

- 7 Site-specifically label the Adora2a_amb using the precharged tRNA Bodipy-TMR-lysine-tRNACUA as described in section 3.3.1 above, steps 3–5.
- 8 Resuspend the microsomal fraction of the Adora2a_amb in PBS. 5 μL aliquots of resuspended Adora2a_amb were mixed with 5 μL adenosine in PBS with final concentrations of [IM] 0 micromolar (μM) , [IM] 100 micromolar (μM) , [IM] 1000 micromolar (μM) , and [IM] 5000 micromolar (μM) adenosine (see **Note 13**).
- 9 10 μL samples were applied for the luminescence and fluorescence measurement. In a first step, the luminescence of the Nluc was detected using an OD2 filter. In a second step, the fluorescence of the coupled Bodipy dye, excited by the Nluc emission was detected.
- 10 As a control 5 μL of the resuspended microsomal fraction of the full-length Adora2a protein without any fluorescent label is treated with the same concentrations of

adenosine (**step 8**) to determine background fluorescence caused by the broad emission spectrum of the Nluc and possible interactions of adenosine with the Nluc.

11 The BRET ratio is calculated as follows:

$$BRET_{ratio} = \frac{Fluorescence (sample)}{Luminescence (sample)} - \frac{Fluorescence (control)}{Luminescence (control)}$$

12 Herein, the calculated BRET ratio (Fig. 3) showed a change in the relation of the fluorescence of the Bodipy in comparison to the Nluc luminescence after the addition of different adenosine concentrations. Only a minimal increase of the BRET ratio can be seen after addition of higher adenosine concentrations (above 100 micromolar (μ M)) indicating that at lower concentrations all receptors are occupied with adenosine. The result indicates a conformational change of the helix III and the connected third intracellular loop that is expected for the Adora2a.

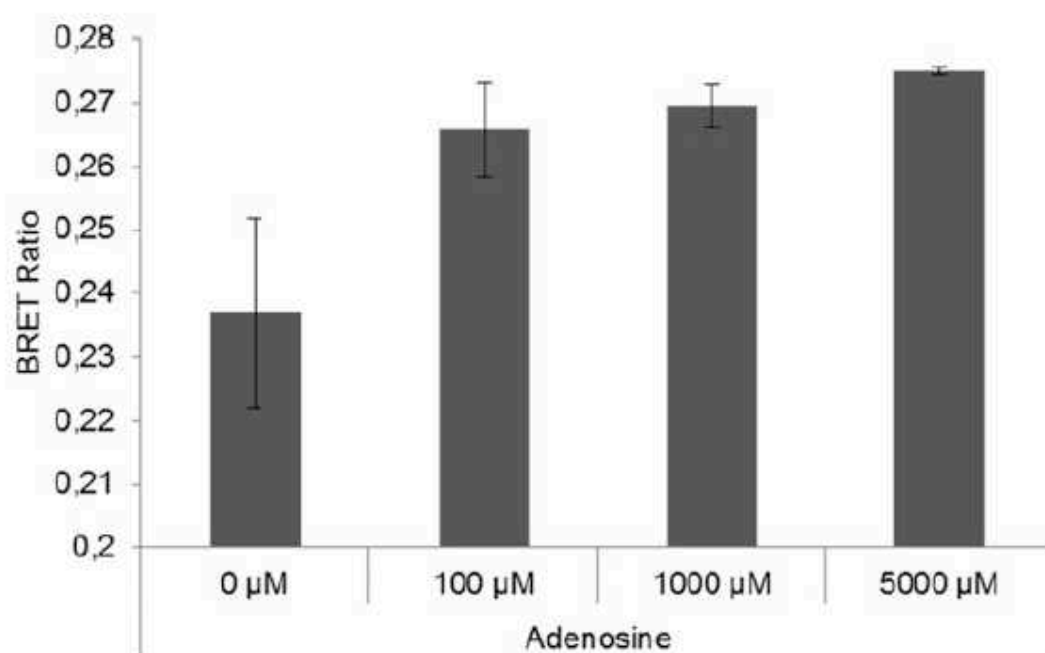


Fig. 3 BRET signal of the fluorescently labeled Adora2a_amb after adenosine binding. Cell-free synthesized and fluorescently labeled Adora2a_amb was subjected to luminescence and fluorescence analysis in presence of different adenosine concentrations (0–5000 μ M). The ratio of the Bodipy fluorescence to the luminescence of the Nluc was calculated and subtracted by a control ratio

3.3.3 Site-Specific Incorporation of a Non-canonical Amino Acid with Subsequent Fluorescent Labeling and Microscopic Analysis

4h



- 13 Additional components are required for the recharging of the suppressor tRNA and a subsequent incorporation of a non-canonical amino acid. Therefore, add the p-propargyloxy-L-phenylalanine, tRNA^{Tyr}CUA, and eAzFRS in a specific order (Table 2) (see **Note 14**).

A	B	C
Order	Components	Final concentration
1	Water	
2	PolyG	10 μ M
3	Translation mix	1×
4	pPa	2 mM
5	DNA-template	40 nM
6	Lysate	40%
7	tRNA ^{Tyr} CUA	3 μ M
8	eAzFRS	3 μ M
9	¹⁴ C-leucine	66.67 dpm/pmol
10	T7-RNA-Polymerase	1 U/ μ l
11	Energy mix	1×

Table 2

Pipetting order of a standard cell-free reaction with orthogonal components for the incorporation of non-canonical amino acids

- 14 Incubate the prepared cell-free reaction at 27 °C for 03:00:00 by gentle shaking at 600 rpm . Keep the reaction in the dark (see **Note 15**).
- 15 The membrane protein is translocated and integrated into the microsomal membrane during the cell-free reaction. Therefore, separate the microsomal fraction by centrifugation at 16000 x g, 4°C, 00:15:00 . Resuspend the pellet fraction in PBS. Use an equal volume of the cell-free reaction for resuspension.
- 16 For the labeling reaction prepare the labeling mix as follows: combine 200 micromolar (μ M) **CuSO₄** with 600 micromolar (μ M) THPTA , 5 millimolar (mM) NaAsc , PBS and a final concentration of 3 micromolar (μ M) Sulfo-Cy5-azid to a final volume of 5 μ L . Add 5 μ L of the resuspended microsomal fraction to the labeling mix. Incubate the labeling reaction at Room temperature for 01:00:00 . Keep the reaction dark (see **Note 16**).



3h



1h





- 17 Centrifuge the labeling reaction for  16000 x g, 4°C, 00:15:00 . Discard the supernatant and resuspend the pellet in  10 µL PBS . This step removes excess fluorescent dye to decrease the background signal in the subsequent fluorescent analyses.
- 18 The analysis of the synthesis of the full-length protein by using the Adora2a construct with or without an amber stop codon was performed by autoradiography and a reporter protein assay (Fig. 4). The autoradiography shows for both synthesis reactions a similar band pattern (Fig. 4a). Interestingly, the incorporation of pPa led to a comparable band signal as obtained for the full-length protein translated from the DNA construct without an amber stop codon. This result implicates a high incorporation efficiency of the non-canonical amino acid. In addition, no termination product is detected in the autoradiograph. The high incorporation efficiency is the basis for a further coupling reaction to a fluorescent dye. In addition, this result is supported by the Nluc assay (Fig. 4b). The measured luciferase activity of the suppression product reaches up to 80% of the luciferase activity of the full-length product. The protein yield and protein distribution (Fig. 4c) is comparable to the previously described results (Fig. 2, see section 3.3.1 above "Fluorescent Labeling with Bodipy-TMRLysine", **step 6**).

15m



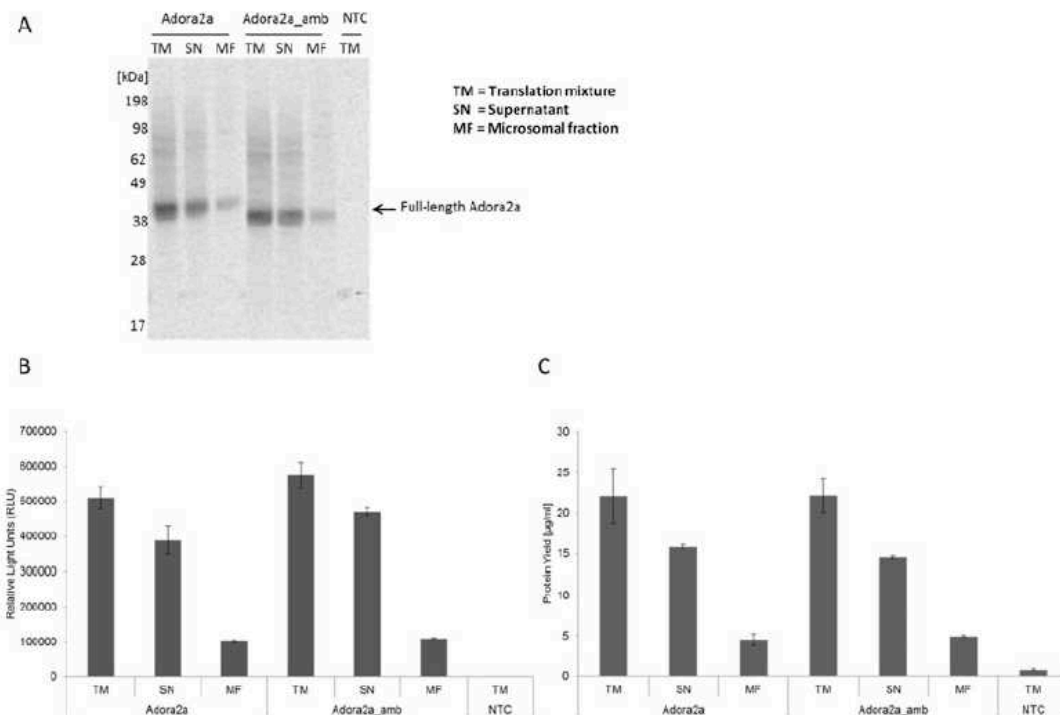


Fig. 4 Analysis of incorporation efficiency using an orthogonal system. **(a)** Autoradiography of synthesized Adora2a after incorporation of ^{14}C -leucine. Translation mixture (TM) was fractionated into supernatant (SN) and microsomal fraction (MF). **(b)** Schematic illustration of subsequent labeling reaction after reaching a high incorporation of the non-canonical amino acid. **(c)** Analysis of the translation of a reporter protein (Nluc) fused to the C-terminus of Adora2a by measuring the luminescence of the luciferase. NTC = no template control. **(d)** Protein yield of synthesized Adora2a, determined by scintillation counting

- 19 Fluorescently labeled GPCRs should be detectable in microsomal structures (Fig. 5). It is recommended to visualize the labeled sample first by in-gel-fluorescence (Fig. 5, left panel). The synthesis reaction in the presence of all orthogonal components led to a specific band at the expected molecular weight. The control reaction without addition of the modified synthetase resulted in no visible band. The success of the microscopic analysis highly correlates to the quality of the in-gel-fluorescence. High background fluorescence during ingel-analysis often results in unspecific staining of the microsomes. The microscopic analysis clearly shows a difference in the fluorescence intensity of the labeled Adora2a and the unspecific staining of the microsomes (right panel).

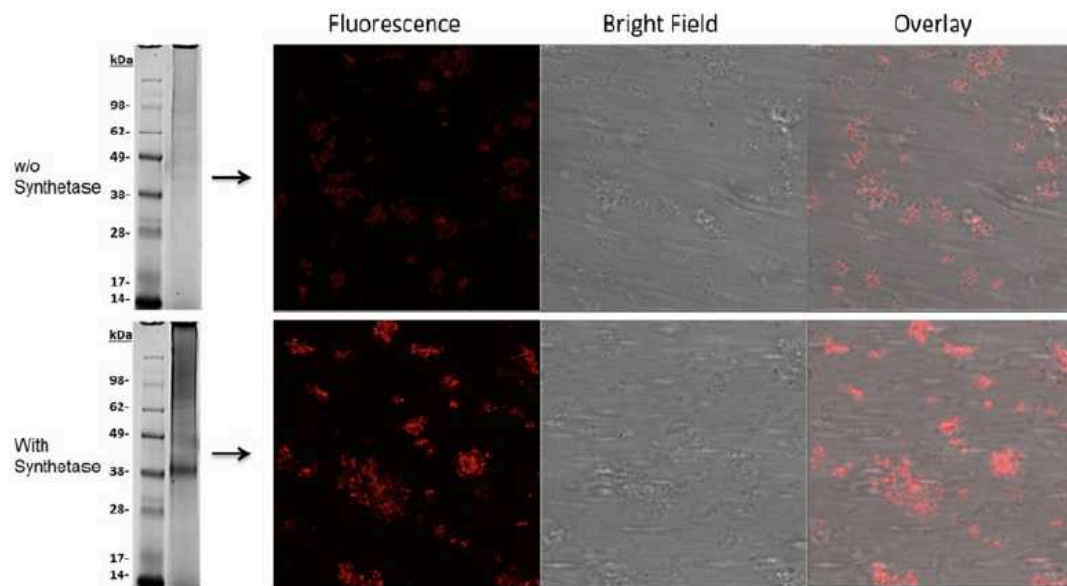


Fig. 5 Fluorescence analysis of labeled Adora2a. Left side: In-gel-fluorescence of synthesized and with Sulfo-Cy5-azide labeled Adora2a in absence or presence of the modified synthetase during the cell-free protein synthesis reaction. Right side: confocal laser scanning microscopy of the control sample (without addition of the modified synthetase during cell-free protein synthesis) and the labeled Adora2a (cell-free protein synthesis in presence of the synthetase)