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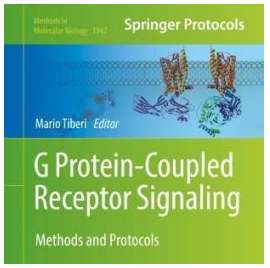
Analysis of De Novo Synthesized Proteins



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Abstract

This is part 3.4 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

2.4 Materials for Analysis of De Novo Synthesized Proteins

1. Trichloroacetic acid (TCA).
2. Water bath.
3. Glass fiber filters.
4. Acetone.
5. Scintillation vials.
6. Scintillation cocktail.
7. Scintillation counter.
8. SDS-PAGE Sample buffer: 1× LDS buffer containing 106 mM Tris–HCl, 141 mM Tris base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G, 0.175 mM Phenol Red, pH 8.5 with 50 mM DTT.
9. SDS-PAGE gels.
10. Fluorescently labeled protein ladder for SDS-PAGE.
11. Fluorescence/phosphorimager.
12. Gel dryer.
13. Phosphorscreens.
14. Adenosine.
15. Nano-Glo® Luciferase Assay System.
16. 96-well microtiter plate.
17. Multimode Microplate Reader Mithras^2 LB 943.
18. µ-Ibidi-Slide (µ-Slide 18 well, flat, Ibidi).
19. Confocal laser scanning microscope.

Troubleshooting

Safety warnings

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

3.4.1 TCA Precipitation and Scintillation Counting

1 After the reaction is completed collect 2 × 3 µL translation mixture. Centrifuge the remaining mix at 16000 x g, 4°C, 00:15:00 and collect 2 × 3 µL supernatant. Resuspend the microsomal fraction in an equal volume of PBS in comparison to the volume of the translation mixture. Collect 2 × 3 µL microsomal fraction.

2 Mix each aliquot with 3 mL TCA and incubate in a water bath at 80 °C for 00:15:00. Store the aliquots for 30 °C On ice or Overnight at 4 °C. 30m

3 The mixture is applied to a vacuum filtration system to separate non-incorporated ^{14}C-leucine from the radioactively labeled protein. Filters with the collected protein are washed *twice* with TCA and *twice* with acetone. Dry the filters under the hood.

4 The filters are transferred into the scintillation vials and overlaid with 3 mL scintillation cocktail. After an incubation time of 01:00:00 with gentle shaking, scintillation vessels are counted in scintillation counter. 1h

3.4.2 In-Gel- Fluorescence and Autoradiography

5 For preparation of SDS-PAGE samples take a 5 µL aliquot (**part 3.3**, section 3.3.1 "Fluorescent Labeling with Bodipy-TMRLysine", **steps 3–5**) or 10 µL site-specifically labeled aliquot (**part 3.3**, section 3.3.3 "Site-Specific Incorporation of a Non-canonical Amino Acid with Subsequent Fluorescent Labeling and Microscopic Analysis", **steps 13–17**) of each prepared sample.

6 Add 45 µL water and 150 µL cold acetone to the 5 µL or 10 µL aliquots and incubate for 00:15:00 On ice. Keep the fluorescently labeled samples in dark during the whole procedure. Centrifuge the samples at 16000 x g, 4°C, 00:10:00 and discard the supernatant. 15m

7 Dry the pellets for 01:00:00 at 45 °C in a thermo mixer with a shaking speed of 1000 rpm. 1h

8 Resuspend the dried pellets in 20 µL SDS-PAGE sample buffer and load the samples on a prepared [M] 10 % SDS-PAGE gel. Use a ladder with fluorescently labeled bands. Run the gel.

9 Transfer the gel to the fluorescence imaging system and detect the labeled protein bands. For Bodipy-TMR-lysine use a 532 nm laser and a 580 nm emission filter. Sulfo-Cy5 can be detected with extinction at 633 nm and emission at 670 nm.

10 Afterwards dry the gel for 01:00:00 at 70 °C using a unigeldryer. The dried gels are exposed on a phosphorscreen for minimal 3 days and read out using a multi-mode imager. 1h

3.4.3 Confocal Laser Scanning Microscopy

11 For confocal laser scanning microscopy use 5 µL fluorescently labeled protein in the microsomal fraction and dilute the sample in 20 µL PBS. Add the mixture to a µ-Ibidi-Slide.

12 Fix the slide. Use a plan-apochromat objective with a 60× or 100× magnification. Microsomal structures usually have a diameter of 1–10 µm.

13 Adjust the beam path to the coupled fluorescent dye. Standard dyes such as Cy5 and FITC usually have a preset configuration. Cy5 is excited at 633 nm and the emission is detected with a long-pass filter above a wavelength of 670 nm.

14 Adjust the microscope settings (laser intensity, gain master, focus, pinhole) according to the individual sample (*see* **Note 17**).