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Detecting Purinosome Metabolon Formation with Fluorescence Microscopy

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Abstract

A long-standing hypothesis in the de novo purine biosynthetic pathway is that there must be highly coordinated processes to allow for enhanced metabolic flux when a cell demands purines. One mechanism by which the pathway meets its cellular demand is through the spatial organization of pathway enzymes into multienzyme complexes called purinosomes. Cellular conditions known to impact the activity of enzymes in the pathway or overall pathway flux have been reflected in a change in the number of purinosome-positive cells or the density of purinosomes in a given cell. The following general protocols outline the steps needed for purinosome detection through transient expression of fluorescent protein chimeras or through immunofluorescence in purine-depleted HeLa cells using confocal laser scanning microscopy. These protocols define a purinosome as a colocalization of FGAMS with one additional pathway enzyme, such as PPAT or GART, and provide insights into the proper identification of a purinosome from other reported cellular bodies.

Keywords

Purinosome; Metabolon; Purine metabolism; De novo purine biosynthesis; Fluorescence microscopy

1 Introduction

Our current understanding of enzymes can be credited to the tools and techniques of traditional in vitro enzymology. However, the removal of an enzyme from a cellular environment has largely downplayed those regulatory events that might contribute to the innate activity or behavior of an enzyme. These factors could include posttranslational modifications, ancillary protein-mediated allosteric modulation, and spatial organization. Therefore, the generation of intracellular reporters has provided a means to better understand how an enzyme functions within a cell and has brought to light the era of in-cell enzymology.

One way in-cell enzymology has reshaped our knowledge of enzymes is through the spatial organization of sequential metabolic pathway enzymes into supramolecular clusters called metabolons [1]. Since the initial observation of metabolon formation among enzymes in the tricarboxylic acid cycle [2], metabolons have been observed in glycolysis [3, 4], amino acid biosynthesis [5], and the de novo biosynthesis of purines and pyrimidines [6, 7]. Several of these metabolons were hypothesized for decades, but traditional in vitro techniques did not provide compelling evidence for their existence. Ultimately, the translation of commonly

employed fluorescence microscopy techniques rapidly developed a tool belt in which one can effectively study these metabolons [8].

Here, we outline a method for visualizing a metabolon comprising all six enzymes within the de novo purine biosynthetic pathway by confocal laser scanning microscopy. The spatial organization of these enzymes in cells is referred to as a purinosome and has been the subject of recent reviews [9, 10]. Purinosome assembly has shown to be a reversible phenomenon whose phenotype is largely predominant when cellular conditions result in a high purine demand, such as in the G_1-phase of the cell cycle [6, 11]. Cellular conditions favoring purinosome formation were also shown to enhance the metabolic flux of the de novo purine biosynthetic pathway suggesting that the two observations are connected—a generalized hypothesis surrounding metabolon formation in cells [12]. Further characterization of purinosomes has unveiled a high degree of colocalization with cytoskeletal elements [13] and mitochondria [14] as well as interactions with molecular chaperones [15]. The interplay between all these different cellular elements has presented the purinosome as a highly regulated complex, whose composition and cellular localization have started to provide a fresh and more comprehensive perspective on the regulation of purine metabolism otherwise not readily recognized by more traditional means.

2 Materials

The original discovery and characterization of purinosomes were performed in the HeLa CCL-2 cervical carcinoma cell line under purine-depleted growth conditions [6]. Since then, purinosomes have been observed under similar growth conditions in human hepatocarcinoma liver cell line HepG2 [16] and its derivative C3A [15], sarcoma osteogenic cell line Saos-2 [16], human embryonic kidney cell line HEK293 [16], human skin cancer cell line A431 [15], human breast cancer cell line HTB-126 [6], primary human keratinocytes [16], and primary human dermal fibroblasts [11, 17]. The diversity in cell types bearing purinosomes, observed by transient expression of fluorescent chimeras of enzymes and/or immunofluorescence, illustrates that purinosome formation is a generalized phenomenon to likely denote elevated pathway usage and not limited to one cell type or genetic background.

The following list of materials has been validated for detecting purinosomes in purine-depleted HeLa CCL-2 cervical carcinoma cells. For these methods, a purinosome is defined as the colocalization of FGAMS (also referred to as PFAS) with one additional pathway enzyme (PPAT or GART) *(see* **Note 1**). Other combinations of plasmids and antibodies can be used to define a purino- some; however, at least two pathway enzymes should be imaged concurrently. If not, differentiating purinosomes from other cellular bodies, such as a recently discovered inhibitory FGAMS enzyme cluster, void of other pathway enzymes, might not be possible [18].

2.1 Materials for Cell Culture and Imaging

1. General mammalian cell culture disposables and instrumentation.
2. HeLa CCL-2 cervical carcinoma cell line (American Type Culture Collection).

3. 35 mm glass bottom tissue culture-treated culture dish.
4. 1× Dulbecco's phosphate buffered saline (without calcium and magnesium) solution (D-PBS).
5. 0.25% Trypsin with 2.21 mM ethylenediaminetetraacetic acid (EDTA).
6. Purine-depleted complete growth medium: Roswell Park Memorial Institute (RPMI) 1640 supplemented with 300 mg/L L-glutamine and 10% (v/v) dialyzed fetal bovine serum (FBS) *(see* **Note 2**).
7. Olympus Fluoview 1000 confocal laser scanning microscope equipped with appropriate lasers and filters for the selected fluorescent dyes and proteins.
8. ImageJ image analysis and visualization software [19].

2.2 Detection of Purinosomes in Living Cells Using Transient Expression of Fluorescently Labeled Protein Chimeras

1. Gibco™ Opti-MEM™ reduced serum medium or Eagle's Minimum Essential Medium (MEM) without fetal bovine serum.
2. Lipofectamine® 2000 transfection reagent.
3. Endotoxin-free plasmids encoding genes for FGAMS-EGFP and PPAT-mCherry *(see* **Note 3**).
4. Hank's Balanced Salt Solution (HBSS): 8.0 g/L sodium chloride, 400 mg/L potassium chloride, 140 mg/L calcium chloride, 1 g/L glucose, 60 mg/L potassium phosphate monobasic, 48 mg/L sodium phosphate dibasic anhydrous, 350 mg/L sodium bicarbonate, 98 mg/L magnesium sulfate anhydrous.
5. Optional: Hoechst 33342 (2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1 H-benzimidazole trihydrochloride trihydrate) counterstain. Prepare a 1 μg/mL solution diluted in complete growth medium *(see* **Note 4**).

2.3 Immunofluorescence Detection of Endogenous Purinosomes in Fixed HeLa Cells

1. Fixative solution: 4% (v/v) electron microscopy grade paraformaldehyde in l× D-PBS.
2. Permeabilization solution: 0.1% (v/v) Triton X-100 in l× D-PBS.
3. Wash buffer (PBST): 0.1% (v/v) Tween-20 in l× D-PBS.
4. Blocking buffer: 5% (v/v) normal donkey serum (serum of secondary antibody host) in PBST.
5. Primary antibody solution: 1:500 dilution of PFAS rabbit polyclonal antibody (Bethyl Laboratories) and 1:1000 dilution of GART mouse monoclonal antibody (Novus Biologicals) prepared in blocking buffer.
6. Secondary antibody solution: 1:1000 dilution of CF488A-conjugated donkey anti-rabbit immunoglobulin and 1:1000 dilution of CF568-conjugated donkey anti-mouse immunoglobulin prepared in blocking buffer.

7. Optional: DAPI (4′,6-diamidino-2-phenylindole) counterstain: 1:1000 dilution of a 300 μM DAPI solution prepared in 1× D-PBS into the secondary antibody solution.

3 Methods

The following methods serve as a starting point for detecting purinosome formation in purine-depleted HeLa CCL-2 cervical carcinoma cells. Optimization of transfection or immunostaining experimental conditions may be warranted for best results. Experimental notes have been added to assist in areas where optimization is often suggested. Further tips on optimizing transfection efficiency and/or cell viability post-transfection can be found on manufacturer's websites.

3.1 Detection of Purinosomes in Living Cells Using Transient Expression of Fluorescently Labeled Protein Chimeras

1. The day before transfection, seed purine-depleted HeLa cells at 0.8–1.0 × 10^5 cells per 35 mm glass bottom tissue culture-treated dish. Incubate the cells overnight at 37 °C under 5% CO2 in purine-depleted growth medium.
2. The next day, check the cell confluency under an inverted microscope. Optimal results (transfection efficiency and cell viability) are obtained when cells are approximately 70–80% confluent the day of transfection.
3. Add 2.0 μg of endotoxin-free pFGAMS-EGFP and 2.0 μg of endotoxin-free pPPAT-mCherry (4.0 μg total) plasmids to 50 μL of Opti-MEM™ reduced serum medium in a microcentrifuge tube. Pipet up and down to mix. Let sit for 5 min at room temperature.
4. Add 4 μL of 1 mg/mL Lipofectamine® 2000 solution to 50 μL Opti-MEM™ reduced serum medium in a microcentrifuge tube. Pipet up and down to mix. Let sit for 5 min at room temperature.
5. After 5 min incubation of both the plasmid and Lipofectamine® 2000 solutions, add the Lipofectamine® 2000 solution to the plasmid solution. Pipet up and down to mix *(see* **Note 5**).
6. Carefully remove growth medium and wash the cells once with 1× D-PBS.
7. Add 1 mL of Opti-MEM™ medium to the 35 mm glass bottom dish. Swirl to cover the entire bottom of the dish.
8. Add the lipid:DNA mixture (100 μL) to the cells dropwise. Gently swirl to mix.
9. Incubate the cells for 4 h at 37 °C (5% CO_2).
10. After 4 h, carefully remove all Opti-MEM™ medium from the culturing dish. Replenish cells with enough purine-depleted growth medium to cover the bottom of the dish (1–2 mL) *(see* **Note 6**).
11. Incubate the cells at 37 °C (5% CO_2) for an additional 16–18 h.

12. Remove the cells from the incubator and look for adherence under an inverted microscope. No significant cell death (>25%) should be observed.

13. Optional: Carefully remove growth medium and replace with a 1 μg/mL Hoechst 33342 solution prepared in purine- depleted complete growth medium. Incubate for 20 min at 37 °C (5% CO_2) for an effective nuclear counterstain.

14. Carefully remove the purine-depleted growth medium from the cells, and wash the cells once with HBSS.

15. Carefully aspirate the HBSS from the cells and replace with enough HBSS to cover the bottom of the dish (1–2 mL) *(see* **Note 7**).

16. Immediately image cells using a confocal laser scanning microscopy using filters appropriate for detecting EGFP and mCherry fluorescent proteins. If time-lapse imaging is desired, use a live cell imaging medium such as FluoroBrite DMEM medium instead of HBSS. Representative images are shown in Fig. 1 *(see* **Note 8** for further definition of the purinosome based on image analyses).

3.2 Immunofluorescence Detection of Endogenous Purinosomes in Fixed HeLa Cells

1. The day before fixation, seed purine-depleted HeLa cells at 6.0–8.0 × 10^4 cells per 35 mm glass bottom tissue culture-treated dish. Incubate the cells overnight at 37 °C under 5% CO_2 in purine-depleted growth medium.

2. The next day, verify cell adherence under an inverted microscope.

3. Carefully aspirate the medium away from the cells and wash the cells twice with enough 1× D-PBS to cover the bottom of the dish (1–2 mL).

4. Add 200 μL of fixative solution dropwise to the cells *(see* **Note 9**).

5. Incubate the samples in the fixative solution covered for 10 min at room temperature.

6. Carefully aspirate the fixative solution from the cells and wash the cells three times with enough 1× D-PBS to cover the bottom of the dish (1–2 mL). Each wash should last at least 5 min and be carried out on an orbital shaker at room temperature *(see* **Note 10**).

7. Add 200 μL of permeabilization solution dropwise to the cells.

8. Incubate the samples in the permeabilization solution for 10 min at room temperature on an orbital shaker.

9. Carefully aspirate the permeabilization solution from the cells, and wash the cells three times with enough 1× D-PBS to cover the bottom of the dish (1–2 mL). Each wash should last at least 5 min and be carried out on an orbital shaker at room temperature.

10. Block the cells with 200 μL of blocking buffer for 1 h at room temperature on an orbital shaker.

11. Carefully aspirate the blocking buffer, and add 200 μL primary antibody solution. For co-staining of FGAMS and GART, use a 1:500 dilution of PFAS rabbit polyclonal antibody and a 1:1000 dilution of GART mouse monoclonal antibody prepared in blocking buffer.

12. Incubate the samples in the primary antibody solution overnight at 4 °C on an orbital shaker. It is best practice to keep the samples covered to maintain dish humidity and minimize evaporation *(see* **Note 11**).

13. The next day, carefully aspirate the primary antibody solution from the cells and wash the cells four times with enough wash buffer (PBST) to cover the bottom of the dish (1–2 mL). Each wash should last at least 5 min and be carried out on an orbital shaker at room temperature.

14. Carefully aspirate the blocking buffer, and add 200 μL secondary antibody solution. For co-staining of FGAMS and GART, use a 1:1000 dilution of CF488A-conjugated donkey anti-rabbit IgG and a 1:1000 dilution of CF568-conjugated donkey anti-mouse IgG prepared in blocking buffer.

15. Optional: Add DAPI (300 nM final solution) to the same secondary antibody solution for an effective nuclear counterstain.

16. Incubate the samples in the secondary antibody solution for 2 h at room temperature on an orbital shaker. From this point on, all samples should be covered to prevent any photobleaching of the fluorescently labeled secondary antibodies.

17. Carefully aspirate the secondary antibody solution from the cells, and wash the cells four times with enough wash buffer (PBST) to cover the bottom of the dish (1–2 mL). Each wash should last at least 5 min and be carried out on an orbital shaker at room temperature.

18. Carefully aspirate the wash buffer from the cells and wash twice with enough 1× D-PBS to cover the bottom of the dish to remove any excess Tween-20. Each wash should last at least 5 min and be carried out on an orbital shaker at room temperature.

19. Add 1 mL of 1× D-PBS to the fixed cells.

20. Image the cells using a confocal laser scanning microscope equipped with filters appropriate for detecting CF488A and CF647 fluorescent dyes. Representative images are shown in Fig. 2.

4 Notes

1. Historically, FGAMS (also referred to as PFAS) has been used as the intracellular marker to denote purinosomes. While all the pathway enzymes have been shown to colocalize with FGAMS, the best combinations, based on reagents available, are between FGAMS and one of the other “core” purinosome proteins, PPAT, and GART [20].

2. HeLa cells are grown under purine-depleted growth conditions for at least two to three passages prior to purinosome detection for optimal results. Note that the doubling time of purine-depleted HeLa cells is approximately 28–32 h compared to HeLa cells cultured under normal growth conditions (approximately 20–24 h). Dialyzed FBS is prepared by extensively dialyzing FBS against 0.9% (w/v) sodium chloride prepared in water using a 10 kDa molecular weight cutoff dialysis membrane.

3. Other plasmids may be used for the detection of purinosomes; however, we strongly recommend FGAMS (PFAS) as one of the transient expressing proteins. All fluorescent protein chimeras of pathway enzymes are C-terminal fusions with the exception of ATIC, where N-terminal fusions are required to prevent disruption of dimerization and enzyme activity. Molecular cloning details can be found in [6].

4. While both DAPI and Hoechst 33342 are popular counterstains for nuclei, Hoechst 33342 works best for live cells, whereas DAPI works best when cells have been fixed and permeabilized. Therefore, we recommend using Hoechst 33342 for live cell imaging (transient transfection-based methods) and DAPI for immunofluorescence-based detection of purinosomes.

5. Older manufacturer's (Invitrogen) protocols suggest a 20 min incubation with both solutions prior to adding the lipid:DNA mixture to the adherent cells. Newer protocols have eliminated the need for this incubation step. Both methods have been used with no detectable difference in transfection efficiency or number of purinosome-positive cells.

6. Opti-MEM™ is a modified form of Eagle's Minimum Essential Medium (MEM) that contains hypoxanthine. Long-term incubation of purine-depleted HeLa cells in this medium may result in loss of purinosome formation. Therefore, Opti-MEM™ must be swapped out with purine-depleted growth medium to achieve optimal results. If this is a concern or does not yield appropriate purinosome formation, try MEM without FBS instead of Opti-MEM™ medium.

7. At this point, the cells can be fixed to preserve purinosome complexation *(see* **steps 2-5** in Subheading 3.2) until imaging by confocal laser scanning microscopy can be performed. Be aware that extended period of time post-fixation might result in decreased fluorescence intensity of the fluorescent protein chimeras. It is best to perform the imaging immediately.

8. Caution must be taken when defining the purinosome metabolon in transient transfected models. Extraction of physical parameters from areas of high colocalization has provided a way to properly identify the purinosome from other well characterized non-membrane-bound cytoplasmic cellular bodies such as processing bodies (P-bodies), stress granules, and aggresomes [10]. These parameters include the overall purinosome diameter and density or number of purinosomes in a cell. Based on an analysis of over 200 purinosome containing HeLa cells, a purinosome has been defined as a cellular body showing

colocalization between FGAMS and another pathway enzyme (such as PPAT or GART), having an FGAMS particle diameter between 0.2 and 0.9 μm and encompassing 50–1000 purinosomes per cell [11]. These features can be extracted from images collected and processed through an analysis and visualization software like ImageJ as previously described [11]. Alternatively, co-transfection of other cellular markers (GFP-G3BP, GFP170*, GFP250) can be used to differentiate the purinosome from known stress granules and aggresomes in a similar fashion as outlined in Subheading 3.1 [15].

9. Paraformaldehyde is a neurotoxin and should be handled only in a biosafety cabinet or hood with appropriate personal protective equipment. Paraformaldehyde is also light sensitive and will degrade over time, so to prevent degradation during storage or use, cover all aliquots and samples in aluminum foil.

10. For weakly adherent cells, agitation during the washing steps could result in detachment of the cells. In those cases, carefully add the wash buffer to the side of the 35 mm glass bottom dish dropwise and do not perform washes on an orbital shaker.

11. Incubation of fixed cells with primary antibodies targeting FGAMS and GART can also be performed at room temperature for 3–4 h without detectable differences in immunostaining.

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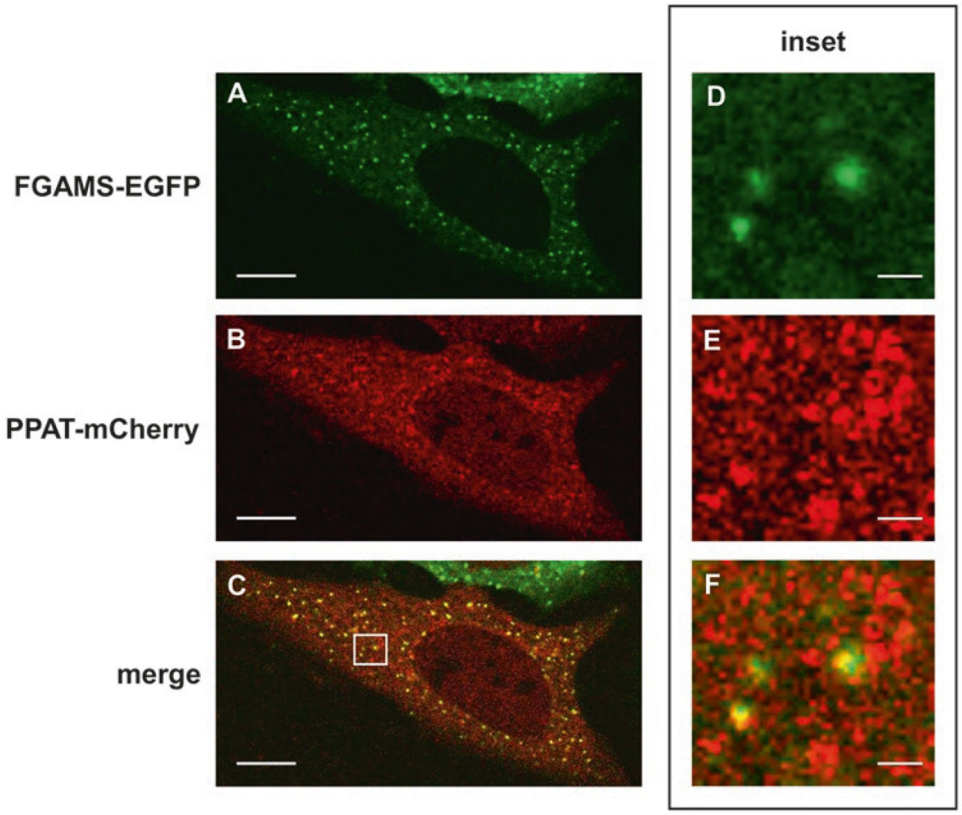

Fig. 1.

Colocalization of transiently expressed FGAMS-EGFP and PPAT-mCherry to visualize purinosomes in purine-depleted HeLa cells. Purine-depleted HeLa cells were transiently transfected with plasmids encoding FGAMS-EGFP and PPAT-mCherry and allowed to express for 16 h prior to live cell imaging in HBSS using a 100× oil objective on an Olympus Fluoview 1000 confocal laser scanning microscope. Sequential imaging of EGFP and TRITC channels resulted in clustering of (**a**) FGAMS-EGFP (green) with (**b**) PPAT-mCherry (red), respectively. (**c**) Merging of the individual channels resulted in proper identification of purinosomes (yellow) as observed through the colocalization of FGAMS-EGFP with PPAT-mCherry. Inset shows an enlarged view of the individual EGFP (**d**), TRITC (**e**), and merged (**f**) channels. Scale bar: 10 μm (**a**-**c**) and 1 μm (**d**-**f**)

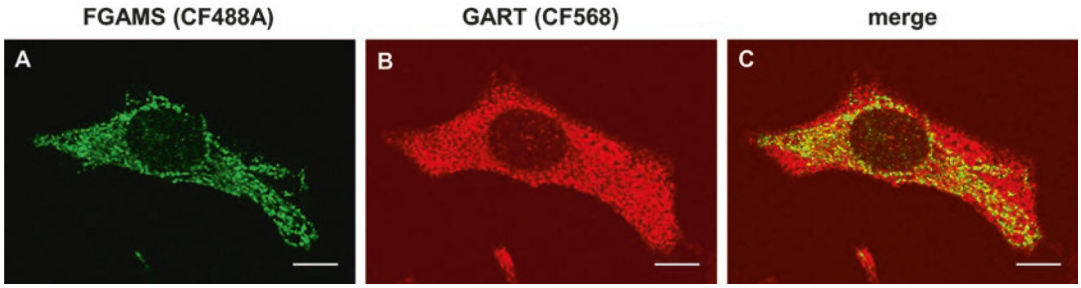

Fig. 2.

Colocalization of endogenous FGAMS and GART for visualization of purinosomes by immunofluorescence. Purine-depleted HeLa cells were fixed and permeabilized prior to being probed for with FGAMS rabbit polyclonal antibody and GART mouse monoclonal antibody. Fluorescently labeled secondary antibodies CF488A-conjugated donkey anti-rabbit and CF568-conjugated donkey anti-mouse were used to visualize the expression and localization of FGAMS and GART, respectively. A representative image of an individual cell was captured using a 100× oil objective on an Olympus Fluoview 1000 confocal laser scanning microscope. Sequential imaging of CF488A and CF568 showed colocalization of (**a**) FGAMS with (**b**) GART as represented by the yellow puncta present in (**c**) the merged image. Scale bar: 10 μm