TECHNICAL BRIEF

A nascent proteome study combining click chemistry with 2DE

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To investigate the dynamic cellular response to a condition change, selective labeling of the nascent proteome is necessary. Here, we report a method combining click chemistry protein labeling with 2D DIGE. To test the relevance of the method, we compared nascent proteomes of actively growing bacterial cells with that of cells exposed to protein synthesis inhibitor, erythromycin. Cells were incubated with methionine analog, homopropargyl glycin, and their nascent proteome was selectively labeled with monosulfonated neutral Cy3 and Cy5 azides specially synthesized for this purpose. Following fluorescent labeling, the protein samples were mixed and subjected to standard 2D DIGE separation. The method allowed us to reveal a dramatic reduction of newly synthesized proteins upon erythromycin treatment, while the total proteome was not significantly affected. Additionally, several proteins, whose synthesis was resistant to erythromycin, were identified.

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Proteins are constantly synthesized by the ribosome and decay via various proteolytic pathways. Investigation of dynamic response of any kind with the standard methods of proteomics meets some limitations. Cells respond to environmental changes, but only newly synthesized proteins reflect immediate response of the cell to the change of conditions.

There is a number of existing methods to study nascent proteome, all of them suffer from some inconveniencies. 2DE [1, 2] is a simple method for proteome analysis, however differential sample labeling with lysine-specific NHS esters of Cy3 and Cy5 [3] does not allow to distinguish newly synthesized proteins from the proteins present in the cell before a condition change. Radioactive pulse labeling was a standard method for studying of dynamic events in the cellular proteins [4], however its application for labeling living cells requires highly specialized laboratories authorized for radioactive material usage. Now the standard method for nascent proteome study (BONCAT) includes labeling with azide-containing aminoacid analog azidohomoalanine, followed by attachment of biotin via copper-catalyzed cycloaddition (click chemistry) [5], followed by affinity purification of nascent proteins, hydrolysis of total nascent proteins, liquid chromatography, and identification of nascent proteins by mass spectrometry [6, 7]. To compare two samples of nascent proteomes using BONCAT method in addition to the biotin attachment, either labeling of cells with stable isotopes could

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Abbreviation: HPG, homopropargylglycin

be applied [5] or labeling of proteins with iTRAQ reagents [7]. Both labeling methods combined with biotin labeling and affinity purification makes this method suitable for a limited number of laboratories.

Here, we report a new and much simpler method for comparative nascent proteome analysis. Nascent proteins in the two samples are labeled by addition of methionine analog, homopropargylglycin (HPG), to the media, followed by its inclusion into proteins [8]. Alkyne group react with azide moiety in a copper (I) catalyzed [3 + 2] cycloaddition (CuAAC) [9]. This reaction, known as "click chemistry," is used directly for fluorescent label attachment. Previous attempts to use this method were unsuccessful due to shifting the pI of the labeled proteins due to the charged nature of fluorophores, substituted for the neutral methionine residue. *Escherichia coli* protein synthesis inhibition by erythromycin was used as an extreme example of environmental conditions change. To make the method suitable for 2DE we synthesized monosulfonated neutral Cy3 and Cy5 fluorophores, which do not alter the charge of proteins upon modification.

The dyes were prepared using one pot cyanine core assembly [8] followed by 3-azidopropylamine attachment (Supporting Information Fig. 1) (for details see Supporting Information).

To demonstrate principal suitability of a combination of HPG inclusion into nascent proteins followed by CuAAC for fluorescent labeling of proteome, we incubated methionine analog, HPG with bacterial cells in the culture (for details see Supporting Information). To one sample erythromycin in a concentration causing complete inhibition of *E. coli* growth (250 μg/mL) was added, while another one was left without the inhibitor. After 5 min of incubation the mixture of HPG and methionine (concentrations are indicated on the Fig. 1), was added to both samples. Reaction was stopped by harvesting cells after 1, 10, or 60 min of growth.

After total proteins separation we used monosulfonated Cy3 and Cy5 azides for copper (I) catalyzed nascent protein labeling (for details see Supporting Information). Efficient inclusion of HPG into bacterial proteins and efficient fluorescent labeling of HPG-containing proteins was observed (Fig. 1A).

Short incubation times after HPG addition to the bacterial media would lead to diminished labeling efficiencies while overlabeling may result in inclusion of multiple HPG groups into the single protein that is undesired. We varied incubation times and HPG-to-methionine ratio to find an optimal labeling conditions. After incubation with HPG, cells were harvested and total proteins were prepared. HPG-containing proteome was modified with monosulfonated Cy3 and Cy5 azides and subjected to 1D separation on denaturing protein gel. Relative labeling efficiencies were determined by scanning of a gel with fluorescence scanner (Fig. 1B). Relative intensity of labeling is shown below Fig. 1B. Total protein composition of bacterial cells was unaffected by methionine:HPG ratio (Fig. 1D). As a result we can suggest 2:1 HPG to methionine ratio as optimal. Growth time with HPG might be started from 5 min for high protein synthesis rate (Fig. 1C), which was observed for the samples without erythromycin addition. Longer, 60 min incubation time might be suggested for low protein synthesis rates, like those observed in the presence of translation inhibitor erythromycin. The superiority of nascent proteome analysis versus total proteome is obvious if one compares nascent Fig. 1C (nascent proteome) and Fig. 1E (total proteome). Almost no change in total proteome is evident upon erythromycin treatment, while nascent proteome is reduced dramatically upon protein synthesis inhibition.

The main problem for the 2D gel separation of fluorescently labeled proteins is alteration of protein charge influencing protein position after the isoelectric focusing. Lysine derivatization by NHS esters of positively charged Cy3 and Cy5 dyes does not alter protein pI because substitution of positively charged lysine amino group with neutral amido group is compensated by an introduced positive charge of Cy3 and Cy5 dyes. This procedure became a standard method in proteome analysis [3], but it was not suitable to distinguish newly synthesized proteins. Substitution of neutral methionine residue by a charged fluorophore is highly inconvenient since it results in a protein spot shifting and multiplication. To avoid this problem, we synthesized neutral monosulfonated Cy3 and Cy5 dyes in a form of azido derivatives. For a comparison, we used standard positively charged Cy3 and Cy5 azides. Protein synthesis inhibition by erythromycin was chosen as a model for testing the suitability of the system.

Bacterial cells in a liquid culture were subjected to erythromycin at a concentration completely inhibiting growth. Incubation for 5 min with antibiotic was followed by addition of HPG to the media in a 2:1 proportion to methionine. After 60 min incubation cells were harvested and complete proteome was labeled with monosulfonated Cy5 azide by the "click chemistry" reaction. The control sample was obtained similarly without addition of erythromycin and labeled with monosulfonated Cy3 azide. Labeled proteins were separated by a standard 2D gel electrophoresis (for details see Supporting Information) (Fig. 2A). Proteomes similarly labeled with standard positively charged Cy3 and Cy5 azides were used for comparison (Fig. 2B). Significant reduction of spots multiplication was observed for the samples labeled with neutral monosulfonated cyanine dyes (Fig. 2A).

Silver staining [10] of the gel (Fig. 2C, D) and modification of total proteomes with Cy3 and Cy5 NHS esters (Fig. 2E) allowed us to compare positions of the fluorescently labeled spots of nascent proteins with that of bulk protein spots. First of all, an evidently more clear image corresponds to the nascent proteome labeled with neutral monosulfonated cyanine dyes (Fig. 2C) compared to positively charged cyanine dyes (Fig. 2D). This result was expected due to alteration of proteins pI by attachment of charged fluorophores to the neutral methionine analog residues. Total proteome labeling with Cy3 and Cy5 NHS esters was efficient but inadequate to distinguish dynamic proteome changes (Fig. 2E). Overall distribution of protein spots labeled by "click chemistry," NHS esters and silver staining were directly comparable.

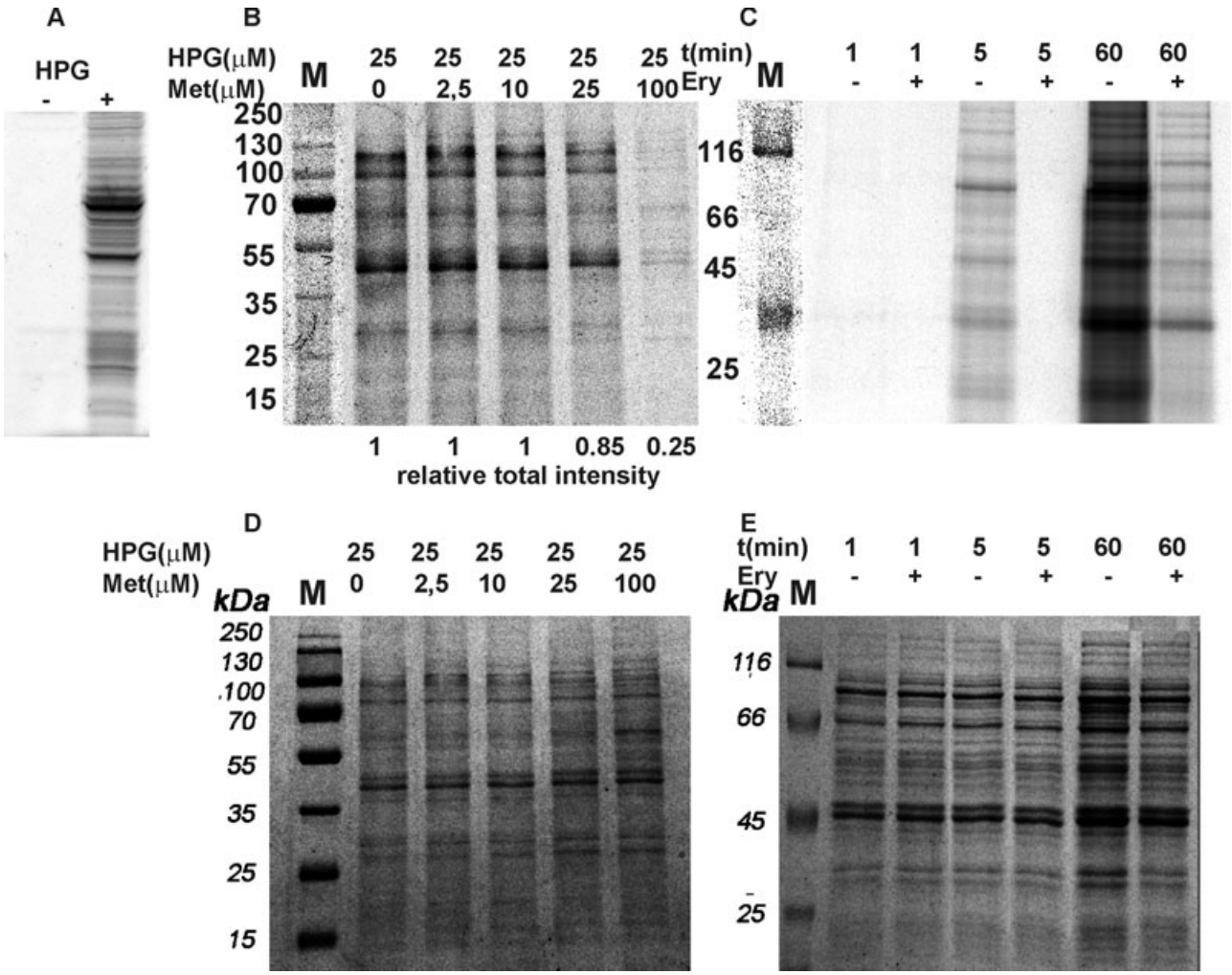

Figure 1. Conditions for nascent proteome labeling. (A) Inclusion of HPG into nascent proteins of *E. coli*. Proteome was labeled with Cy5 azide. Presence of HPG in the growth media is indicated above the gel. (B) HPG inclusion efficiency upon cell growth in the media containing various HPG to methionine ratios. Methionine and HPG concentrations are shown above the gel. (C) Efficiency of HPG inclusion as a function of time and presence of translation inhibitor, erythromycin. Incubation time with HPG and erythromycin presence is indicated above the gel. (D) and (E) show the same gels as on the panels (B) and (C) but stained with Coomassie blue. M is the molecular weight markers lanes, corresponding molecular masses are indicated on the left. Prestained nonfluorescent markers are barely visible upon scanning of fluorescence, so for the sake of convenience the sensitivity was increased for marker's lanes on the panes B and C.

Synthesis of the majority of proteins was completely shut upon addition of erythromycin (Fig. 2A, compare green and red spots). However, several proteins are resistant to erythromycin inhibition (Fig. 2A, yellow spots). We identified the proteins whose biosynthesis was not affected by erythromycin (Fig. 2A, identified spots are marked by red arrows). As a control we also identified a spot whose intensity was highly inhibited by erythromycin (Fig. 2A, marked by green arrow). After silver staining, the protein spots were identified by trypsinolysis/MALDI-TOF (for details see Supporting Information). We revealed a number of translation-related factors and a single energy metabolism protein complex, pyruvate dehydrogenase (Supporting Information Table 1).

Antibiotics affecting the translation present an ideal model for nascent proteome investigation. Action of macrolides on translation was extensively studied in model systems [11–13]. It is known that macrolide-binding site is located in the ribosomal peptide tunnel [14, 15]. Binding of macrolides caused narrowing of the tunnel and experiments with in vitro translation model system demonstrated that macrolides cause peptidyl-tRNA drop-off [12]. Nascent proteome study allowed us to reveal several genes whose expression was resistant to erythromycin treatment. This subset of genes is clearly related to translation and the phenomenon observed here might constitute a compensatory mechanism of a cell for translation inhibition.

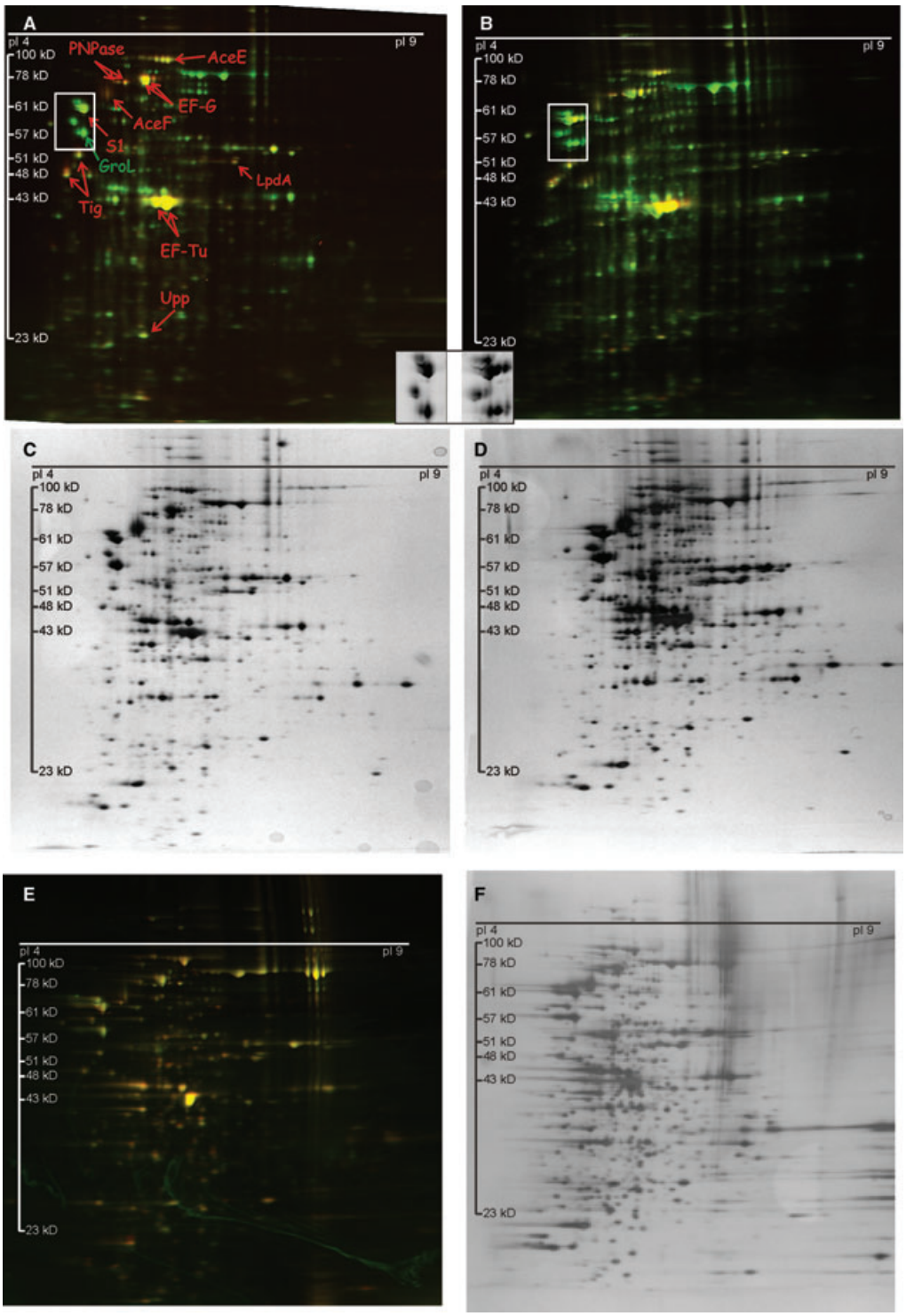

Figure 2. Resolution of nascent proteome on 2D gel. (A) 2D protein gel of a nascent proteome mixture derived from untreated cells (neutral monosulfonated Cy3 labeled, green fluorescence) and erythromycin treated cells (neutral monosulfonated Cy5 labeled, red fluorescence). (B) shows the same gel as (A) but labeled with standard positively charged Cy3 and Cy5 azides. An insert on the bottom line of panels A and B shows a magnified boxed part of the gels to demonstrate clearly reduction in spot multiplication. (C) and (D) show the same gels as (A) and (B) but silver stained. Identified protein spots, corresponding to proteins whose synthesis is resistant to erythromycin are indicated by red arrows. A control spot, whose intensity was highly diminished by erythromycin is indicated by green arrow. (E) 2D protein gel of a total proteome mixture derived from untreated cells (NHS Cy3 labeled, green fluorescence) and erythromycin treated cells (NHS Cy5 labeled, red fluorescence). (F) is the same as gel shown on (E), but silver stained.

The method presented is quite simple and can supplement steady-state proteome comparison in cases where dynamic proteome change is of interest. Incorporation of HPG into proteins and following CuAAC of neutral monosulfonated Cy5 and Cy5 fluorophores reduces spot multiplication and proved to be a useful method for native proteome study.

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