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Localization of Mycobacterial Antigens by Immunofluorescence Staining of Agarose Embedded Cells

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Abstract

Here we report the adaptation of an immunofluorescence staining method for mycobacterial antigens. Our approach is based on agarose embedding followed by thin sectioning of the bacterial samples. We demonstrate that this technique has great potential for protein localization studies, without the use of fluorescence tagged fusion proteins, which is of special interest for slow growing mycobacterial species.

Keywords: Agarose embedding; Antigen localization; Immunofluorescence assay; Mycobacteria

Introduction

A commonly used technique for studying the cellular localization of proteins is based on the use of genetically encoded fluorescence tagged (i.e. GFP) fusion proteins [1,2]. This requires a study organism easily accessible to genetic modifications. However, this may not be practical for certain mycobacterial species due to slow growth rate, strong aggregate formation as well as a low recovery rate upon transformation [3,4].

An alternative well-established method for localizing proteins within a bacterial cell is based on indirect immunofluorescence (IF) staining using antibodies (Ab) specific for the protein of interest. The primary Ab is then detected by a fluorochrome-labeled secondary Ab. During this process a signal amplification occurs, due to the binding of several secondary Abs to individual primary Ab molecules [5,6].

In order to optimize the antigen localization in mycobacterial cells, we adapted an agarose embedding protocol originally developed for thin sectioning of eukaryotic cells.

Materials and Methods

Mycobacteria (*M. tuberculosis* H37Rv and *M. ulcerans* NM20/02) were grown in BacT® medium (bioMérieux, Marcy l'Etoile, France) to an OD600 of 0.8, pelleted (10'000xg,10 min) and fixed by adding 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, US) for 24h at RT. The fixative was removed by spinning the samples at 2'000xg for 7min. The pellet was resuspended in 500µl 1.5% low melting agarose (BioWhittaker Lonza, Basel Switzerland) in PBS, transferred into 07x07x06 mm cryomodules (Applied BioSystems, Foster City, CA, US) and placed on ice for 10 min. Agarose blocks were embedded in paraffin, cut into 3µm sections using an HM 335 E rotary microtome (Microm International GmbH, Boise, ID, US), and retrieved on Superfrost Plus (Thermo Scientific,Waltham,MA,US) slides. The sections were deparaffinized, rehydrated and subsequently pretreated with boiling 1 mM EDTA (Sigma-Aldrich, St. Louis, MO, US) buffer (pH=8.0) for heat induced epitope retrieval. Unspecific binding was prevented by incubation for 1h in 1.5% blocking serum in PBS matching the secondary Ab host. Primary monoclonal Abs DD3.6 (anti-MUL2232), JD3.2 (anti-MUL3720), F126-2 (anti-Ag84) and polyclonal rabbit anti-*M. tuberculosis* Ag85B Abs (Abcam, Cambridge, UK) were incubated at a final concentration of 2 µg/ml for 1 h in PBS containing 0.1% Tween 20 (PBS-T) at RT or alternatively o/n at 4°C. Slides were washed five times in PBS-T. Secondary goat anti-mouse/rabbit total IgG (H+L) Alexa 488 labeled detection Abs (Life Technologies, Carlsbad, CA, US) diluted 1/200 in 1.5% goat serum in PBS was added for 30min. For double stainings, goat anti-mouse IgG1 Alexa 594 and anti-mouse IgG2b Alexa 488 subclass specific secondary Abs (Life Technologies, Carlsbad, CA, US) were used. After washing the slides five times in PBS-T and once with ddH2O the slides were mounted using ProLong® Gold Antifade Reagent containing DAPI (Life Technologies,Carlsbad,CA,US). Image acquisition was performed on a confocal laser microscope (Carl Zeiss, Axiovert 200M equipped with a LSM510 Meta laser scanning module) (Carl Zeiss, Oberkochen, Germany). Images were analyzed using the freeware Fiji [7].

Western blotting was performed essentially as previously described by [8]. 5µg bacterial lysate was loaded per lane.

Results

In a first series of experiments we applied this method for the localization of Ag84 and Ag85B in *M. tuberculosis*. Ag84 is a DivIVA-like protein involved in cell division and cell wall synthesis. Our IF staining method allowed to demonstrate the anticipated polar localization of the antigen (Figure 1A) with a resolution comparable to the one obtained using genetically modified bacteria expressing Ag84-GFP [9]. Ag85B is a mycolyltransferase involved in fibronectin binding as well as in the final stages of cell wall assembly [10,11]. The protein showed the expected cell surface localization [12] with an elevated expression towards the bacterial poles (Figure 1A). Such a polar localization is also found for other proteins implicated in bacterial growth [13].

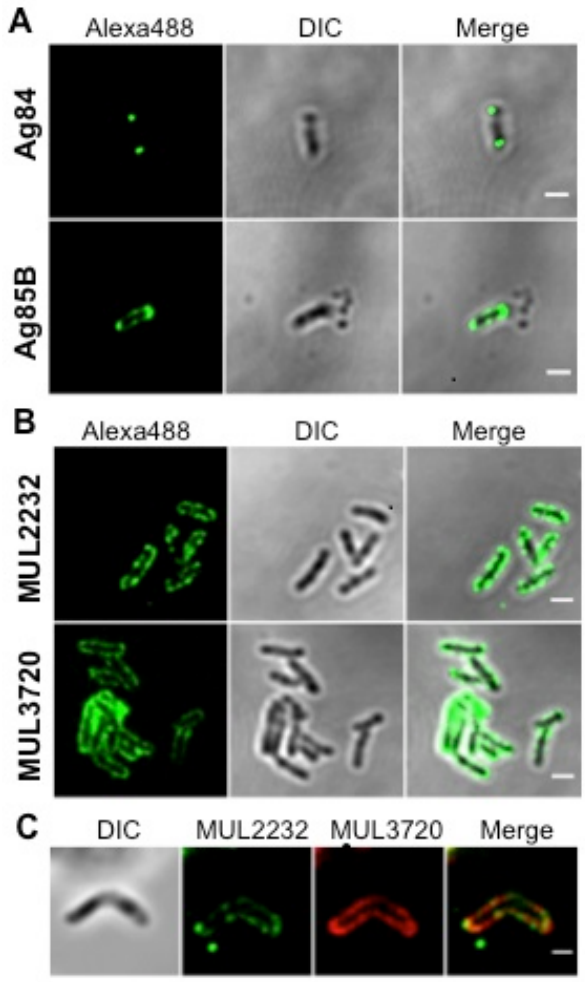

Figure 1: Protein localization in mycobacteria using the agarose embedding based IF method. Monoclonal antibodies were used to stain the antigens Ag84 and Ag85B in *M. tuberculosis* H37Rv (A) and MUL2232 and MUL3720 in *M. ulcerans* NM20/02 (B) in formalin fixed agarose embedded paraffin sections. For MUL2232 / MUL3720 double staining (C), IgG subclass specific secondary antibodies were used. Scale bar: 2 µm.

In a next step we applied our method to localize antigens of *M. ulcerans*, a mycobacterium which displays an abundant extracellular matrix and has a strong tendency to aggregate [14]. We found that both MUL2232, a 18kDa small heat shock protein [15] and MUL3720, a 21kDa protein with putative lectin and peptidoglycan-binding domains were localizing at the bacterial surface. While MUL2232 seemed to be heterogeneously distributed, for MUL3720 a homogenous surface staining was observed (Figure 1B).

Furthermore, our IF assay protocol was also suitable for carrying out co-localization studies confirming the presence of both antigens at the bacterial surface (Figure 1C). Intracellular DNA staining with DAPI confirmed the previously observed surface localization for MUL3720 in DIC micrographs (Figure 3).

In addition, the specificity of the IF staining was analyzed using a *M. ulcerans* mutant strain representing a functional knockdown of MUL2232. No MUL2232 IF staining was observed for the mutant (Figure 2A), which was also negative for MUL2232 in Western blot analysis as compared to the wild type strain (Figure 2B).

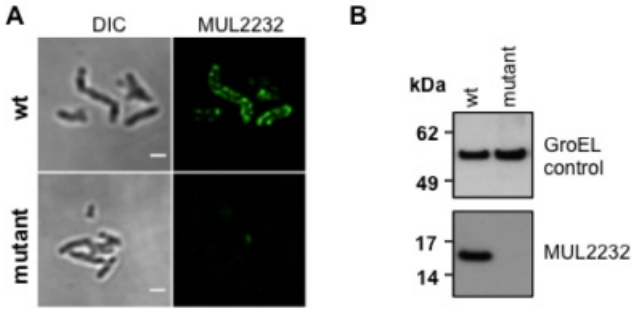

Figure 2: Specificity assessment by comparing IF assay and Western blotting results for the detection of MUL2232. NM 20/02 wild type *M. ulcerans* was compared to a mutant strain overexpressing HspR_2, a transcriptional repressor of MUL2232, resulting in a functional knockdown of MUL2232. The mutant strain was generated from NM20/02 using a HspR_2 harboring plasmid previously described [18]. GroEL served as a loading control. All micrographs were acquired using the same exposure time (950 ms) and detector gain (=1). Scale bar: 2µm

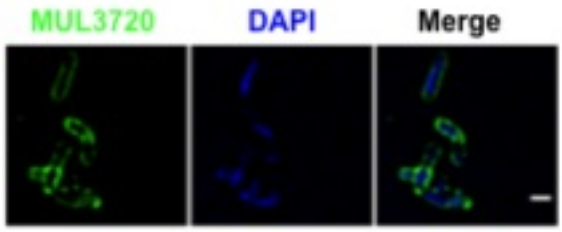

Figure 3: Surface localization of MUL3720 was confirmed by intracellular DNA staining using DAPI. This micrograph was acquired on an API DeltaVision (Applied Precision, Issaquah, WA, US) fluorescence microscope containing a DAPI filter set. Scale bar: 2µm

Discussion

To our knowledge, this is the first report describing an agarose embedding procedure combined with an IF assay for the analysis of the subcellular localization of proteins in mycobacteria. This newly adapted technique harbors two main advantages over IF staining protocols involving the direct fixation of bacteria on the microscopy slides: i) the preparation of thin sections leads to a better accessibility of the Abs to their target; ii) the possibility to perform an epitope retrieval by breaking down the formalin cross-linking, facilitates Ab binding in formalin fixed paraffin embedded sections [16]. Based on these advantages, it was possible to confirm the sequence-predicted surface localization of MUL2232 and MUL3720 by IF. Furthermore,

agarose embedding allowed significant improvement for the localization of Ag85B in *M. tuberculosis* as compared to Rambukkana and colleagues [12].

In addition, the possibility to study the localization and expression of proteins of interest by IF staining without the need to genetically modify the microorganism is of great benefit in particular for slow growing mycobacteria such as *M. ulcerans* [17], for which genetic modification is extremely time consuming. Furthermore, the IF staining method allows studying the protein of interest in its natural context in the absence of heterologous GFP co-expression, which in some cases can lead to mislocalizations [1]. Last, the here presented method is inexpensive and easy to perform, in case histopathology equipment is accessible.

Taken together, we successfully adapted and validated a reliable IF based method to localize proteins within disease relevant mycobacteria, which might be of general use for basic research and the validation of potential vaccine candidates.

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References

1. Crivat G, Taraska JW (2012) Imaging proteins inside cells with fluorescent tags. Trends Biotechnol 30: 8-16.
2. SHIMOMURA O, JOHNSON FH, SAIGA Y (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. J Cell Comp Physiol 59: 223-239.
3. Jacobs WR Jr, Kalpana GV, Cirillo JD, Pascopella L, Snapper SB, et al. (1991) Genetic systems for mycobacteria. Methods Enzymol 204: 537-555.
4. Lamrabet O, Drancourt M (2012) Genetic engineering of Mycobacterium tuberculosis: a review. Tuberculosis (Edinb) 92: 365-376.
5. Coons AH, Creech HJ, Jones RN (1941) Immunological Properties of an Antibody Containing a Fluorescent Group. Exp. Biol. Med. 47: 200–202.
6. J. Paul Robinson, Jennifer Sturgis, George L. Kumar (2009) in IHC Stain. Methods 61–65 (Dako,).
7. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, et al. (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9: 676-682.
8. Dreyer AM, Beauchamp J, Matile H, Pluschke G (2010) An efficient system to generate monoclonal antibodies against membrane-associated proteins by immunisation with antigen-expressing mammalian cells. BMC Biotechnol. 10: 87.
9. Nguyen L, Scherr N, Gatfield J, Walburger A, Pieters J, et al. (2007) Antigen 84, an effector of pleiomorphism in Mycobacterium smegmatis. J Bacteriol 189: 7896-7910.
10. Abou-Zeid C, Ratliff TL, Wiker HG, Harboe M, Bennedsen J, et al. (1988) Characterization of fibronectin-binding antigens released by Mycobacterium tuberculosis and Mycobacterium bovis BCG. Infect Immun 56: 3046-3051.
11. Belisle JT, Vissa VD, Sievert T, Takayama K, Brennan PJ, et al. (1997) Role of the major antigen of Mycobacterium tuberculosis in cell wall biogenesis. Science 276: 1420-1422.
12. Rambukkana, A et al. (1991) Subcellular distribution of monoclonal antibody defined epitopes on immunodominant Mycobacterium tuberculosis proteins in the 30-kDa region: identification and localization of 29/33-kDa doublet proteins on mycobacterial cell wall. Scand. J. Immunol. 33: 763–775.
13. Hett EC, Rubin EJ (2008) Bacterial growth and cell division: a mycobacterial perspective. Microbiol Mol Biol Rev 72: 126-156, table of contents.
14. Marsollier L, Brodin P, Jackson M, Korduláková J, Tafelmeyer P, et al. (2007) Impact of Mycobacterium ulcerans biofilm on transmissibility to ecological niches and Buruli ulcer pathogenesis. PLoS Pathog 3: e62.
15. Diaz D, Döbeli H, Yeboah-Manu D, Mensah-Quainoo E, Friedlein A, et al. (2006) Use of the immunodominant 18-kiloDalton small heat shock protein as a serological marker for exposure to Mycobacterium ulcerans. Clin Vaccine Immunol 13: 1314-1321.
16. Shi SR, Key ME, Kalra K L (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J. Histochem. Cytochem. 39, 741–748.
17. Walsh DS, Portaels F, Meyers WM (2008) Buruli ulcer (Mycobacterium ulcerans infection). Trans R Soc Trop Med Hyg 102: 969-978.
18. Pidot SJ, Porter JL, Tobias NJ, Anderson J, Catmull D, et al. (2010) Regulation of the 18 kDa heat shock protein in Mycobacterium ulcerans: an alpha-crystallin orthologue that promotes biofilm formation. Mol Microbiol 78: 1216-1231.