Enhanced methodologies for detecting phenotypic resistance in mycobacteria

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Summary

Lipid droplets microscopic organisms, found in in algae and other have become of interest to many researchers partially because they carry the capacity to produce bio-oil for the mass market. They are of importance in biology and clinical practice because their presence can be a phenotypic marker of an altered metabolism, including reversible resistance to antibiotics, prompting intense research

A useful stain for detecting lipid bodies in the lab is Nile red. It is a dye that exhibits solvatochromism; its absorption band varies in spectral position, shape and intensity with the nature of its solvent environment, it will fluoresce intensely red in polar environment and blue shift with the changing polarity of its solvent. This makes it ideal for the detection of lipid bodies within *Mycobacterium* spp. This is because mycobacterial lipid bodies'

primary constituents are non-polar lipids such as triacylglycerols but bacterial cell membranes are primarily polar lipid species. In this chapter we describe an optimal method for using Nile red to distinguish lipid containing (Lipid rich or LR cells) from those without lipid bodies (Lipid Poor or LP). As part of the process we have optimised a method for separating LP and LR cells that does not require the use of an ultracentrifuge or complex separation media. We believe that these methods will facilitate further research in these enigmatic, transient and important cell states.

1. Introduction

In recent years there has been an increasing interest in mycobacteria within which lipid bodies are seen[1-3]. This is due to the important association with low metabolic state and phenotypic resistance to key anti-tuberculosis antibiotics [4-7]. As the goal of improving tuberculosis treatment remains frustratingly out of reach, it is important that we understand what the true susceptibility of *M. tuberculosis* is as it is clear there is a significant difference in the susceptibility of cells with lipid bodies present in comparison with those that are not [8]. It is of considerable importance, therefore, to be able to reliably separate and quantify mycobacterial cells in different cells state. Previously published methods are effective but often complex and may result in metabolic alteration in the cells studied.

Separation of particles based on their buoyant density has practiced since at least 1919 [9]. Differences in buoyant density can be used to separate particles [10-13], and the density-dependent cell sorting (DDCS) method has been applied to laboratory cultured bacteria [14]. Cells in different physiological states have been successfully separated using this approach [15] because physiological changes alter cellular components and the subsequent buoyant density. The DDCS method has been applied mostly to pure cultures [16]. It can be used as a purifying process for differential centrifugation. For mycobacteria, methods to permit separation of LR and LP cells have been described [17,18]. Equilibrium sedimentation classically uses a gradient of a solution such as sucrose to separate particles based on their individual densities. These usually require extended centrifugation, ultra-centrifugation or the use of complex separation media such as sucrose or Percoll [19]. Very

little is known about the effect of these processes on the metabolism of mycobacteria, which is often the purpose of the experiments. Sucrose separation gradients can provide a carbon source for mycobacteria that are not fastidious and can utilise almost all simple carbohydrate carbon sources including sucrose [20].

Isopycnic centrifugation refers to a method wherein a density gradient is either pre-formed or forms during high speed centrifugation[21]. After the gradient is formed particles move within the gradient to the position having a density matching their own [22]. To improve our capacity to study mycobacteria in different cell states we describe a simple isopycnic technique to separate lipid rich and lipid poor mycobacteria based on their density. Our methodology was based upon isopycnic centrifugation with or without a centrifugation step [23]. This technique can produce very pure "single state" mycobacteria at good yield for use in further experimentation.

Another advantage of the method is that a solution of D_2O and pure water has no difficulties caused by evaporation. For other methods such as sucrose density centrifugation, a solution of sucrose and water will change its density if left uncovered overnight at room temperature due to the water evaporating off leaving comparatively more sucrose behind. Stability is another advantage as D_2O is atomically and there will be no change in solutes from precipitation mid-experiment caused by a change in the density of the media.

Confirmation and quality checking of the lipid-state of separated sub-populations can be obtained by use of the Nile red staining technique above and we report a simple method that assists the quantification of the LR and LP fractions.

To stain a bacterial culture or to grow it on differential and/or selective media is a standard and simple method for differentiating between genera of bacteria [24]. It is rapid and effective and remains an important part of everyday microbiology practice.[24,25] Staining methodologies can also be adapted by adding more complex manipulations such as flow cytometry [26], cell counting & sorting and biomarker detection amongst other applications [27].

Nile red has been used for many years to visualise intracellular lipids. In this chapter we have adapted the methodology to provide a simple and reproducible technique to reliably visualise and quantify lipid bodies within mycobacterial cells. Importantly, the method can be adapted to flow cytometry. It is possible to further adapt our described Nile red staining protocol to a high throughput screening method to allow for rapid quantification of the lipid body load in a particular sample. Figure 1 below demonstrates what a lipid rich cell from a few mycobacterial species looks like and how they were identified microscopically.

When the preparation is visualised at 527nm non-polar lipid fluoresce green. Polar lipid such as those found in the cell wall and membranes appear as bright red. This clear visual separation allows for easy counting so that the proportion of lipid rich (LR) and lipid poor (LP) cells can be quantified accurately. This property can also be used by flow cytometry to rapidly quantify LR and LP cells in a mycobacterial culture [8] from a single staining step. This obviates the need for two stains: an acid-alcohol method to identify the mycobacterium followed by destaining followed by Nile red staining to classify the lipid content.

2 Materials

All solutions should be made prior to beginning this procedure. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Buoyant density separation

1. Tubes –list all and the suppliers

2. Heavy water (Sigma Aldrich)

3. Centrifuge (Beckman Coulter J6-MI)

4. Media (Sigma Aldrich)

5. Micro-centrifuge tubes (Axygen)

6. Pipettes (Thermo Scientific, Finnpipette F2)

7. Glass Pasteur pipette (Thermo Scientific)

8. Parafilm (Bemis, Parafilm)

9. Large centrifuge tubes (Cellstar)

2.2 Nile red staining

1. Nile red stain: Nile red power (Sigma Aldrich) (see **Note 1**)
2. Clean microscope slides (Thermo Fisher)
3. DMSO (Sigma Aldrich)
4. Microscope (see **Note 2**)
5. Water bath

3. Method

3.1 Buoyant density separation (One *g* separation)

1. Take a 1mL aliquot of bacterial cells is harvested from culture
2. Washed three times by micro-centrifugation (20,000g for 3 minutes) with filter-sterilised water
3. R-suspend the washed cells in 200µL of filter sterilised dH_2O
4. The full 200µL is aliquoted into an uncharged (or de-static) sterile plastic vessel (see **Note 3**)
5. Add 600µL of D_2O to give final volume of 800µL. (D_2O : dH_2O; 3:1). (see **Note 4 and 5**)
6. Seal the D_2O/dH_2O solution and leave to equilibrate for 24 hours without agitation.
7. After 24 hours take 100µL of solution from within 1mm of the meniscus using a 200 µL pipette.
8. Store the cells in a sterile micro-centrifuge tube.

9. Remove the material from within 1mm of the bottom of the tube with a 200 µL pipette (see **Note 6**).
10. Remove 100µL from this layer and stored in a sterile Micro-centrifuge tube (see **Note 7**)

3.2 Micro-centrifuge (~200*g*) Separation (See **Note 8**)

1. Cells for centrifugation were prepared as described in above.
2. Take an anti-static micro-centrifuge tube is prepared.
3. Aliquot 1mL mixture of washed cells in a 3:1 D_2O:H_2O into the tube.
4. Seal the tube and centrifuge for 5 minutes at 200 *g*.
5. Take the fractions in same way as noted above in 3.1

3.3 High volume preparation (200g) separation (see **Note 8**)

1. Take a standard short nosed glass pipette and heat in a Bunsen burner whilst gripping the end of the pipette tip with forceps.
2. When the glass of the pipette tip begins to soften twist the forceps are and pulled to sever the end of the pipette tip and seal it in one movement.
3. Use this sealed and shortened pipette as the separation vessel.
4. Prepare a 5mL solution of cells and 3:1 D_2O:H_2O is prepared as above and added to the sealed glass pipette.
5. Seal the pipette with parafilm at the opening and place into a 15mL centrifuge tube that has been padded with absorbent white tissue.
6. Ensure a good seal by adding more tissue paper around the pipette and above it before the cap of the large centrifuge tube is sealed.
7. Centrifuge the assembly at 200 *g* for 5 minutes.

3.4 Staining Cells in liquid phase

1. Dissolve Nile red in DMSO (Sigma Aldrich) to a final concentration of 2mg/mL (see **Note 9)**.

2. Nile red solution can be added directly into media containing cells at 1:10 ratio (final concentration of 100µg/mL)

3. The Nile red sample should be incubated at room temperature with constant agitation for 20 minutes.

4. The sample is centrifuged for 3 minutes at 20,000*g* to pellet the cells.

5. The supernatant is removed and discarded and 200µL of 100% ethanol is added. Vortex to mix

6. The sample is centrifuged for 3 minutes at 20,000*g* and the supernatant discarded.

7. 100µL of PBS is added to the sample and vortexed for 1 minute.

8. The stained sample (10µL) can be applied to a clean glass slide and heat-fixed.

9. Bacterial preparations can be viewed by fluorescence microscopy (see Note10) or quantified by flow cytometry (see **Note 11**)

3.5 Staining cells in solid phase (see **Note 12)**

1. Nile red is prepared as above to 100µg/mL.
2. Sample bacterial cells using a sterile plastic loop
3. prepare a thin smear on a clean glass slide).
4. Heat fix the smear
5. Nile red bath is prepared with enough solution to flood the entire slide.
6. Place the prepared slide in a Nile red bath.
7. Bath and slide are incubated at room temperature for 30 minutes in the dark.
8. Remove the slide from bath and drain excess stain (see Note B)
9. Slide has excess stain drained from it onto absorbent towelling.
10. Slide is rinsed once with deionised water, 3 seconds.
11. Slide is rinsed with 70% ethanol, 5 seconds.
12. Slide is rinsed again with deionised water and allowed to dry at room temperature in the dark (see note C).

4. Notes

1. Nile red is a benzophenoxazone dye and is highly soluble in ethanol but is negligibly soluble in water which makes its use in biological situations difficult. This can be overcome by bathing the sample to be stained in a highly polar substance. This can damage or change to properties of the sample under investigation so is generally avoided. An alternative is to use DMSO (dimethysulphoxide) as the solvent for the dye. Nile red is readily soluble in DMSO and DMSO will aid in the carriage of Nile red across biological membranes.

2. Any fluorescent microscope fitted with a 100X oil emersion lens and a >8mega-pixel camera will suffice. The crucial elements that it must possess are filter cubes that fall within a fine range. We use Texas red and Bodipy FL cubes as these have a narrow spectral range, ±40nm of the stated wavelength.

3. This is achieved using an antistatic gun (Milty).

4. Deuterium oxide is a stable oxide of deuterium. Pure D_2O has a specific gravity of 1.11 g/cm^3. Pure water has a specific gravity of 1.00 g/cm^3. This means that a solution of D_2O from 1% - 99% could have the range of specific gravities from 1.01 g/cm^3 - 1.10 g/cm^3. Previous work has shown that the density of lipid rich mycobacterial cells lies within this range (Lipworth, Gillespie, unpublished).

5. In order to effectively separate particles it is necessary to know the specific gravity of the particles in question. This can be established by performing several BDS' at a range of different specific gravities. It was found that lipid rich cells are separated at approximately 1.08 g/cm^3. Another cell type present in the sample (lipid poor cells) had a specific gravity of approximately 1.1 g/cm^3. In order to create a separation medium with a specific gravity similar to the density of lipid rich cells a mixture of D_2O and pure H_2O was used. Given the above figures on the relative densities of pure H_2O (1.00 g/cm^3) and D_2O (1.11 g/cm^3) a 3:1 solution of D_2O: H_2O gave a specific gravity of 1.08325 g/cm^3. This is slightly more dense than the lipid rich cells under investigation. With a solution density of 3:1 D_2O: H_2O a population of exclusively lipid rich cells gathered at the meniscus of the D_2O solution whereas all other cells sink to the bottom of the separation vessel.

6. Take the sample with bubbling through the D_2O/H_2O mixture until the correct depth was reached to prevent cells from other layers entering the pipette tip and contaminating the separated material.

7 When separations failed to achieve sufficient purity by fluorescent microscopic evaluation (see figure 1) such samples can be subjected to a further round of buoyant density separation.

8 For a micro-centrifuge tube the maximum safe volume of liquid to be used is 1200µL when centrifuging a sample. For a glass pipette, it is possible to use up to 5mL of liquid.

9**.** Stain can be reused for subsequent staining up to a maximum of 5 times if stored in the absence of light or if used with 1 week of preparation

10. For optimal clarity of separation we use an excitation frequencies of 480/40 and 540/40. We detect emission at 527/30, and 645/75. In our lab we use the Leica CTR 5500 DM microscope.

11. Preparations can be quantified using flow cytometry. Cells stained by the method noted as above in liquid phase are loaded into flow cytometry vessel and processed as normal. Microscopic analysis of Nile red stained preparations is recommended to validate the results of flow cytometric analysis.

12. The same Nile red protocol that is used for solid phase cultures can be applied to *ex vivo* samples such as biopsy or *post mortem* sections. Slides must be de-waxed and distained if originally prepared thus. *Ex* vivo slides can then be processed as for solid phase, above.

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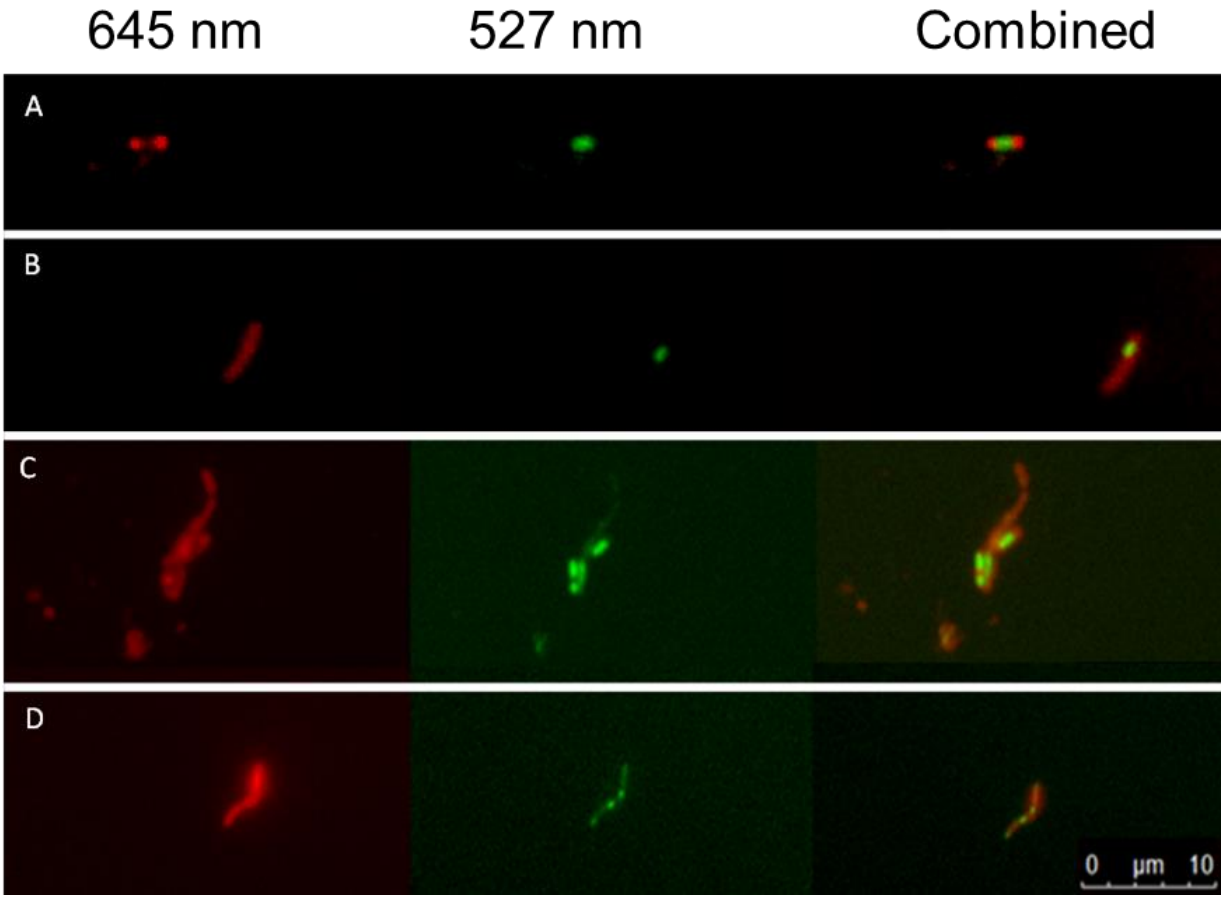

Figure 1 Left to right; Nile red fluorescence of polar lipids at 645 nm, Nile red fluorescence of non-polar lipids at 527 nm, composite image lipid rich cells extracted from D_2O separation top layer. A; *M. marinum,* B; BCG, C; *M. smegmatis*, D; *M. fortuitum*. It can be seen that in *M. marinum* the lipid body is single and large in the centre of the cell. In BCG the lipid body (or bodies) is found at the polar end of the cell. *M. smegmatis* is similar to *M. marinum* in that it will have large lipid bodies situated in the centre of the cell but *M. smegmatis* regularly displays more than one lipid body. *M. fortuitum* is similar again to BCG as it shows lipid bodies at the poles of the cells but much more commonly there will be two lipid bodies rather than one.

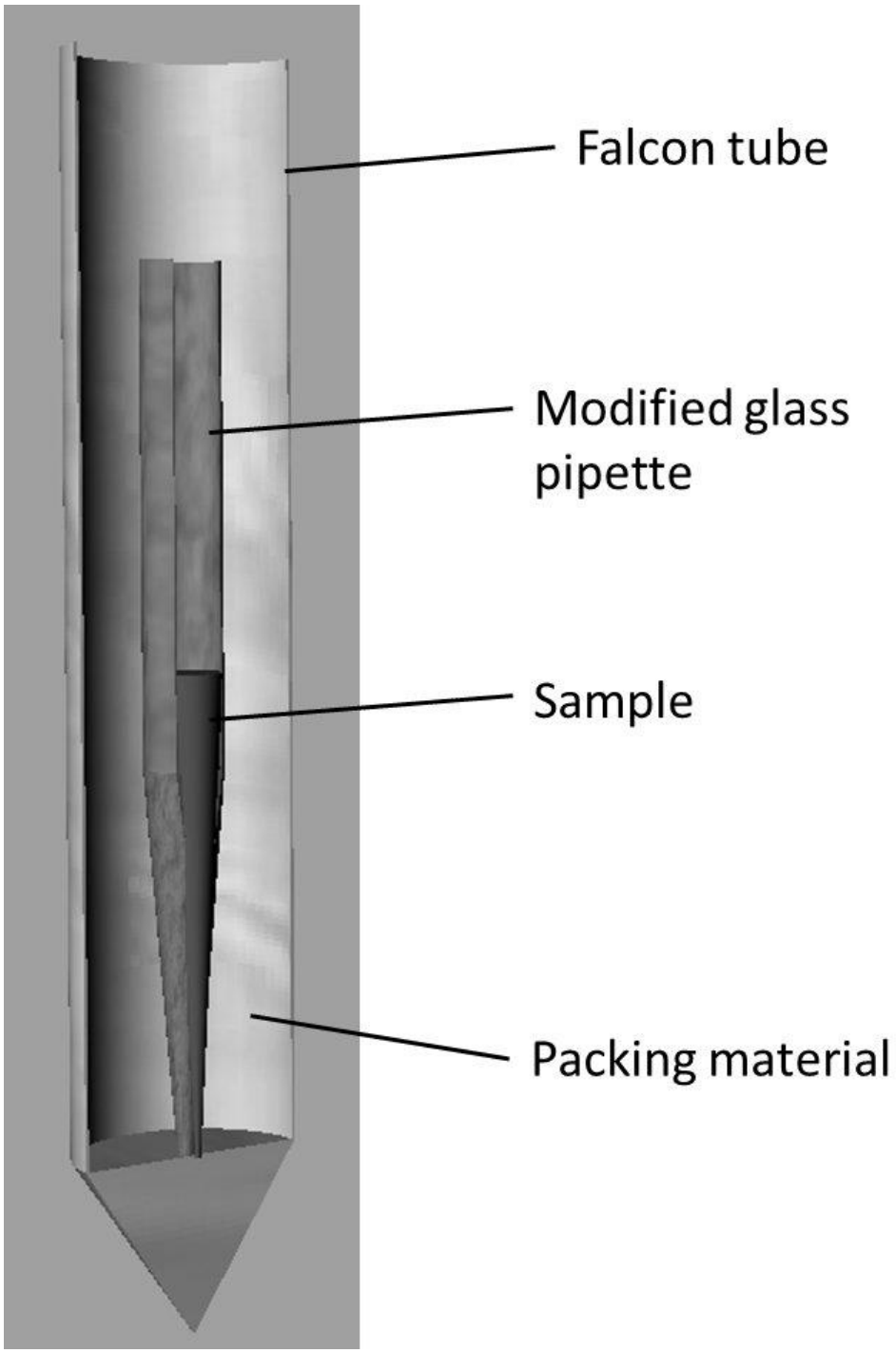

Figure 2 Schematic diagram of the assembly used to centrifuge D_2O and bacterial samples for separation