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Percoll discontinuous density gradient centrifugation method for the fractionation of the subpopulations of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* from *in vitro* cultures ☆

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ABSTRACT

Bacterial populations in the *in vitro* laboratory cultures, environment, and patients contain metabolically different subpopulations that respond differently to stress agents, including antibiotics, and emerge as stress tolerant or resistant strains. To contain the emergence of such strains, it is important to study the features of the metabolic status and response of the subpopulations to stress agents. For this purpose, an efficient method is required for the fractionation and isolation of the subpopulations from the cultures. Here we describe in detail the manual setting up of a simple, easy-to-do, reproducibly robust Percoll discontinuous density gradient centrifugation for the fractionation of subpopulations of short-sized cells (SCs) and normal/long-sized cells (NCs) from *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* cultures, which we had reported earlier. About 90-98% enrichment was obtained respectively for SCs and NCs for *M. smegmatis* and 69-67% enrichment was obtained respectively for the SCs and NCs for *M. tuberculosis*.

- The Percoll discontinuous density gradient centrifugation helps the fractionation and isolation of mycobacterial subpopulations that differ in density.
- The method offers a consistently reproducible high enrichment of the subpopulations of SCs and NCs from the *in vitro* cultures of *M. smegmatis* and *M. tuberculosis*.
- Our earlier reports on the consistency in the differential response of the subpopulations, enriched using the method, to oxidative, nitrite, and antibiotic stress proves its validity.

☆ **Related research article** S. Vijay, R.R. Nair, D. Sharan, K. Jakkala, N. Mukkayyan, S. Swaminath, A. Pradhan, N. V. Joshi, P. Ajitkumar, Mycobacterial cultures contain cell size and density specific sub-populations of cells with significant differential susceptibility to antibiotics, oxidative and nitrite stress. Front. Microbiol. 8 (2017): 463. doi:10.3389/fmicb.2017.00463.

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Resource availability:	**Reagents, Materials, and Equipment** **Reagents** (volumes required for Analytical method and Preparative method **)** 1. Percoll (Sigma) (sterile) to be stored at 4°C (9 ml; 27 ml) 2. 1.5 M NaCl (sterile) – freshly prepared (1 ml; 3 ml) 3. 0.15 M NaCl (sterile) - freshly prepared (4 ml; 12 ml) 4. Middlebrook 7H9 broth (sterile) – freshly prepared (50/100 ml; 200/400 ml) 5. 0.5% v/v Tween 80 (sterile) – freshly prepared (7 ml; 45 ml) 6. Phosphate buffered saline (PBS) (sterile) – freshly prepared (20 ml; 65 ml) 7. 10% Albumin-dextrose supplement (5-10 ml; 20-40 ml) **Materials** 1. Conical flasks (250 ml for analytical scale and 1000 ml for preparative scale) 2. Eppendorf tubes 1.5 ml volume (sterile) 3. Corex glass tube 35 ml capacity (from Sorval) 4. Syringe (0.60 × 25 mm) 2 ml (sterile) 5. Needle 23 gauge (sterile) 6. Sorvall tubes (35 ml capacity) for harvesting analytical scale cultures 7. GSA bottles (250 ml capacity) for harvesting preparative scale cultures 8. Ultraclear polyallomer ultracentrifuge tubes (Beckman Coulter Cat. No. 344059; 13.2 ml capacity) for analytical scale fractionation) 9. Polycarbonate capped bottles (Beckman Coulter, Cat. No. 357002; 50 ml capacity for preparative scale fractionation) 10. Pipettman (20 µl, 100 µl, 200 µl, 1000 µl) **Equipment** 1. Vortex mixer 2. Sorvall RC 5B centrifuge or Kubota 6000 centrifuge 3. Beckman Coulter SW41 rotor for analytical scale fractionation 4. Beckman Coulter JS13.1 rotor for preparative scale fractionation 5. Beckman L8-70M ultracentrifuge for analytical scale fractionation 6. Beckman J-30I centrifuge for preparative scale fractionation 7. Upright Carl Zeiss AXIO Imager M1 microscope with 100X objective and DIC. 8. Sonicator (Vibra-Cell; Sonics & Materials, Inc., USA) with microprobe

Method details

In vitro grown cultures of different *M. smegmatis* [1], *M. xenopii, M. tuberculosis*, and sputum of pulmonary tuberculosis patients broadly contain two subpopulations wherein the short-sized cells (SCs) arise mainly due to asymmetric division of about 20% of the cells undergoing septation, generating SCs and normal/long-sized cells (NCs) from every division [2,3]. The fractionation of the subpopulations was facilitated by the difference in their buoyant density, with the SCs having lower buoyant density and the NCs having higher buoyant density. We had earlier reported the fractionation in brief as part of our publication on the characterisation of the subpopulations [4]. About 90-98% enrichment was obtained respectively for SCs and NCs for *M. smegmatis* and 67-69% enrichment was obtained respectively for the SCs and NCs for *M. tuberculosis* was achieved through the fractionation method.

Most of the earlier Percoll density gradient centrifugation methods, which were designed for the preparation of sperm cells, mitochondria, X- and Y-bearing sperms [5–10], used Percoll continuous density gradient. Percoll discontinuous density gradient centrifugation was used for the rapid isolation and purification of functional platelet mitochondria [11]. The basic principle of the continuous and discontinuous gradient centrifugation remains the same, but the fractionation would differ because the final density in each unit volume of the linear continuous density gradient and of the discontinuous density gradient would be different. For example, the Percoll continuous density gradient prepared by us using a gradient mixer failed to give enriched fractionation of the mycobacterial subpopulations. Hence, we standardised a Percoll discontinuous density gradient centrifugation method for the fractionation of the mycobacterial subpopulations, and it gave enrichment of mycobacterial subpopulations. Thus, enriched fractions of the mycobacterial subpopulations could be obtained only by using the manually layered discontinuous density gradient, and not by the linear continuous gradient formed using gradient mixer.

Earlier studies have demonstrated that the buoyant density of *M. tuberculosis* cells correlates with the lipid content, when fractionated on a Percoll density gradient [12]. The lipid composition of the outer membrane of *M. smegmatis* and *M. tuberculosis* differs [13], with *M. tuberculosis* having a richer lipid layer than *M. smegmatis*. Both *M. smegmatis* and *M. tuberculosis* have been found to possess an external electron transparent layer [14]. However, pathogenic mycobacteria like *M. tuberculosis* have been found to possess a thick prominent outermost capsule-like layer as compared to non-pathogenic bacteria like *M. smegmatis* [15–17]. The capsular polysaccharides have been reported to be upregulated upon exposure to stress in both *M. smegmatis* and *M. tuberculosis* [18], suggesting similar pathways in the regulation of capsular production in both the species. However, the different lipid composition and/ or the difference in the thickness of the capsular layer between both the strains, might have caused the necessity to fractionate *M. tuberculosis*

cells at a lower Percoll density fraction (60% + 62%) as compared to *M. smegmatis* cells (64% + 66%). This observation correlates with the report on the influence of the presence of capsule on the differential Percoll discontinuous density gradient fractionation demonstrated in the case of capsulated and non-capsulated bacteria, such as *Klebsiella pneumoniae* and *Streptococcus pneumoniae* [19].

Bacterial strains and culture conditions

1. *M. smegmatis* mc^2155 [1] cultures were grown in Middlebrook 7H9 broth containing 0.05% v/v Tween 80, at 37°C, with shaking at 170 rpm, till the $OD_{600\ nm}$ of the culture reached 0.6 (mid-log phase, MLP).
2. *M. tuberculosis* $H_{37}R_a$ [obtained from the National JALMA Institute of Leprosy and Other Mycobacterial Diseases, Agra, India] was cultured in Middlebrook 7H9 medium containing albumin-dextrose supplement and 0.05% Tween 80 at 37°C, with shaking at 170 rpm, till the $OD_{600\ nm}$ of the culture reached 0.6 (mid-log phase, MLP).
3. These MLP cultures are used for the fractionation of the subpopulations. We had earlier reported the existence of two subpopulations of **s**hort-sized **c**ells (**SC**s) and the **n**ormal-sized **c**ells (**NC**s) in MLP cultures [2,3]. Genomic content of SCs and NCs was inferred to be same as they both independently gave rise to a whole MLP type population on re-inoculation [2–4].
4. The distribution of *M. smegmatis* MLP cells based on their cell-size showed a clear demarcation of a subpopulation of cells (~10%) between the size range of 2-3 μm. The average cell-size of this subpopulation of cells was found to be 2.60 ± 0.25 μm and was called the short-sized cells (SCs) [4]. On a similar note, TEM images of *M. tuberculosis* mother cells undergoing asymmetric cell division revealed the average cell-size of the shorter portion to be ~0.73 μm [2]. Thus, the size cut-off for SCs was that the cells with size ≤ 2.6 μm were called short cells in the case of *M. smegmatis* [3,4] and the cells with size ≤ 0.73 μm were called short cells in the case of *M. tuberculosis* [2]. Therefore, *M. smegmatis* cells > 2.6 μm and *M. tuberculosis* cells > 0.73 μm were the respective NCs.

Percoll Discontinuous Density Gradient Centrifugation

The method was designed for the enrichment and isolation of mycobacterial subpopulations by fractionation at analytical scale (50-100 ml) and preparative scale (200-400 ml). Firstly, to understand the morphological features of the SCs and NCs, the *M. smegmatis* MLP cells were fractionated at analytical scale and then processed for Scanning and Transmission Electron Microscopy [4]. Subsequently, the differential stress response of the SCs and NCs was determined post-exposure to antibiotic, oxidative, and nitrite stress [4]. However, an increased number of the SCs/NCs were required to address the molecular reasons for their differential stress-susceptibility. Hence the *M. smegmatis* MLP cells were subjected to preparative scale Percoll discontinuous density gradient centrifugation to obtain enriched fractions of SCs/NCs and processed, as previously described [20], to determine the levels of Reactive Oxygen Species (ROS) and Fe (II). Further, the preparative scale fractionation of SCs/NCs was also required: (i). for the gene expression analyses of NADH Oxidase and other antioxidant genes, and (ii). to find out the benefit of the differential levels of ROS and Fe (II) in terms of the antibiotic-resister generation potential of the enriched fractions [21].

Analytical scale

1. 50 ml cultures grown in 250 ml flasks (It is important to maintain the culture to flask volume for reproducible population heterogeneity).
2. For the fractionation of SCs and NCs from mycobacterial cultures, discontinuous gradients, at 2% increment of the gradient, from 64% to 80% for *M. smegmatis* cultures and from 60% to 76% for *M. tuberculosis* cultures, were set up manually every time.

(We had tried to prepare the Percoll density gradient using gradient mixers, however the gradient obtained did not fractionate the mycobacterial subpopulations. This outcome would have been due to the differences in the final density gradient obtained by using the gradient mixer and by the manual method)

3. With practice, a single person can set up in parallel a maximum of 3 independent analytical or preparative scale gradients without the gradients of different Percoll concentrations getting mixed up.
4. The **SCs** containing **f**raction was called the **SCF** and the **NCs** containing **f**raction was called the **NCF**.
5. The SCF was found distributed into two consecutive low-density fractions, which we called SCF1 and SCF2. In the case of *M. smegmatis* samples, the 64% and 66% fractions were called SCF1 and SCF2, whereas for *M. tuberculosis* samples, the 60% and 62% Percoll fractions constituted SCF1 and the 64% fraction was SCF2.
6. The NCs got fractionated mostly into one of the higher density fractions. The 78% NCF Percoll fraction contained >80% NCs of *M. smegmatis* while the 66% fraction of *M. tuberculosis* samples (NCF) contained >80% NCs [4].

The assignment of the SCF1, SCF2, and NCF fractions were based on the above-mentioned size cut-off of the SCs and the NCs in the case of these two mycobacterial species. Thus, the fractionation gave an enrichment of the cells of the respective sizes.

Analytical scale fractionation of the subpopulations

Preparation of *M. smegmatis* samples: All the procedures, except centrifugations and sonications, were conducted in laminar airflow hood, for maintaining sterility.

Table 1
Percoll discontinuous density gradient profile for the fractionation of SCF and NCF from *M. smegmatis* (*Msm*) and *M. tuberculosis* (*Mtb*) cultures.

Msm Percoll fraction (%)	Before centrifugation Density (g/mL)	After centrifugation Density (g/mL)	*Mtb* Percoll fraction (%)	Before centrifugation Density (g/mL)	After centrifugation Density (g/mL)
64	1.064	0.936	60	1.036	1.036
66	1.074	1.023	62	1.052	1.041
68	1.078	1.034	64	1.061	1.044
70	1.082	1.041	66	1.072	1.046
72	1.086	1.052	68	1.077	1.049
74	1.092	1.058	70	1.079	1.050
76	1.096	1.059	72	1.084	1.066
78	1.098	1.061	74	1.088	1.068
80	1.100	1.064	76	1.093	1.069

1. *M. smegmatis* cells, cultured in Middlebrook 7H9 medium, grown to 0.6 $OD_{600\ nm}$ (mid-log phase), in 50 ml volume in 250 ml flask or in 100 ml volume in 500 ml flask were harvested in Sorvall tubes at ~4300 x g (8000 rpm), 25˚C, for 15 min in Sorvall or Kubota centrifuge.
2. Five ml of sterile 0.5% Tween 80 was used to wash the *M. smegmatis* cell pellet, which was collected in Sorvall tube from 50 ml or 100 ml culture. After washing by resuspension, the cells were collected by centrifugation at ~4300 x g, at 25˚C for 15 min in Sorvall or Kubota centrifuge. This step was repeated.
3. The washed pellet was again resuspended in one ml of 0.5% sterile Tween 80 and transferred to an Eppendorf tube. The cells were dispersed well by vortexing for one min and then syringed aseptically 15 times for removing clumps, if any, using one ml syringe with needle size 0.45 × 13 mm, 26 gauge. Clumps, if any, if not removed, would affect fractionation.

We verified that the syringing does not cause cell death and hence does not reduce CFU [see (ii) under NOTES]. Disruption of all clumps was confirmed using microscopy.

Preparation of *M. tuberculosis* samples

1. *M. tuberculosis* was cultured in 100 ml Middlebrook 7H9 medium in 500 ml flask. The culture at 0.6 $OD_{600\ nm}$ was harvested by pouring into Sorvall tubes. The cells were centrifuged down at ~4300 x g, 25˚C, for 15 min in Sorvall or Kubota centrifuge.
2. Five ml of sterile 0.5% Tween 80 was used to resuspend the cell pellet and wash by dispersing the cells in the pellet, followed by centrifugation at ~4300 x g, 25˚C for 15 min.
3. The cells in the washed pellet were resuspended in one ml of 0.5% sterile Tween 80. The cells were subjected to sonication aseptically at 22% amplitude for 2 secs for removing clumps, if any.
4. Disruption of all clumps was confirmed using microscopy.

The Percoll discontinuous density gradient preparation

Standardisation of the Percoll discontinuous density gradient setup was performed with reference to the continuous density gradient reported [12]. In this report, the fractionation of *M. tuberculosis* cells, post exposure to multiple stress conditions, was found to be ~1.019 – 1.064 g/ml. Using the formula provided in the technical bulletin for Percoll density gradient preparation published by Sigma (Sigma Technical bulletin), discontinuous density gradients of different ranges of percentage of Percoll were tried to arrive at the optimum range. Such exercise was performed independently for the *M. smegmatis* and *M. tuberculosis* strains. Therefore, for different strains of mycobacteria, the optimum range for the fractionation of subpopulations, if they exist, can be found out only by repeatedly trying out by trial-and-error different ranges of Percoll discontinuous density gradients.

V_y = volume of diluting medium in mL
V_i = volume of SIP in mL
ρ_i = density of SIP in g/mL
ρ_y = density of diluting medium in g/mL
(Density of 0.15 M NaCl is ~1.0046 g/mL) *
ρ = density of diluted solution produced in g/mL

The entire procedure has been depicted in a flow diagram (Fig. 1). In the case of *M. smegmatis* and *M. tuberculosis*, the best enrichment of the mycobacterial subpopulations was obtained in 64%-80% for *M. smegmatis* and 60%-76% for *M. tuberculosis*. Table 1 describes the densities of the different fractions, before and after centrifugation, for *M. smegmatis* and *M. tuberculosis* cells.

1. A laboratory stand with a clamp was set up. An ultraclear polyallomer ultracentrifuge tube (Beckman Coulter Cat. No. 344059; 13.2 ml tube volume) was clamped vertically to the stand.
2. As per the manufacturer's protocol (Sigma), nine parts of Percoll (Sigma) were mixed with 1 part of sterile 1.5 M NaCl (Sigma) to prepare the Stock Isotonic Percoll (SIP). The SIP must be made fresh every time for the gradient preparation.

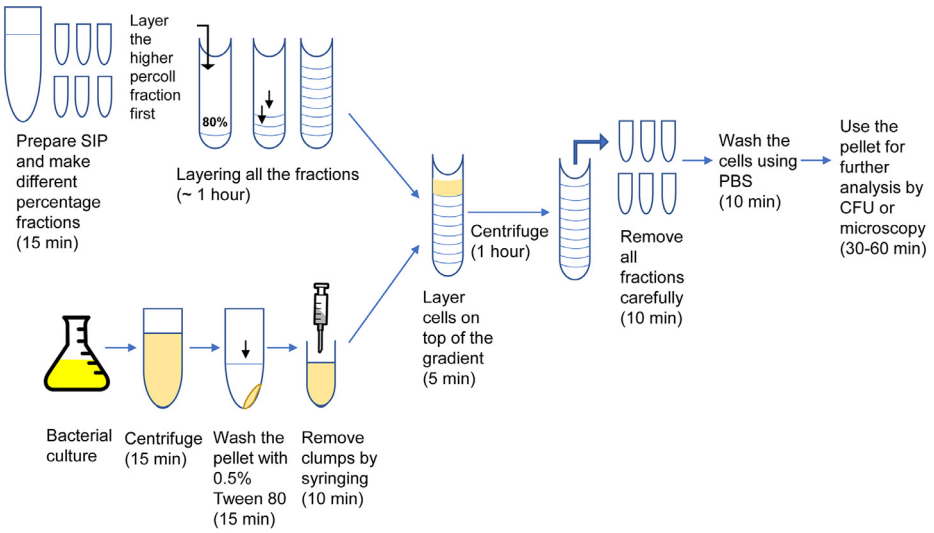

Fig. 1. Summary of the Percoll gradient centrifugation procedure developed to separate bacterial subpopulation with time estimate for each step

3. The different percentages of gradients were made by diluting the SIP further with 0.15 M NaCl. One ml was kept as the final volume of each percentage fraction of Percoll.
4. Accurately, the highest percentage Percoll fraction was first layered manually, 200 µl at a time, carefully and very slowly using Pipetteman, allowing it to go down very slowly along the side of the tube into the bottom of the ultraclear polyallomer ultracentrifuge tube.
5. Once the complete one ml of the highest percentage Percoll fraction was layered, Percoll of next lower percentage was layered onto the top layer of the highest percentage Percoll, with 200 µl at a time, using Pipetteman, without disturbing the existing Percoll layer. The layering of the lower percentage Percoll fraction was performed very carefully and slowly so as to allow the Percoll to slowly run down the inner wall of the tube, pool over the top of the previous density gradient layer without disturbing the gradient interface. In this manner, step by step, the different lower percentages of Percoll were layered onto the existing gradient.
6. The addition of Percoll onto the previous higher percentage of Percoll in the gradient requires immense patience, care, dedication, full attention, and alertness. The layering must be completed in one sitting and used immediately for layering the cells (see below), followed with centrifugation for the fractionation. If the Percoll gradient was left unused, the different percentages of the Percoll gradient may get mixed at the boundaries at a very slow undetectable rate due to Brownian motion. This will adversely affect the fractionation of mycobacterial subpopulations.
7. In the case of *M. tuberculosis* samples also, the cells were immediately layered manually onto the top of the Percoll gradient, which was prepared exactly as performed for *M. smegmatis* cultures, but in the 60% to 76% range of Percoll.

The Percoll discontinuous density gradient centrifugation

1. The tubes containing the Percoll discontinuous gradient, with the cells layered on top, were carefully loaded into SW41 rotor and ultracentrifugation was performed in Beckman L8-70M ultracentrifuge at 385 x g (1500 rpm), 20˚C, for 60 min.
2. After ultracentrifugation, each of the one ml Percoll fraction of specific percentage is aseptically pipetted out slowly and carefully, as 250 µl at a time, into separate tubes. Thus, each one ml of the fraction gets distributed into four tubes. Into each tube, five volumes (1250 µl) of sterile 1x PBS were added and gently vortexed to disperse the cells.
3. From each of the four tubes, 100 µl of the uniform cell suspension was withdrawn and used for plating for CFU determination. We used to determine the CFU of the fractions from at least 10 different preparations from 10 different samples that were cultured independently, to verify the consistency of the yield. We have always got consistent and reproducible yields.
4. The dispersed cells were then centrifuged at ~3900 x g, 25˚C for 10 min. After centrifugation, the cell pellets 64% (SCF1) and 66% (SCF2) Percoll fractions of *M. smegmatis* sample and the 60% & 62% (SCF1) and 64% (SCF2) Percoll fractions of *M. tuberculosis* would be almost invisible in the case of the fractions from analytical preparation but clearly visible in the case of the fractions from the preparative scale.
5. The 78% (NCF) fraction of *M. smegmatis* samples and the 66% (NCF) fraction of *M. tuberculosis* samples would be clearly visible both in the analytical and preparative scale preparations. From each of the four tubes, now containing 1500 µl of supernatant and the cell pellet, the supernatant must be pipetted out very carefully. Decanting of the supernatant should be avoided as the cells would get dislodged from the pellet and lost. Using either 400 µl of 1x PBS or 0.5% Tween 80 or Middlebrook 7H9 medium, the cells from the four tubes were pooled into one tube.

Table 2
CFU determination for the number of cells present in each fraction of SCF and NCF from M. smegmatis (Msm) and M. tuberculosis (Mtb) cultures.

Msm Percoll fraction (%)		No. of cells present in each fraction	*Mtb* Percoll fraction (%)		No. of cells present in each fraction
SCF1	64	5.8 (± 3.2) X 10^6	SCF1	60/62	4.2 (± 2.2) X 10^8
SCF2	66	8.4 (± 4.7) X 10^6	SCF2	64	4.2 (± 3.5) X 10^8
NCF	78	4.5 (± 2.3) X 10^9	NCF	66	2.8 (± 2.3) X 10^8

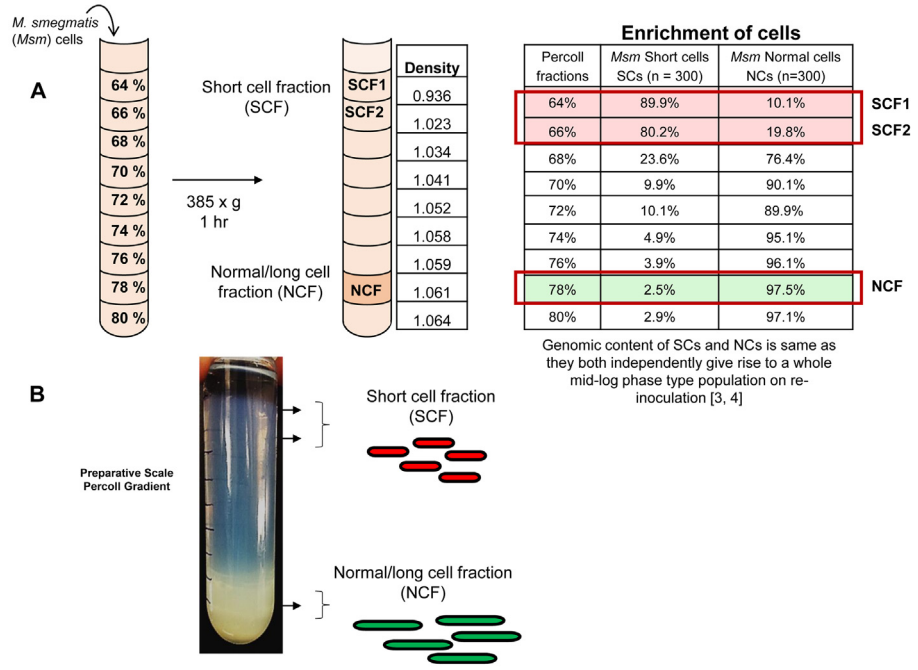

Fig. 2. Percoll discontinuous density gradient centrifugation-based fractionation of SCs and NCs from *Mycobacterium smegmatis* cultures. (**A**) Fractionation steps and the extent of enrichment of NCs and SCs. (**B**) The ultracentrifuge tube after fractionation.

Determination of cell numbers in each fraction: Following the Percoll discontinuous density gradient centrifugation, the enriched fractions were washed with 1x PBS and then resuspended in Middlebrook 7H9 medium. From each of the enriched fractions, an aliquot was taken for CFU determination. Table 2 shows the number of cells present in each of the *M. smegmatis/M. tuberculosis* enriched fractions after the analytical scale Percoll density gradient centrifugation. The calculation (with standard deviation) was performed using the CFU data from 10 independent experiments using 10 independent cultures.

Reproducibility and consistency of the method: The fractions were examined under DIC microscopy (Carl Zeiss AXIO Imager M1 microscope) to determine the presence of SCs and NCs in terms of cell sizes and for measuring their size distribution in each fraction [2–4]. The mycobacterial purity of the preparation was determined by acid-fast staining and examining under bright field. Verification of purity and size distribution were performed every time the fractions were prepared. We always used to get consistent and reproducible yield of SCs and NCs despite resuspending them in either 1x PBS or 0.5% Tween 80 or Middlebrook 7H9 medium. Resuspension in 1x PBS was for the determination of H_2O_2 and Fe (II) levels in the cell lysates of SCF and NCF [20,21], while resuspension in 0.5% Tween 80 was for the determination of enrichment of the SCs and NCs in the SCF and NCF [4], and Middlebrook 7H9 medium was used for resuspension for the exposure to oxidative/ antibiotic stress [4,20,21]. The qualitatively and quantitatively differential and distinct response of the SCF and NCF subpopulations to oxidative, nitrite, and antibiotic stress conditions as reported [4,20,21] was taken as the proof that the subpopulations prepared by the method were homogeneous and not mixed-up subpopulations. We always got consistent and reproducible response of the subpopulations to the stress conditions. The analytical method of fractionation is depicted in Fig. 2. The representative DIC images of a typical preparation of *M. smegmatis* SCF1, SCF2, and NCF samples are given in Fig. 3.

Negligible cell loss during fractionation: To determine the loss of cells during/after the Percoll discontinuous density gradient centrifugation, 100 mL of *M. smegmatis* MLP cells were divided equally into two parts, one part was plated directly (100 µl), and the other part was processed for Percoll discontinuous density gradient centrifugation. After completion of the centrifugation, cells from all the fractions were pelleted down, washed and 100 µl from each fraction was plated on Middlebrook 7H10 agar. The data was calculated from three independent experiments and the percentage recovered after Percoll treatment was found to be 82.33% ± 25.1%.

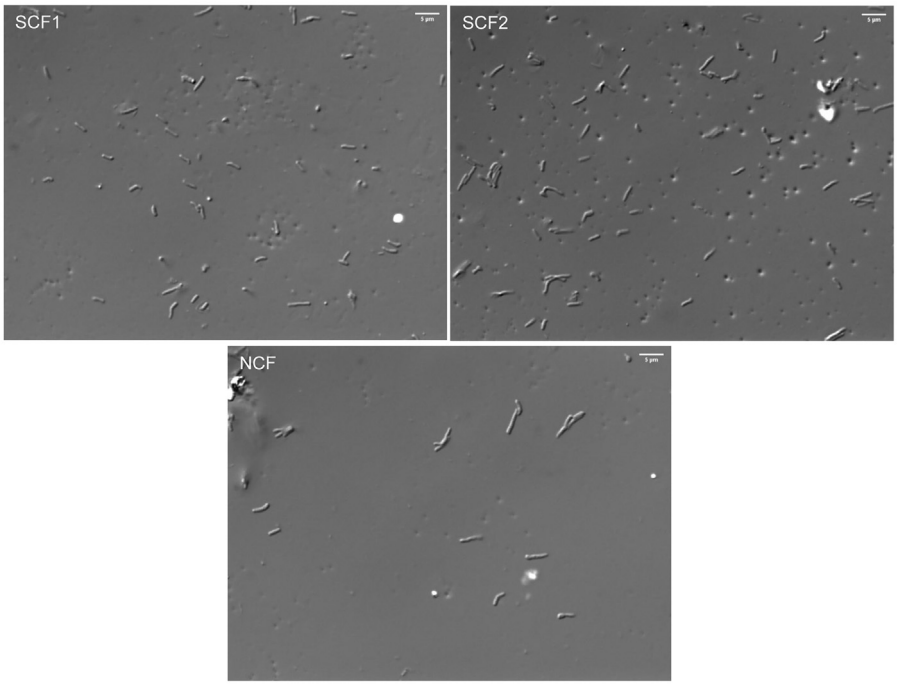

Fig. 3. DIC images of SCF1, SCF2 and NCF from *Msm* cultures after Percoll discontinuous density gradient fractionation. Scale, 5 μm.

Table 3
Major differences between the analytical scale and preparative scale Percoll discontinuous density gradient centrifugation for the preparation of mycobacterial subpopulations.

	Differentiating factor	Analytical Scale	Preparative scale
1.	Volume of bacterial culture required	50-100 ml	200-400 ml
2.	Volume of each Percoll fraction	1 ml	4 ml
3.	Volume of single-cell suspension of cells	1 ml	4 ml
4.	Tubes used to set up Percoll gradient	Ultraclear polyallomer ultracentrifuge tube	50 ml Beckman Coulter polycarbonate capped bottle
5.	Ultracentrifuge used	Beckman L8-70M centrifuge	Beckman J-30I centrifuge
6.	Rotor used for ultracentrifugation	SW41 rotor	JS 13.1 rotor
7.	Volume of PBS required for washing each fraction	5 ml	20 ml

Percoll fractionation does not affect the native state of the cell: We validated that Tween 80 does not affect the mycobacterial cell envelope by scanning and transmission electron microscopy of SCF and NCF, which revealed normal cell envelope ultrastructure and lipid-rich membrane vesicles (MVs) [4]. In addition, *M. smegmatis* SCF and NCF cells (~ 3×10^6 cells/ml) obtained from the Preparative Scale Percoll discontinuous density gradient centrifugation were inoculated at 1% inoculum into fresh Middlebrook 7H9 medium. The cultures inoculated from the enriched fractions reached mid-log phase (~0.6 $OD_{600\ nm}$) after ~18-21 hrs of incubation at 37°C. The unprocessed *M. smegmatis* MLP cells, when re-inoculated at the same cell density as that of the enriched fractions, also reached ~0.6 $OD_{600\ nm}$ after ~18-21 hrs of incubation. This regeneration potential of the enriched fractions to give rise to the same density of MLP population in the same time frame as that of the unprocessed *M. smegmatis* cells showed that the syringing as well as the other procedures followed during the Percoll discontinuous density gradient centrifugation did not affect the viability of the cells [4]. Above all, SCF and NCF showed 99% viability with SYTO9 / propidium iodide staining [4]. These data validate that none of the steps or reagents used in the fractionation procedure does not affect the native state or viability of the cell.

Preparative scale fractionation of the subpopulations

The entire protocol for the preparative scale fractionation is identical to that of the analytical scale fractionation, except for the following differences (Table 3).

1. The Percoll percentage gradients were prepared in 4 ml per percentage of Percoll using SIP and 0.15 M NaCl as described above. From each of the 4 ml of the Percoll gradient percentage, one ml was pipetted at a time using Pipetteman very carefully into 50 ml Beckman Coulter polycarbonate capped bottles (Beckman Coulter, catalogue no. 357002) along the sides, from the highest to the lowest percentage Percoll.

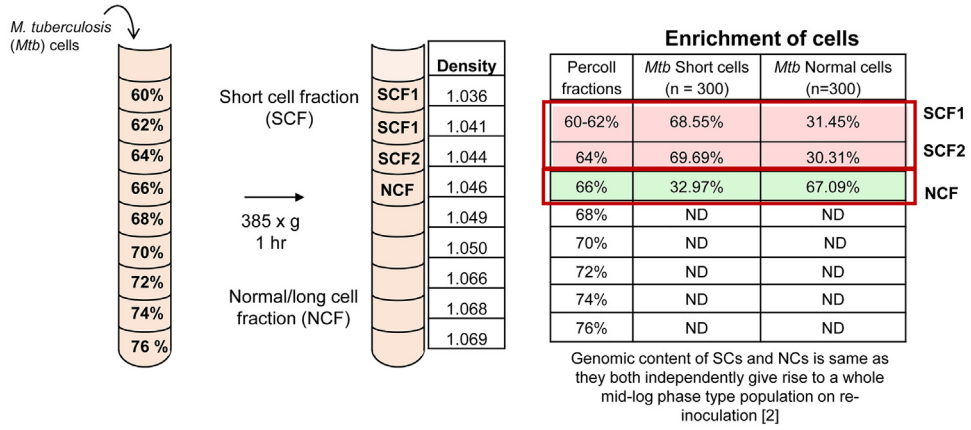

Fig. 4. Percoll discontinuous density gradient centrifugation-based fractionation of SCs and NCs from *Mycobacterium tuberculosis* cultures. Cartoon showing the fractionation steps and the extent of enrichment of NCs and SCs. ND – Very few or no cells.

2. *M. smegmatis* cells were harvested at 0.6 $OD_{600\ nm}$ in GSA bottles from 1 or 2 × 200 ml cultures from one litre flasks (200 ml culture per flask) by centrifugation at ~7100 x g, 25°C, for 15 min. The cells were resuspended in 20 ml of sterile 0.5% Tween 80, transferred to Sorvall tubes, and centrifuged at ~4300 x g, 25°C for 15 min. The cells were washed once more as mentioned above and centrifuged.
3. Using 4 ml of 0.5% sterile Tween 80, the cells were resuspended, transferred to two 2 ml Eppendorf tubes, and dispersed by vortexing for one min. The cell suspension was withdrawn into a 2 ml syringe with 26-gauge needle of 0.60 × 25 mm size. The sample was aseptically syringed 15 times to remove clumps, if any. It was then immediately layered manually on to the top of the gradient, very carefully as described above.
4. The polycarbonate capped bottles containing the gradient with cells on top were placed in JS 13.1 rotor and centrifuged in Beckman J-30I centrifuge at 352 x g (1500 rpm), 20°C, for 60 min.
5. After centrifugation, the Percoll fractions (four ml each) having the cells were pipetted out aseptically using Pipetteman into Corex glass tubes. Sterile 1x PBS (20 ml) was added into the Corex glass tubes, vortexed, and centrifuged at ~4300 x g, 25°C for 10 min.
6. The cell pellets in the 64% and 66% Percoll fractions were found to be loose. From each fraction, the supernatant was pipetted out very carefully without disturbing the cell pellet. From each fraction, the cell pellet was resuspended in 500 µl of 1x PBS, transferred to Eppendorf tubes, and centrifuged at ~3900 x g, 25°C for 10 min, to remove all the residual Percoll. The supernatant was pipetted out from each fraction.
7. The cells in the 64% and 66% (SCF1 and SCF2, respectively) were resuspended in 400 µl each of 1x PBS or 0.5% Tween 80 or Middlebrook 7H9 broth. The 78% Percoll fraction (NCF) gave slightly firm good size pellet, and it was also resuspended in 400 µl of 1x PBS or 0.5% Tween 80 or Middlebrook 7H9 broth.
8. The CFUs of the cells in the SCF1, SCF2, and NCF were determined from at least 10 different fractionations using 10 independently cultured samples. The ratios of the CFUs (and hence the volume of the respective cell suspension) gave the proportion at which SCF1, SCF2, and NCF cells existed. Thus, the proportions of the cells in SCF1, SCF2 and NCF were determined in this manner from the CFU.
9. The purity of the preparation was ensured using acid fast staining and examining under bright field. The size distribution of the cells in the SCF1, SCF2, and NCF was verified under DIC. Carl Zeiss AXIO Imager M1 microscope was used for both purposes.

The fractionation of the SCF and NCF following the preparative scale Percoll density gradient centrifugation is depicted in Fig. 2.

CFU determination of *M. smegmatis* and *M. tuberculosis* SCF1, SCF2 and NCF

The CFUs of *M. smegmatis* cells in the 64% (SCF1), 66% (SCF2) and 78% (NCF), and *M. tuberculosis* cells in the 60% & 62% (SCF1), 64% (SCF2), and 66% (NCF) (Fig. 4) were determined using the fractions from the analytical scale preparation using Percoll discontinuous gradient centrifugation. The cells from these fractions were resuspended in 400 µl of 1x PBS or 0.5 % Tween 80 or Middlebrook 7H9 medium (resuspension in: 1x PBS for the determination of H_2O_2 and Fe (II) levels in the cell lysates of SCF and NCF [5,6]; 0.5% Tween 80 for the determination of enrichment of the SCs and NCs in the SCF and NCF [4]; and Middlebrook 7H9 medium for the subsequent exposure to oxidative/ antibiotic stress [4,20,21]. Due to higher cell yield, the cells in the NCF needed to be diluted five times with Middlebrook 7H9 medium. From each of these samples, 100 µl was taken for serial dilution followed by plating on Middlebrook 7H10 plates to determine their CFU. The number of cells in each fraction was determined from CFU data. It was essentially performed as follows. Once the final cell suspension of SCs and NCs were prepared, 20 µl was taken out and diluted 10 times by adding to 180 µl of PBS/ Middlebrook 7H9 media, this dilution is called as '-1'. This was mixed properly. Next, from this

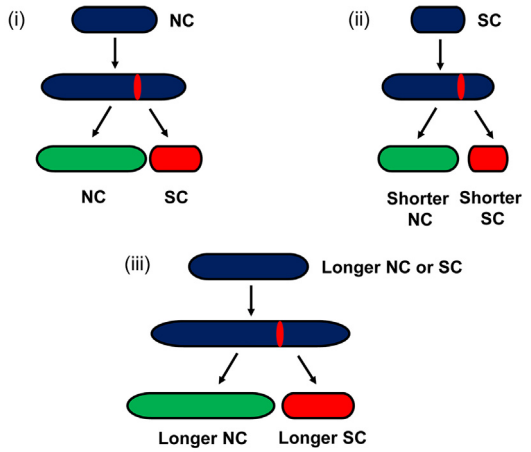

Fig. 5. Three ACD types generate differently sized NCs and SCs. (i). An NC generates a NC and a SC; (ii). An SC generates a short NC and a shorter SC; and (iii). A long-sized NC/SC generates a longer NC and longer SC. NC, Normal/long Cell; SC, Short Cell.

'-1' dilution, 100 µl was taken out and diluted 10 times by adding 900 µl of PBS/ 7H9 media and mixed thoroughly. Likewise, such dilutions were made till '-6' dilution. Dilutions were prepared for all the samples and from each sample, 100 µl was spread plated on Middlebrook 7H10 agar plate. Colonies were counted from each plate after the required incubation time (72 hours for *M. smegmatis* and 21 days for *M. tuberculosis*). This CFU data was used to calculate the number of cells from each sample and this number was compared among the samples.

NOTES: (i). The possibilities for the presence of SC-sized cells in the NC fraction and the NC-sized cells in the SC fraction has been depicted in Fig. 5. Differential buoyant density between the NCs and SCs is the criterion for the fractionation of NCs into higher density and the SCs into lower density. Therefore, the SC-like (short-sized) cells in the NC fraction and the NC-like (normal-sized) cells in the SC fraction are truly NCs and SCs, respectively, in terms of their difference in the buoyant density even though there is deviation from their respective average size. This is possible when a shorter-sized (below average size) NC mother cell divides into NC and SC by asymmetric division where the SC and NC will be shorter in size than that of the average NC and SC cells. Similarly, the presence of longer-sized SCs is possible when a longer-sized (above average size) NC mother cell divides into NC and SC by asymmetric division where the SC and NC will be longer in size than that of the average NC and SC cells. We have seen the generation of longer SCs and shorter NCs while documenting asymmetric division using live-cell imaging microscopy [2,3]. Since we had hardly seen clumps (or multiplets), the possibility of clumps containing differently sized cells contributing to such variation in the NC and SC fractions is minimal. Similar is the case with duplets. In any case, the clumps and duplets/multiplets would have high buoyant density, and hence will invariably settle down along with NCs at higher density of Percoll. Therefore, it cannot explain the presence of longer-sized SCs in the lower buoyant density SCs fraction that we had seen. Hence, the role of duplets, multiplets, or clumps contributing to size variant SCs and NCs is very minimal. Thus, the most probable reason would be the asymmetric division of longer SCs generating short-sized NCs and longer-sized NCs generating longer SCs, which we had observed in live-cell imaging microscopy experiments reported [2,3]. The *M. smegmatis* size outliers (the cells longer than 2.58 ± 0.71 µm in SCF1, longer than 2.73 ± 0.90 µm in SCF2, and the cells shorter than 3.58 ± 1.16 µm in NCF) were found to be 30.96% in SCF1, 26.40% in SCF2, and 27.01% in NCF, respectively [reported in Table S4 in [4]. Whereas the *M. tuberculosis* size outliers (the cells longer than 1.06 ± 0.24 µm in SCF1, longer than 1.41 ± 0.37 µm in SCF2, and the cells shorter than 1.64 ± 0.47 µm in NCF) were found to be as follows: 27.51% in SCF1, 29.03% in SCF2, and 28.46% in NCF [reported in Table S7 in [4]. The mechanistic assumption mentioned above was found to be valid in terms of the qualitatively and quantitatively differential and distinct response of the two subpopulations SCF (includes longer SCs) and NCF (includes shorter NCs) to oxidative, nitrite, and antibiotic stress conditions as reported [4,20,21]. This was taken as proof that the subpopulations prepared by the method were homogeneous and not mixed-up subpopulations.

(ii). Comparing the SCF and NCF pellets obtained after the Percoll density gradient centrifugation, the SCF pellets are smaller in size and loose in texture while the NCF pellets are bigger and firm. The loose pellet of the SCF cells could be due to the higher lipid contents in them as compared to those in the NCF.

(iii). We avoided open needle emulsification of *M. tuberculosis* mid-log phase cell preparation as it could not successfully remove clumps. If clumps are not removed, the clumps will settle down in the gradient, causing inefficient fractionation leading to non-enrichment of the subpopulations. Hence, we used sonication and found that sonication was sufficient for removing the clumps, as clumps were not observed in the microscopy images (Fig. 8D in [4]).

(iv). For obtaining more quantities of the SCs and NCs, 2-3 analytical/ preparative scale Percoll discontinuous density gradients need to be prepared in parallel. For this, one needs to keep the following points in mind:

1. The Percoll discontinuous density gradient fractions for the different gradient setup should be prepared individually, at the suggested scale, and not by increasing the Percoll quantity and tube size.

2. Only after all the different discontinuous density fractions of Percoll are prepared individually, their layering to generate the discontinuous gradient setup should be initiated.
3. Layer the same Percoll density fraction in each of the 2-3 gradient setups, one after the other. Once the same density fraction has been layered in all the gradient setups, then the next lower density Percoll fraction should be layered.
4. The *M. smegmatis/M. tuberculosis* cultures could be pelleted and washed with 0.5% Tween-80, after layering the three higher density Percoll fractions in each gradient setup (for example, 80%, 78% and 76% for *M. smegmatis* cells and 76%, 74%, and 72% for *M. tuberculosis* cells). Subsequently, layer the remaining decreasing density Percoll fractions.
5. The *M. smegmatis/M. tuberculosis* cells should be prepared, syringed, and layered immediately after syringing, in each gradient setup. A small portion of the cells before and after syringing is used for finding out the presence of clumps, if any, using microscopy.
6. While isolating the enriched fractions, the same fraction should be pipetted out from all the gradient setups and then continue with the next density gradient fraction.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Srinivasan Vijay: Conceptualization, Methodology, Data curation, Writing – review & editing. **Rashmi Ravindran Nair:** Conceptualization, Methodology, Data curation, Writing – review & editing. **Deepti Sharan:** Conceptualization, Methodology, Data curation, Writing – review & editing. **Kishor Jakkala:** Conceptualization, Methodology, Data curation, Writing – review & editing. **Parthasarathi Ajitkumar:** Conceptualization, Supervision, Writing – review & editing.

Data availability

No data was used for the research described in the article.

Ethics statements

The work reported here did not involve human subjects, or animal experiments, or collection of data from social media platforms.

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