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Method Article

A simple heat-based alternative method for deparaffinization of histological sections significantly improves acid-fast staining results for Mycobacteria in tissue ☆

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ABSTRACT

Histopathology is the study of how disease alters human and animal tissue and is based on the microscopic examination of stained tissue sections. To maintain tissue integrity, preserving it from degradation, it is initially fixed, primarily with formalin, before being treated with alcohol and organic solvents, allowing the infiltration of paraffin wax. The tissue can then be embedded in a mold and sectioned, usually at a thickness between 3 and 5 μm, before staining with dyes or antibodies to demonstrate specific components.

As the paraffin wax is insoluble in water, it is necessary to remove it from the tissue section before applying any aqueous or water-based dye solution, to allow the tissue to successfully interact with the stain. This deparaffinization/hydration step is normally carried out using xylene, an organic solvent, followed by hydration using graded alcohols.

However, this use of xylene has been shown to have detrimental effects on acid-fast stains (AFS), such as those employed to demonstrate *Mycobacterium*, including the causative agent of tuberculosis (TB), as the integrity of the lipid-rich wall present in these bacteria may be compromised using xylene.

A simple, novel method, Projected Hot Air Deparaffinization (PHAD) removes the solid paraffin from the tissue section without the use of any solvents, which produces significantly improved staining results using AFS. PHAD relies on the projection of hot air onto the histological section to melt and remove paraffin from the tissue, which can be achieved using a common hairdryer.

- PHAD relies on the projection of hot air onto the histological section which can be achieved using a common hairdryer.
- The blowing force is such that melted paraffin is removed from the tissue in 20 min.
- Subsequent hydration allows for using aqueous histological stains with success, such as the fluorescent auramine O acid-fast-stain.

☆ Related research article: Marinho, P. F., Vieira, S. L., Carvalho, T. G., Peleteiro, M. C., Hanscheid, T. (2022). A novel and simple heat-based method eliminates the highly detrimental effect of xylene deparaffinization on acid-fast stains. Am J Clin Pathol 2023 (in press).

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More specific subject area:	*Clinical Pathology*
Name of your method:	*Projected Hot Air Deparaffinization (PHAD)*
Name and reference of original method:	*NA*
Resource availability:	*NA*

Method details

Background

Histology is based on microscopic observation of sectioned animal or human tissue with 3–5 micrometer thickness. Native tissue cannot be sectioned with this thickness without destroying tissue integrity. This has led to the widely used standard technique to embed tissues in paraffin which solidifies at room temperature and thus allows sectioning. Paraffin wax consists of a mixture of hydrocarbons with 20–40 carbon atoms and starts to melt at around 60 ˚C [1]. It is insoluble in water [2] which implies that all paraffin must be removed before the water-based stains can be applied. Protocols to remove paraffin use xylene (an aggressive solvent) and alcohol (ethanol) at various concentrations (Fig. 1).

It has also been demonstrated that the standard xylene-deparaffinization method of formalin-fixed paraffin-embedded tissue sections (FFPE) may have a detrimental effect on stains, especially acid-fast-stains (AFS) for the detection of mycobacteria in tissue, important to establish a diagnosis of tuberculosis (TB) [3,4].

TB causes 10 million infections and 1.5 million fatalities per year on average, of which 16% were extra-pulmonary tuberculosis (EPTB) in 2019 [5,6]. Microbiological diagnosis is often very difficult in EPTB. It is histology that often reveals TB-like tissue lesions, such as granulomas, which suggests a diagnosis of EPTB. However, AFS stained sections are needed for a definitive diagnosis but are often negative for mycobacteria [7–9]. AFS depend on the integrity of the lipid rich bacterial cell wall, which xylene seems to damage [3]. Certainly, xylene deparaffinization has been shown to severely impair bacilli staining and subsequent detection when compared to an alternative heat-based approach [3].

Here we describe in detail a simple, novel method, Projected Hot Air Deparaffinization (PHAD), which relies on the projection of hot air onto the histological section to remove the solid paraffin and which completely bypasses the use of any organic solvent (Fig. 2).

The method uses a common household appliance (hairdryer) and common laboratory equipment. Thus, it is easily possible to substitute any of these components if the specified parameters (temperature, blowing force, distances, etc.) are met. We illustrate

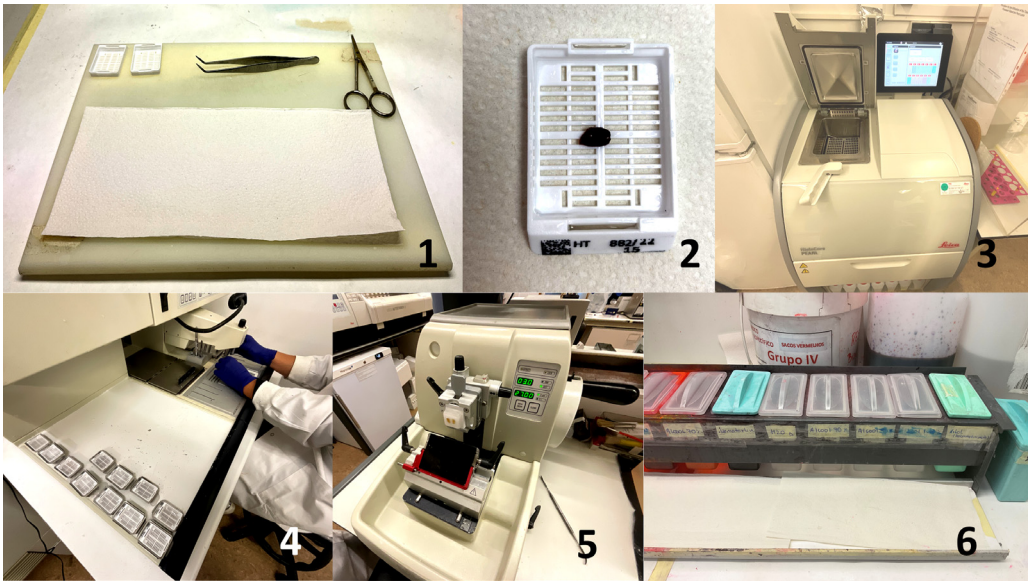
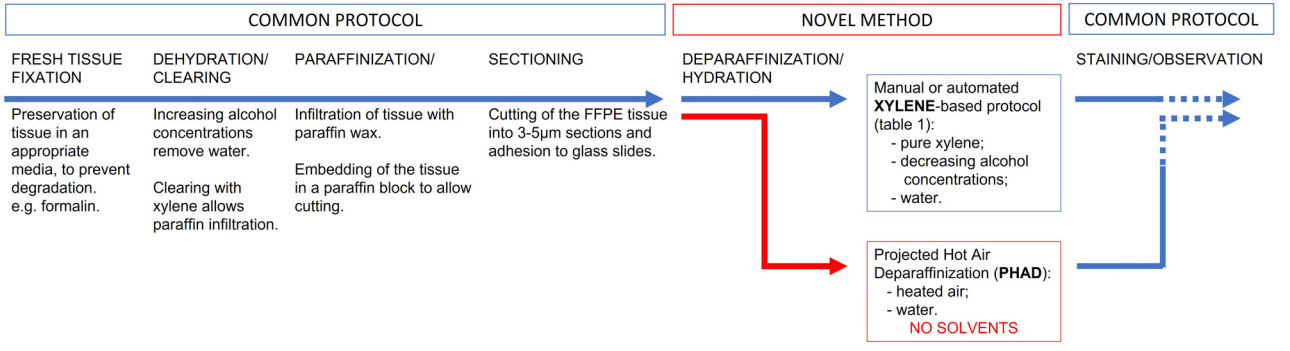


Fig. 1. Standard tissue processing steps, A tissue/organ sample fixed in formalin is prepared and placed in a cassette (1–2); the cassette is fitted in an automated paraffinization machine for dehydration, clearing and inclusion in paraffin into the organ (3); the cassette is dipped in paraffin to generate a large block surrounding the tissue/organ sample, to allow sectioning in the desired planes (4); the sample in a paraffin block is sectioned with a microtome, placed on glass slides and warmed in order to adhere (e.g. 10 min. at 70 ˚C or 60 min. at 37 ˚C) (5); sectioned tissue on glass slides is deparaffinized with xylene and decreasing ethanol concentrations manually (6) or in automated machines (Fig. 2).



STANDARD MANUAL DEPARAFFINIZATION	STANDARD AUTOMATED DEPARAFFINIZATION	PROJECTED HOT AIR DEPARAFFINIZATION (PHAD)
Xylene (100%): 10 minutes	Xylene (100%): 1-2 minutes	Hot air (72,5°C): 20 minutes
Xylene (100%): 10 minutes	Xylene (100%): 2-5 minutes	Water: 5 minutes
Ethanol (100%): 5 minutes	Ethanol (100%): 3 minutes	
Ethanol (95%): 5 minutes	Ethanol (96%): 2 minutes	
Ethanol (70%): 5 minutes	Water: 5 minutes	
Water: 5 minutes		

Fig. 2. Schematic representation of the tissue processing workflow, highlighting the difference of PHAD to traditional methods, Simplified step-by-step workflow illustrating the process from a fresh tissue sample until a final, stainable, FFPE tissue section on a glass slide. In blue: steps which are identical between the traditional method, using xylene deparaffinization; in red: steps which differ when using Projected Hot Air deparaffinization. Table highlighting a protocol and reagents for standard manual or automated xylene deparaffinization (blue) and for PHAD (red).

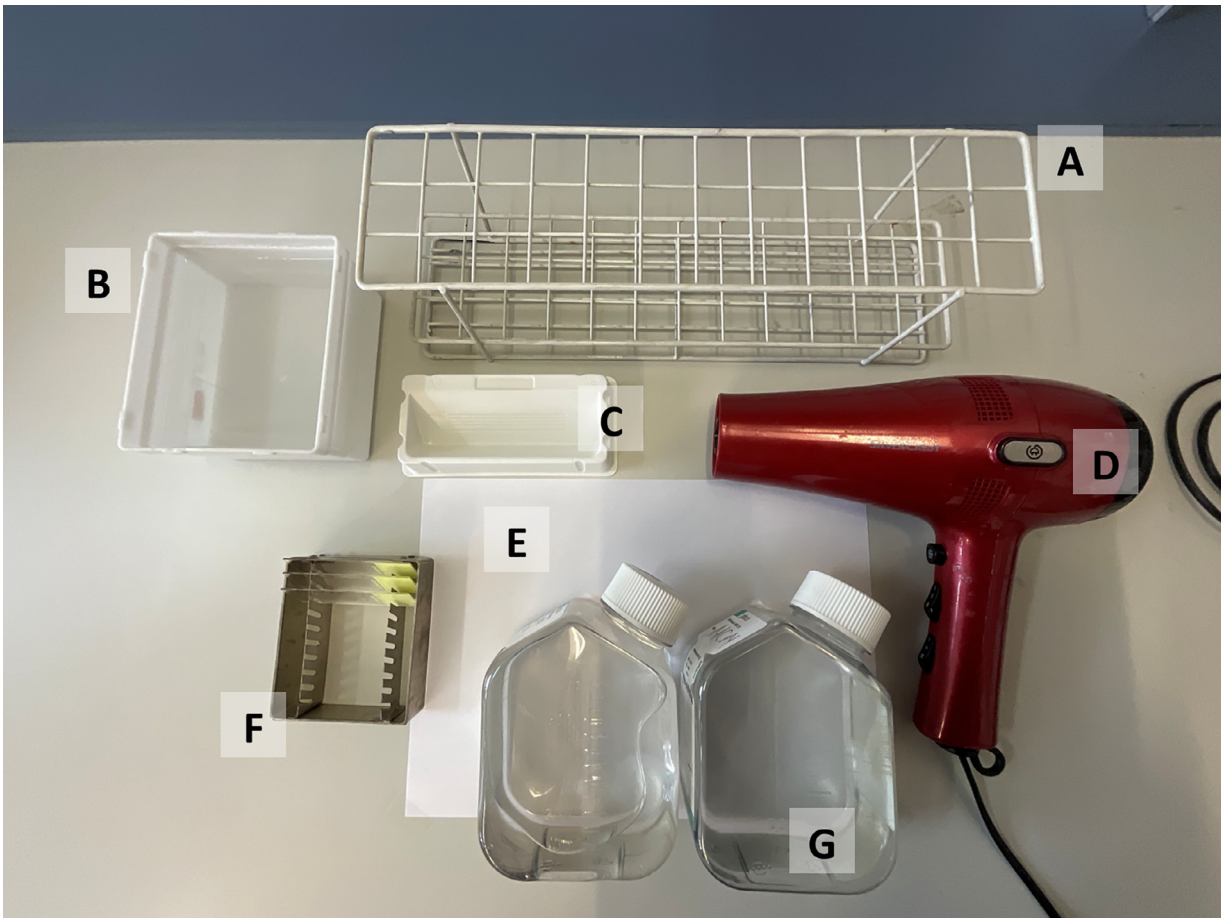


Fig. 3. Materials necessary to replicate the PHAD method set-up, (A) metal grid/tube rack to serve as support for the hairdryer; (B) reservoir for distilled water; (C) pipetting reagent reservoir to serve as the support for the glass slide; (D) hairdryer as source of the heated air; (E) blank sheet of paper to aid set-up; (F) samples of FFPE tissue is histology grade glass slides; (G) heavy weights to stabilize the apparatus A and D.

the superiority of this method for AFS by presenting that significantly more fluorescent bacilli are detected than compared to xylene deparaffinization.

Materials and set-up

The PHAD method relies on two aspects: first a source of heat high enough to melt the paraffin, and second, a directed air stream of sufficient force to remove the melted paraffin ("blowing it off the tissue sample"). Both aspects are found in a common domestic hairdryer, which can achieve the melting point of paraffin and remove it from the tissue sample. Here we describe the necessary materials and the specifications for temperature, air speed, apparatus set-up and duration which provided adequate deparaffinization of sections as shown by successful mycobacterial stainability.

Recreate the set-up for the PHAD method using the following materials (or similar) (Fig. 3):

- A common hairdryer (or any similar apparatus) capable of reaching the specifications for heat and blowing force as detailed bellow.
- A support for the hairdryer (or apparatus) to achieve the right distance from the tissue section at a right angle. We have used a standard metal culture tube rack, weighted down with water filled flasks.
- A support for the glass slide with the FFPE section at about a 90° angle and which catches the removed liquid paraffin. We used a simple pipetting reagent reservoir.
- A reservoir to hold distilled water at room temperature for the washing step.
- The FFPE tissue section to be deparaffinized.
- Any reagents for subsequent (acid-fast) staining.

A common household hair dryer (SilverCrest® SHTK 2000 W B1, Lidl, Neckarsulm, Germany) was fixed in a supporting grid approximately perpendicular to the glass slide (FFPE sample). The center point of the hairdryer nozzle sits at about 21 cm from the

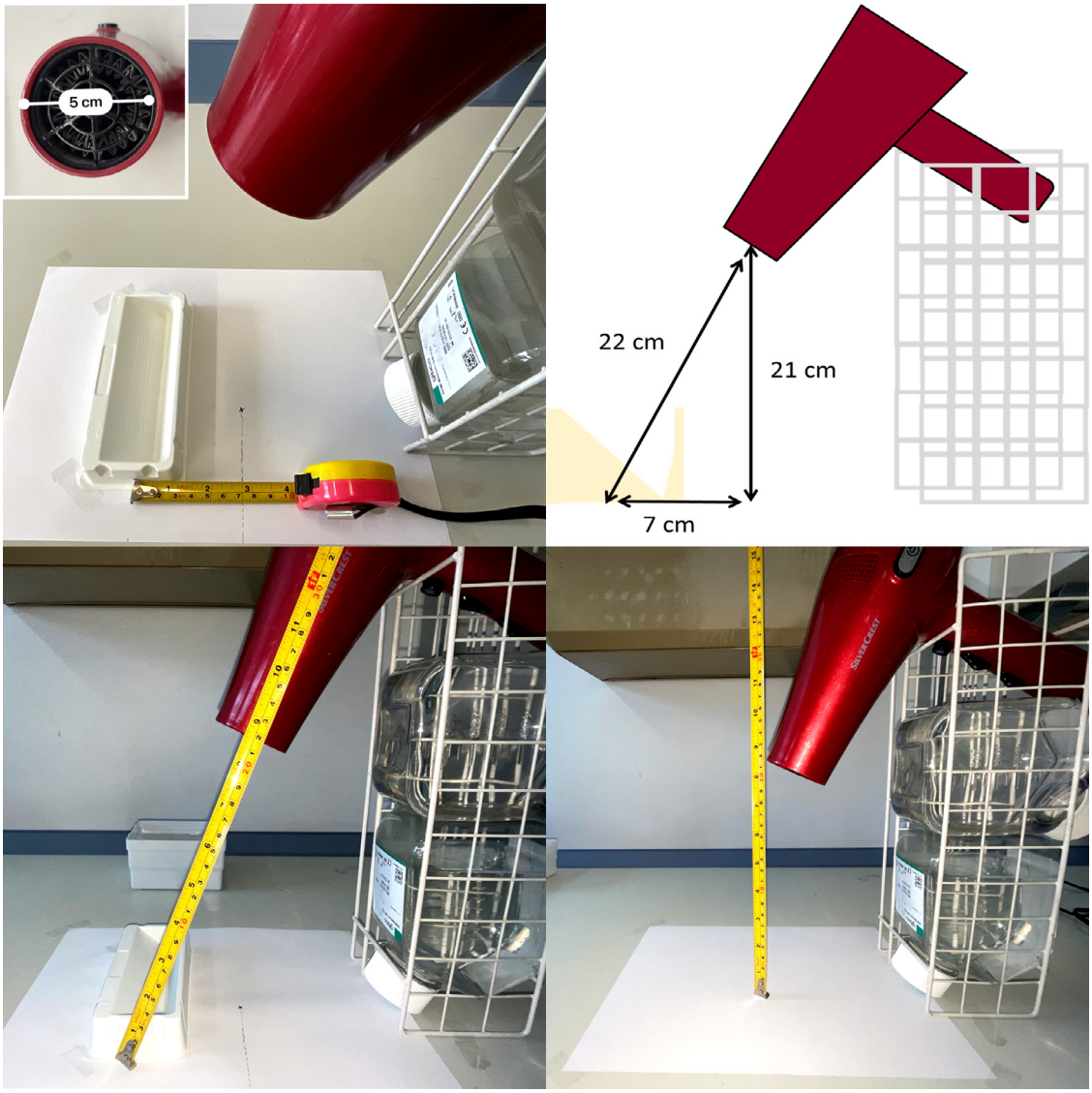


Fig. 4. Set-up of the deparaffinization apparatus for the PHAD method, with approximate measurements.

benchtop facing the slide holder (a pipetting reagent reservoir), centered at 7 cm from the base point, as illustrated in Fig. 4 and video 1. To facilitate the set-up, we recommend it to be prepared using a sheet of paper which easily allows the marking of measurements and assures that the set-up can be reliably reproduced.

The glass slide (with the FFPE tissue section) is placed in the reservoir, facing the nozzle. We recommend that it should stand in a tilted position (around 45°), to facilitate that the melted paraffin can easily run off (video 1). This particular hairdryer was then turned on at maximum speed and temperature settings.

Using an anemometer UT363BT (*UNI-T*, Dongguan, China), a low-cost instrument available to measure air speed, the measured air speed at the nozzle was 13 ± 0.5 m/s and at the surface level of the glass slide it was 7.5 ± 0.5 m/s. This is congruent with reported measurements from the manufacturer of the hairdryer, which gave 12.9 m/s. If you do not possess an anemometer, we provide a simple alternative to assess the blowing force, shown in a video (video 2) to illustrate what it would look like if projected air is directed onto a sheet of 80 g/m^2 printing paper cut in 1 cm wide strands, at a distance of 1 m. The temperature variation was measured for the duration of the procedure and remains at an average of around 72.5 °C, more than enough to melt histology grade paraffin (Fig. 5).

The protocol was tested with several durations, using *Mycobacteria* spp. infected FFPE sections, deparaffinized with hot air from under 10 min up to 40 min. Deparaffinized tissue was stained with a standard auramine O acid-fast stain[10] and scored by measuring the fluorescence intensity of the mycobacteria. It was noted that 2 or 5 min deparaffinization was not enough to produce stainable sections. Deparaffinization between 10 and 20 min produced best results, whereby the value of fluorescence was similar. However,

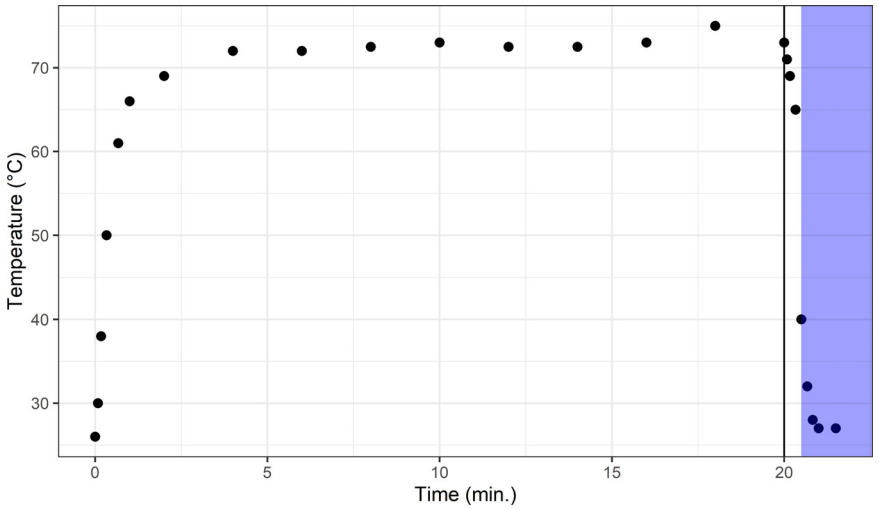


Fig. 5. Temperature at the level of the sample during the duration of PHAD treatment, Temperature profile observed at the level of the slide under the heat source of the PHAD method. The vertical black line represents the shut-off time of the hairdryer at 20 min and the blue rectangle represents the immersion of the sample in water. The temperature during deparaffinization was approximately 72.5 °C.

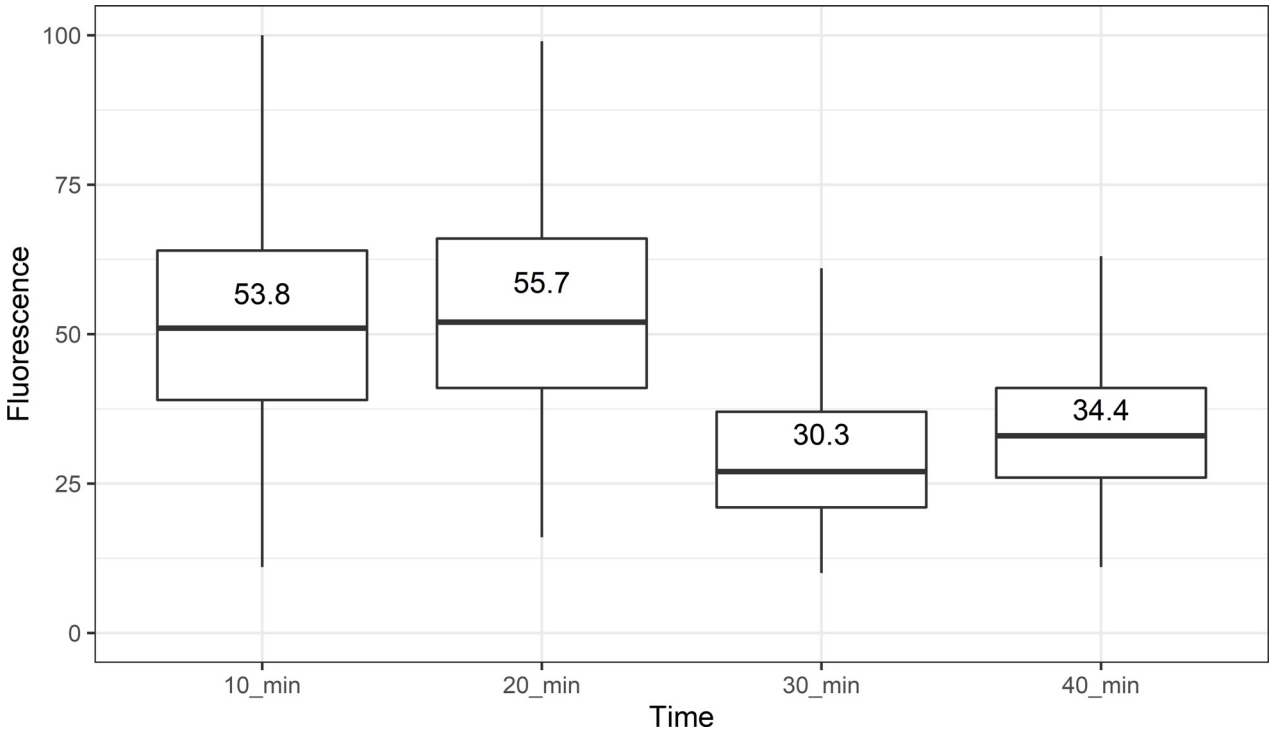


Fig. 6. Outcomes of different durations of deparaffinization with the PHAD method, Comparison of several durations of heat application in the PHAD method, as assessed by the fluorescence of acid-fast-stained mycobacteria in tissue sections. The box represents the values from the first to third quartile, with a horizontal line marking the second quartile/median with the corresponding value. The whiskers extend to the minimum and maximum values (number of observations: n10=616, n20=818, n30 =1011, n40=461). Y-axis shows fluorescence intensity units in a scale of 0–240 of brightness, as determined by the "measure" tool of the imageJ software.

deparaffinization of 30 min or longer let to a reduction in fluorescence (Fig. 6). Based on these results we suggest using 20 min, however, it is possible that slightly shorter times may produce equally satisfactory results.

Deparaffinization and staining protocol

The step-by-step instructions of the PHAD method:

1. Center one or two glass slides with FFPE tissue sections opposite to the hairdryer. If more than two glass slides are used, they are no longer centered in the air flow and may not produce satisfactory results with the used equipment (and nozzle).
2. Turn on the hairdryer at the required settings and leave it running for no more than 20 min.
   Note: if the glass slides become unbalanced due to the force of the air, rest a small heavy object at the bottom of the reservoir against the glass, but not covering the tissue sample, such as a pencil eraser.
3. At the end of the 20 min immediately dip the slides into the reservoir of distilled water and leave for 5 min.
   Note: this step is important to rehydrate the tissue to allow later staining, but also to solidify any paraffin which may be present outside of the tissue. The glass slide will be hot at this step: handle with caution!
4. Proceed with the desired stain. For example: auramine O acid fast staining (see next step)
5. Auramine O acid-fast-stain:
   (A) move the slides from the water and immediately flood for 15 min with 3.3 mM auramine O (0.5 g auramine O, Merck, Darmstadt, Germany in 50 ml of 95% ethanol mixed with 420 ml of distilled water containing 15 g phenol, AppliChem, Darmstadt, Germany)
   (B) Rinse by gently applying tap water to the edge of the slide and letting it run over the sample;
   (C) Use 0.5% acid-alcohol solution to decolorize the sample. (0.5 ml of hydrochloric acid in 100 ml of 70% ethanol). Apply drop-by-drop until no more dye is expelled.
   (D) Rinse again as in step 5;
   (E) Flood with 1% methylene blue (Alfa Aesar, Karlsruhe, Germany) in distilled water for 2 min as a counterstain.
   (F) Rinse again as in step 5;
   (G) Air-dry and proceed with microscopic observation.
   Note: we did not use any coverslips/mounting media for observation, however they may be used.

For a demonstration of the procedure, you may watch video 1, where the run-through of the method is shown and the melting and removal of paraffin is clearly visible.

Method validation

Samples of animal tissue infected with *Mycobacteria* were deparaffinized with the PHAD method as described or with the standard manual xylene deparaffinization method and compared for their effect on bacilli fluorescence after auramine O staining. There is a significant increase in the fluorescence of bacilli when tissue is deparaffinized with PHAD (Fig. 7), having a large effect size compared

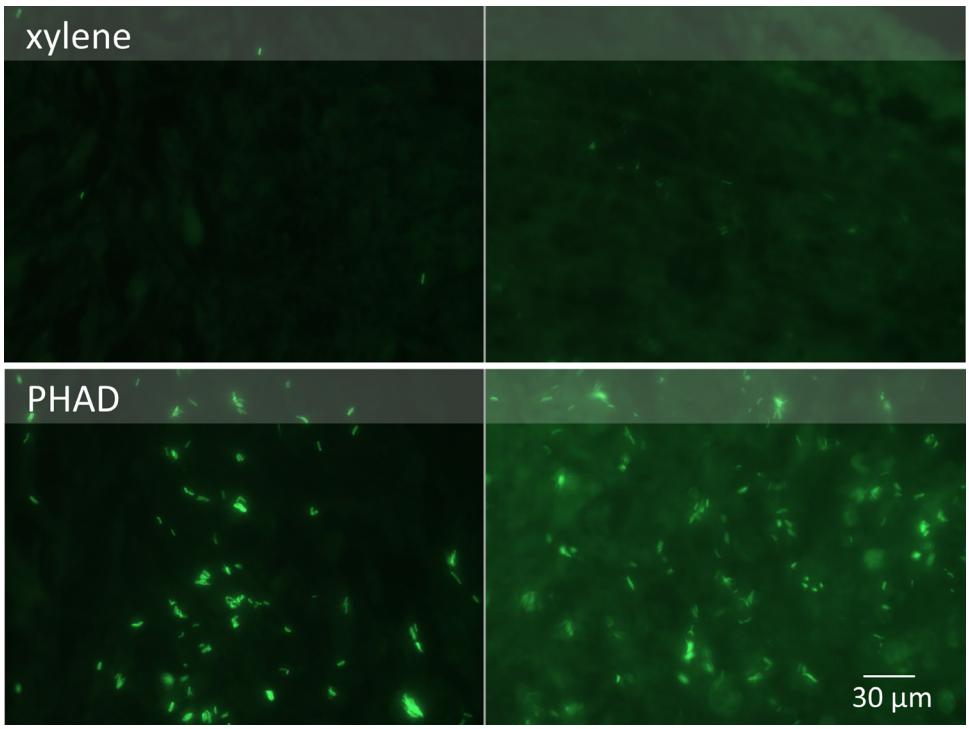


Fig. 7. Representative image of xylene or PHAD deparaffinized tissue, Muscle tissue infected with Mycobacteria spp. and stained with the auramine O method show a noticeable difference in fluorescence after standard xylene deparaffinization (top) or after Projected Hot Air Deparaffinization (PHAD) (bottom). (1000x).

to xylene in different tissues, as we have demonstrated elsewhere [3]. This difference can be larger than a twofold increase in median fluorescent value. It was also noted that bacilli become easier to find and appeared more numerous (Fig. 7). We have also shown that PHAD can produce superior staining with the Ziehl-Neelsen method, resulting in much more detectible *Mycobacteria* bacilli, as well as unaltered morphology and tissue integrity with the H&E stain [3].

Declaration of Competing Interest

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

Pedro F. Marinho: Conceptualization, Methodology, Software, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Thomas Hanscheid:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2023.102079.

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