Effects of Postnatal Hyperoxia Exposure on Cardiac Muscle Examined with 3D Serial Block Face SEM using Rapid Automated Sample Preparation

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Premature birth is associated with increased risk for cardiac dysfunction and failure throughout life. Postnatal hyperoxia exposure in rodents is a well-established model of chronic lung disease of prematurity which recapitulates the pulmonary vascular and cardiovascular phenotype of premature birth, including decreased skeletal muscle fatigability, mitochondrial oxidative capacity, and altered morphology [1]. In the present study, we sought to investigate the effect of postnatal hyperoxia on cardiac (right ventricle) muscle including mitochondrial morphology.

Serial block-face 3D electron microscopy (SBEM) creates new possibilities for life science discovery. While many repetitive aspects of SBEM imaging and analysis are now computer-controlled, sample preparation remains a hurdle. SBEM preparation uses chemical fixation, staining, and resin embedding based on methods developed for TEM, but with additional toxic and reactive steps. While TEM preparation typically requires 1-2 days, SBEM preparation requires nearly a week. Previously, the mPrep ASP-1000™ (ASP, Microscopy Innovations™) has demonstrated tissue preparation for TEM in 1-3 hours [3]. Herein we report the preparation of cardiac tissue for SBEM where all reagent exchanges were automatically performed using the mPrep ASP-1000™ in one working day.

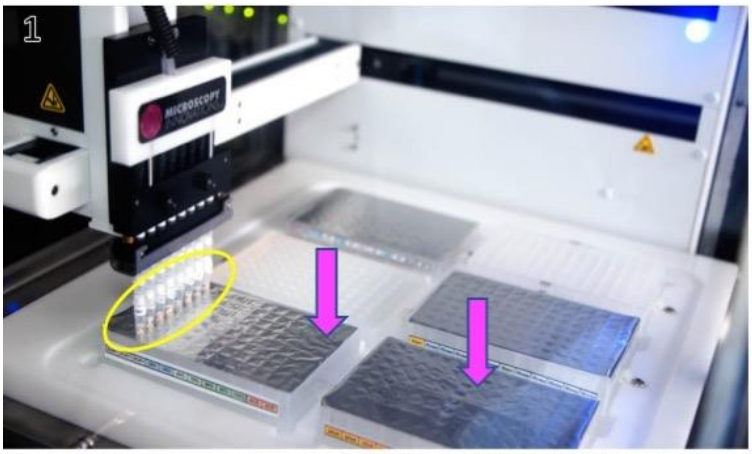
Pups from timed-pregnant Sprague Dawley rats were randomized to normoxia or hyperoxia (F_IO_2 0.85) for their first 21 days of life. Pups were then anesthetized and the heart was perfusion-fixed with buffered glutaraldehyde-paraformaldehyde. The right ventricle cardiac muscle was then excised and immersed in buffered fix and stored at 5C. Specimens (~0.5 x 2 mm) were then entrapped in mPrep/s capsules, mounted onto the ASP, and reagents dispensed into microwell trays and then covered (Fig. 1). The ASP aspirated successive reagents into each capsule for programmed times, including: Buffer to remove aldehyde fix, potassium ferrocyanide–OsO_4, thiocarbohydrazide, OsO_4, uranyl acetate, lead aspartate, graded ethanols, acetone, and epoxy. Epoxy-infiltrated specimens in capsules were removed from the ASP and cured at 60C. A Thermo Scientific™ VolumeScope™ SEM performed SBEM imaging at 2.5 kV, 0.1 nA under low vacuum using a lens-mounted backscattered detector. Over 1000 slices were acquired for each sample with 10 nm pixels and 50 nm slices. Image post-processing used Thermo Scientific™ Amira Software.

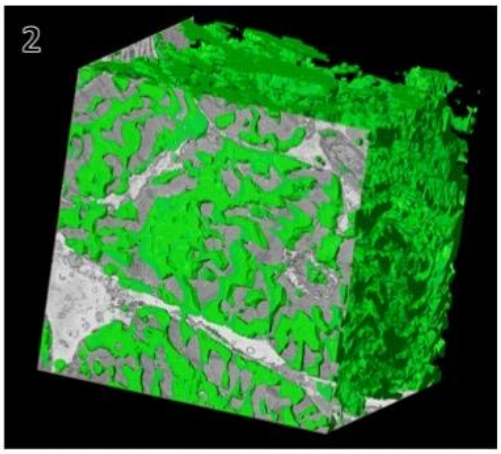
Figures 2-4 show that rapid sample preparation provided the required properties for SBEM imaging, including complete infiltration, good sectioning, conductivity and staining intensity sufficient for determining mitochondrial morphology and density using image analysis, as shown in a segmented image of a 25 um^3 region of a normoxic ventricle (Fig. 2). Comparison of the normoxic and hyperoxic tissues shows a marked difference in this preliminary study. Normoxic mitochondria (Fig. 3) appear

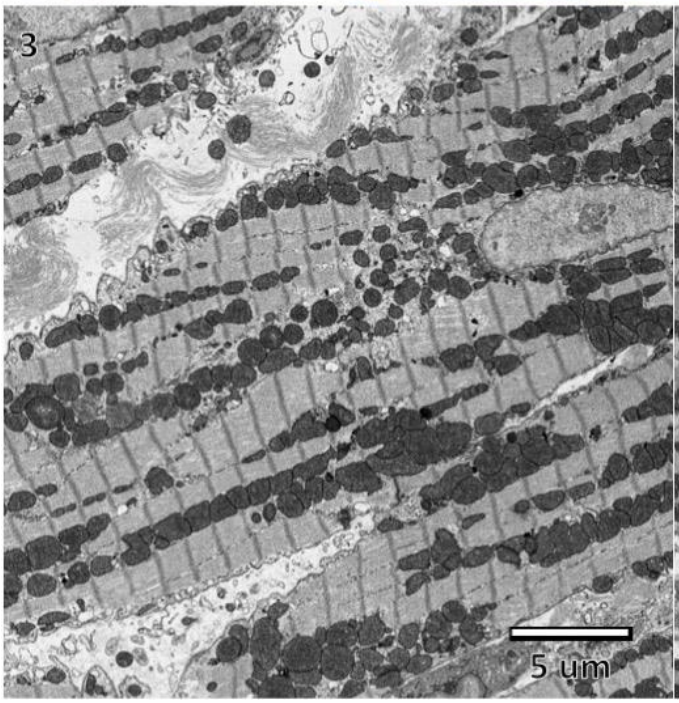
larger and more rounded than hyperoxic mitochondria (Fig. 4). The volume fraction of mitochondria per tissue volume appears greater for the normoxic right ventricle compared to the hyperoxic (52.71% vs. 48.56%), although significance determination will require more thorough analysis. By using mPrep ASP SBEM preparation, the work-flow for such studies is eased and accelerated. Further, ASP protocols are readily modified to alter staining or change any other parameter. Also, because each mPrep/s capsule can be processed simultaneously with a different reagent sequence, one study can simultaneously use multiple protocols, for example, to develop methods, or prepare controls.

References

[1] LH Tetri, et al. Frontiers Physiology 9 (2018) p. 326.
[2] KN Goss, et al. Am J Physiol – Lung 308 (2015) p. L797.
[3] TE Strader, NR Stewart, *et al*, Microsc. Microanal 24 (2018) 1284-5.







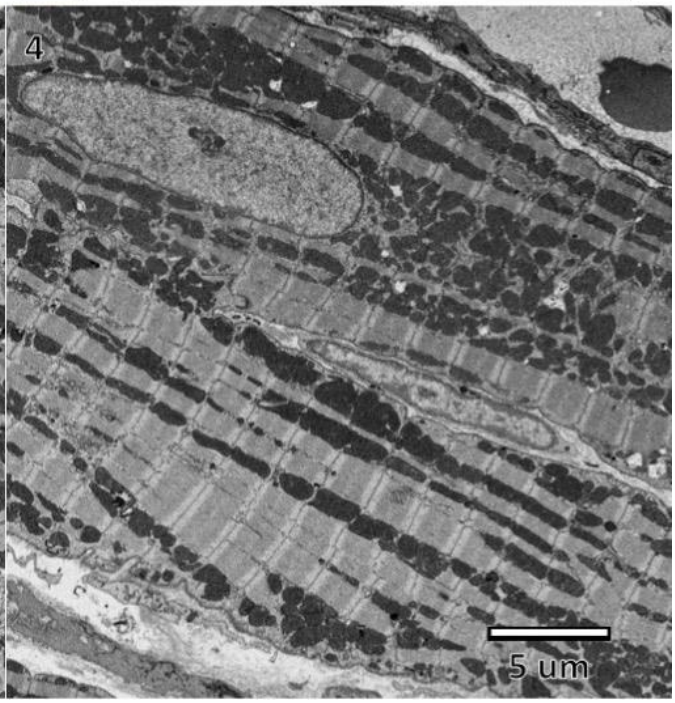


Figure 1. Specimens in mPrep/s capsules (circled) on ASP. Reagents in microwell plates (arrows).
Figure 2. Normoxic right ventricle. Segmented to show mitochondria. 25 um^3 cube.
Figure 3. Normoxic right ventricle representative SEBEM section. **Figure 4:** Hyperoxic right ventricle.

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Introduction

Premature birth is associated with increased risk for cardiac dysfunction and failure throughout life. Postnatal hyperoxia exposure in rodents is a well-established model for chronic lung disease of prematurity that recapitulates the pulmonary vascular and cardiovascular phenotype of premature birth. Disease symptoms include skeletal muscle fatigue, decreased mitochondrial oxidative capacity, and altered skeletal muscle mitochondrial morphology [1]. We have investigated the effect of postnatal hyperoxia on right ventricle cardiac muscle including mitochondrial morphology [2]. Herein we use 3D electron microscopy to examine right ventricle muscle morphology and mitochondria in hyperoxic and control neonate rats.

Serial block-face 3D electron microscopy (SBEM) creates powerful possibilities for life science discovery. SBEM imaging, sectioning and analysis workflows are largely automated. However, SBEM sample preparation requires nearly a week of manual reagent changes with noxious chemicals. The mPrep ASP-1000™ (ASP, Microscopy Innovations™) has demonstrated the acceleration of tissue preparation for TEM from 1-2 days to a few hours [3]. Herein we report automated preparation of for SBEM in one working day.

Experimental

Newborn Sprague Dawley rat pups were randomized to hyperoxia (F_IO_2 0.85) or normoxia (F_IO_2 0.21) for for their first 14 days (Fig. 1). Pups were euthanized at 21 days, and hearts perfusion-fixed with cacodylate buffered glutaraldehyde-paraformaldehyde. Right ventricles were excised, immersed in fix and stored until processing. Tissue was oriented, entrapped in mPrep/s capsules, and mounted onto the ASP for processing. The ASP aspirated successive reagents into capsules from covered microwells following the protocol (Table below) using repeated reagent flow cycles to accelerate diffusion into specimens. Epoxy-infiltrated specimens in capsules were removed from the ASP and cured at 60°C for 1 day.

Reagent	Reagent repeats	Mixing cycles	Time (min)
Karnovsky fix	Perfusion		Refrigerated
Buffer	1	50	5
OsO4 - KFeCN	1	610	61
H2O	3	10/50/100	16
1% Thiocarbohydrazide	1	600	60
H2O	3	10/50/100	16
OsO4	1	310	31
H2O	3	10/50/100	16
2% Uranyl Acetate	1	600	60
H2O	3	10/50/100	16
Lead Aspartate	1	300	30
H2O	3	10/50/100	16
50% EtOH	1	100	10
70% EtOH	1	100	10
80% EtOH	1	10	1
90% EtOH	1	25	2.5
95% EtOH	1	25	2.5
100% EtOH	2	30/30	6
Acetone	2	30/30	6
25% epoxy	1	50	5
50% epoxy	1	50	5
75% epoxy	1	50	5
100% epoxy	3	50/100/100	25
	Total time (includes ASP motion & operations)		~7.5 Hours

Specimen blocks were trimmed, then mounted for SEBM in a Thermo Scientific™ VolumeScope™ SEM. Imaging at 2.5 kV, 0.1 nA under low vacuum using lens-mounted backscattered detector. Over 1000 slices were acquired for each sample with 10 nm pixels and 50 nm slices. Images were post-processed with Thermo Scientific™ Amira software.

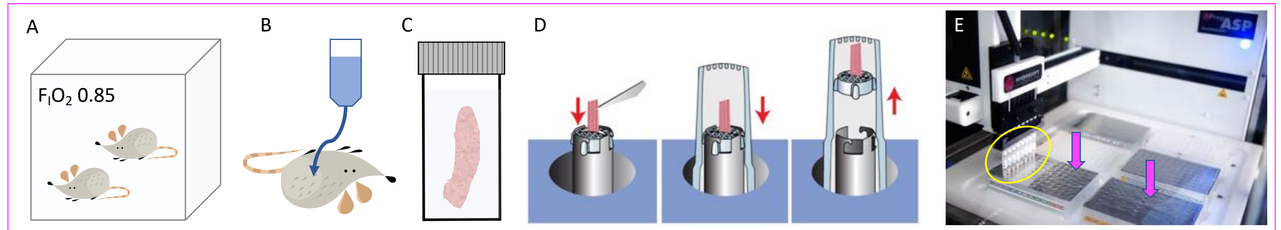

Figure 1: A) Neonate rat pups in hyperoxic "ICU incubators" 14 days, & controls. B) Perfusion fix heart (21 days old). C) Right ventricle excised & immersed in fix at 5°C. D) Right ventricle cut to ~2 x 0.5 mm, oriented & entrapped in mPrep/s capsules using mPrep Workstation. E) Eight specimens in mPrep/s capsules (circled) on ASP-1000 robotic arm with reagents dispensed into microwell plates and sealed with pierceable covers (arrows).

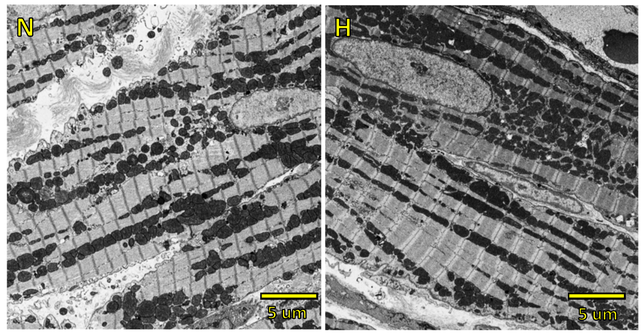

Figure 2: Normoxic (N) and Hyperoxic (H) right ventricle – SEM plane view.

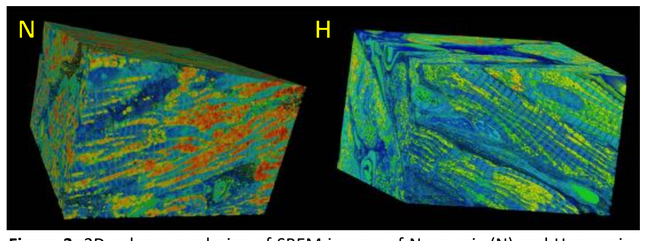

Figure 3: 3D volume rendering of SBEM images of Normoxic (N) and Hyperoxic (H) ventricles. Normoxic = 55 x 36 x 60 µm (1205 slices). 43 hr image acquisition. Hyperoxic = 59 x 38 x 50 µm (1009 slices). ~36 hr image acquisition.

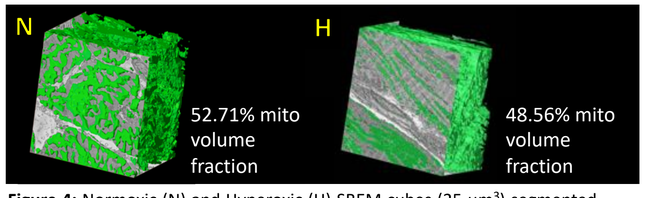

Figure 4: Normoxic (N) and Hyperoxic (H) SBEM cubes (25 μm^3) segmented to determine the volume of mitochondria (green) per total muscle volume.

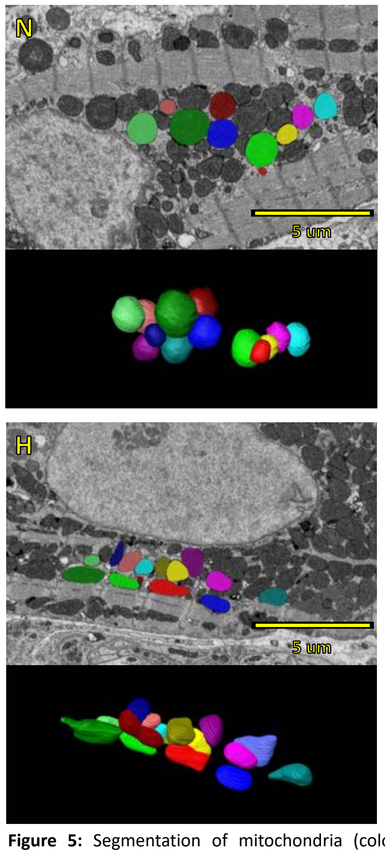

Figure 5: Segmentation of mitochondria (colors) from Normoxic (N) and Hyperoxic (H) tissue. Lower images show 3D rendering of segmented mitochondria.

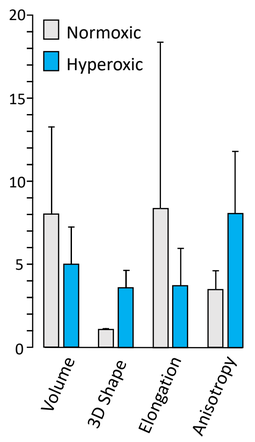

Figure 6: Shape analysis from 15 SBEM segmented mitochondria per condition: mean ± sd:

- Volume: μm^3
- 3D Shape: 1 = sphere
- Elongation: Low = long & skinny
- Anisotropy: Deviation from sphere.

Results and Discussion

SBEM provides informative two-dimensional (Fig. 2) and comprehensive 3D datacube images of right ventricle cardiac muscle (Fig. 3). Segmental analysis measurement of the mitochondria volume fraction per non-interstitial tissue volume shows that the mitochondrial volume fraction for the normoxic ventricle (52.71%) is greater than the hyperoxic ventricle (48.56%) (Fig. 4). Segmental analyses of 15 individual mitochondria per condition (Fig. 5) shows normoxic mitochondria are larger than hyperoxic mitochondria (0.8 ± 0.53 um^3 vs. 0.5 ± 0.22, respectively), and that their 3D morphologies are substantially different (Figs. 5-6).

The smaller mitochondrial tissue volume fraction, the lower volume of individual mitochondria, and their altered morphology may provide insight into the reduced aerobic capacity and pulmonary disease of hyperoxic neonates. These morphological difference are more clearly represented and quantified with SBEM, compared to single section views (Fig. 2) that do not provide volume information.

Workflow

The ASP-1000 Automated Specimen Processor improves the 3D SBEM workflow in comparison to manual preparation methods:

- Specimens processed automatically in 1 day, instead of a week
- Operator time reduced to ~1 hour
- Reduces handling and exposure to toxic reagents
- Eliminates specimen handling including messy transfers in resin
- Provides automated consistency and reproducibility
- ASP-1000 prepared SBEM specimens have staining, conductivity, and good sectioning for 3D imaging & segmentation analysis
- Protocols are readily modified to perform immuno-gold labeling, change stains, or alter other parameters.

With the ASP-1000, SBEM workflows are automated throughout:

Specimen preparation → SBEM imaging → Analysis

How is the ASP-1000 process speed so fast?

Chemical fixation, staining, and embedding are largely diffusion limited reactions. The ASP-1000 and mPrep/s capsules accelerate diffusion into specimens using gentle alternating fluid flow, with mixing cycles that can be repeated as rapidly as every second.

References

[1] LH Tetri, et al. Sex-specific skeletal muscle fatigability and decreased mitochondrial oxidative capacity in adult rats exposed to postnatal hyperoxia. Frontiers Physiology 9 (2018) p. 326.

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