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1   **TITLE**
2   Measurement of Protein Import Capacity of Skeletal Muscle Mitochondria
3
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22   **SUMMARY:**
23   Mitochondria are key metabolic organelles that exhibit a high level of phenotypic plasticity in
24   skeletal muscle. The import of proteins from the cytosol is a critical pathway for organelle
25   biogenesis, essential for the expansion of the reticulum and the maintenance of mitochondrial
26   function. Therefore, protein import serves as a barometer of cellular health.
27

28   **ABSTRACT:**
29   Mitochondria are key metabolic and regulatory organelles that determine the energy supply as
30   well as the overall health of the cell. In skeletal muscle, mitochondria exist in a series of complex
31   morphologies, ranging from small oval organelles to a broad, reticulum-like network.
32   Understanding how the mitochondrial reticulum expands and develops in response to diverse
33   stimuli such as alterations in energy demand has long been a topic of research. A key aspect of
34   this growth, or biogenesis, is the import of precursor proteins, originally encoded by the nuclear
35   genome, synthesized in the cytosol, and translocated into various mitochondrial sub-
36   compartments. Mitochondria have developed a sophisticated mechanism for this import process,
37   involving many selective inner and outer membrane channels, known as the protein import
38   machinery (PIM). Import into the mitochondrion is dependent on viable membrane potential and
39   the availability of organelle-derived ATP through oxidative phosphorylation. Therefore its
40   measurement can serve as a measure of organelle health. The PIM also exhibits a high level of
41   adaptive plasticity in skeletal muscle that is tightly coupled to the energy status of the cell. For
42   example, exercise training has been shown to increase import capacity, while muscle disuse
43   reduces it, coincident with changes in markers of mitochondrial content. Although protein import
44   is a critical step in the biogenesis and expansion of mitochondria, the process is not widely

45 studied in skeletal muscle. Thus, this paper outlines how to use isolated and fully functional
46 mitochondria from skeletal muscle to measure protein import capacity in order to promote a
47 greater understanding of the methods involved and an appreciation of the importance of the
48 pathway for organelle turnover in exercise, health, and disease.
49
50 **INTRODUCTION:**
51 Mitochondria are organelles that exist in complex morphologies in different cell types and are
52 recognized to possess an increasing array of functions that are critical for cellular health. As such,
53 they can no longer be whittled down merely to energy-producing organelles. Mitochondria are
54 key metabolic regulators, determinants of cell fate, and signaling hubs, the functions of which
55 can serve as useful indicators of overall cellular health. In skeletal muscle cells, electron
56 microscopy studies reveal the presence of geographically distinct subsarcolemmal (SS) and
57 intermyofibrillar (IMF) mitochondria, which exhibit a degree of connectivity[1–4] that is now
58 recognized to be highly dynamic and adaptable to changes in skeletal muscle activity levels, as
59 well as with age and disease. Mitochondrial content and function in muscle can be assessed in
60 numerous ways[5,6], and traditional methods of organelle isolation have been applied to better
61 understand the respiratory and enzymatic capacities (Vmax) of mitochondria distinct from the
62 influence of the cellular milieu[7,8]. In particular, these traditional methods have revealed subtle
63 biochemical distinctions between mitochondria isolated from subsarcolemmal and
64 intermyofibrillar regions, belying possible functional implications for metabolism in these
65 subcellular regions[8–11].
66
67 The biogenesis of mitochondria is unique in requiring the contribution of gene products from
68 both nuclear and mitochondrial DNA. However, the vast majority of these are derived from the
69 nucleus since mtDNA transcription only leads to the synthesis of 13 proteins. Since mitochondria
70 normally comprise >1000 proteins involved in diverse metabolic pathways, biogenesis of the
71 organelle requires a tightly regulated means of import and assembly of precursor proteins from
72 the cytosol into the various mitochondrial sub-compartments to maintain proper stoichiometry
73 and function[12,13]. Nuclear-encoded proteins destined for mitochondria normally carry a
74 mitochondrial targeting sequence (MTS) that targets them to the organelle and facilitates their
75 sub-compartmental localization. Most matrix-bound proteins contain a cleavable N-terminal
76 MTS, while those destined for the outer or inner mitochondrial membrane usually have internal
77 targeting domains[14]. The import process is carried out by a set of diverse channels that provide
78 multiple avenues for entry into the organelle[13]. The translocase of the outer membrane (TOM)
79 complex shuttles precursors from the cytosol into the intermembrane space, where they are
80 recognized by the translocase of the inner membrane (TIM) complex. This complex is responsible
81 for importing them into the matrix, where proteases cleave the N-terminal targeting
82 presequence. Proteins destined for the outer membrane can be directly inserted into this
83 membrane through the TOM complex, while those destined for the inner membrane are inserted
84 by a TIM protein, specifically TIM22. Following their import, proteins are further processed by
85 resident proteases and chaperones and often combine to form larger complexes, such as those
86 found in the electron transport chain.
87

88 Mitochondrial protein import itself also serves as a measurement of mitochondrial health, as this
89 process relies on the presence of membrane potential and a source of energy in the form of ATP[15].
90 For example, when the membrane potential is dissipated, protein kinase PINK1 cannot be taken
91 up by the organelle, and this leads to phosphorylation signals that trigger the onset of the
92 degradation of the organelle through a pathway called mitophagy[16,17]. Under similar
93 circumstances, when the import is impeded, the protein ATF5 cannot enter the organelle, and it
94 subsequently translocates to the nucleus, where it serves as a transcription factor for the up-
95 regulation of UPR gene expression[18,19]. Thus, measuring protein import efficiency can provide
96 comprehensive insight into the health of the organelle, while the gene expression response can
97 be used to indicate the degree of retrograde signaling to the nucleus.
98
99 Despite its obvious importance for the biogenesis of mitochondria and for cellular health in
100 general, the import pathway in mammalian mitochondria is remarkably understudied. In this
101 report, we describe the specific steps involved in measuring the import of precursor proteins into
102 skeletal muscle mitochondria and provide data to illustrate the adaptive response of the import
103 system to changes in muscle and disuse, illustrating the contribution of the protein import to the
104 adaptive plasticity of skeletal muscle.
105
106 **PROTOCOL:**
107
108 All animals used in these experiments are maintained in the animal care facility at York University.
109 The experiments are conducted in accordance with the Canadian Council on Animal Care
110 guidelines with approval from the York University Animal Care Committee (Permit: 2017-08).
111
112 **1.   Functional isolation of subsarcolemmal and intermyofibrillar mitochondria from**
113 **skeletal muscle**
114
115 1.1.   Reagent preparation:
116
117 1.1.1. Prepare all the buffers and media as mentioned in **Table 1**.
118
119 1.1.2. Set the buffers to pH 7.4 and store at 4 °C (up to 2 weeks), except nagrase protease.
120
121 1.1.3. Prepare fresh nagarse protease (**Table 1**) each time.
122
123 1.2.   Tissue removal and mincing
124
125 NOTE: A flowchart of the steps below for the isolation of mitochondria is provided in **Figure 1.**
126
127 1.2.1. Fill a glass scintillation vial with ∼ 20 mL of Buffer 1 (**Table 1**) and place it on ice.
128
129 1.2.2. While the mouse is under anesthetic, harvest skeletal muscle (tibialis anterior, quadriceps,
130 gastrocnemius etc.), approximately 500–1000 mg, and place the muscle in the scintillation vial
131 containing chilled Buffer 1.

132

NOTE: Gaseous isoflurane is used as an anesthetic at 2.5% at a flow rate of 0.4 L of O_2/min. A pinch test is performed to ensure that the animal is non-responsive.

1.2.3.   Following tissue collection, euthanize the animal via cervical dislocation.

1.2.4.   Pre-chill a watch glass on ice. Remove any fat or connective tissue from the muscles and mince the tissue on the watch glass until it is a homogenous slurry.

1.2.5.   Place the minced tissue in a pre-chilled 50 mL plastic centrifugation tube (see **Table of Materials**) and record the exact weight.

1.2.6.   Dilute the minced tissue 10-fold with Buffer 1 + ATP (**Table 1**).

1.2.7.   Homogenize the muscle sample using an 8 mm twin-blade homogenizer (see **Table of Materials**) at a power output of 9.8 Hz for 10 s, ensuring no visible chunks of muscle remaining.

1.2.8.   Centrifuge the samples at 800 x g for 10 min using a high-speed centrifuge (see **Table of Materials**).

NOTE: The purpose of this step is to separate SS and IMF mitochondrial fractions. The supernate contains SS mitochondria, while the pellet contains IMF mitochondria.

1.3.   SS mitochondrial isolation

1.3.1.   Filter the supernate through a single layer of cheesecloth into another set of 50 mL plastic centrifugation tubes to remove any large debris or contaminants.

1.3.2.   Centrifuge the supernate at 9,000 x g for 10 min at 4 °C using a high-speed centrifuge.

1.3.3.   Discard the supernate and resuspend the pellet in 3.5 mL of Buffer 1 + ATP.

NOTE: Resuspend with a P1000 and do this carefully. Mitochondria are fragile organelles. Perform gently and avoid touching the pellet with the pipette tip.

1.3.4.   Centrifuge the sample at 9,000 x g for 10 min at 4 °C using a high-speed centrifuge.

1.3.5.   Discard the supernate unless further processing to isolate a cytosolic fraction devoid of nuclei, mitochondria, and other organelles is desired. Resuspend the pellet carefully in roughly 95 µL of the resuspension medium (**Table 1**) using a P200 pipette.

NOTE: Similar to step 1.3.3, avoid touching the pellet with the pipette tip. The volume of the resuspension medium can be altered depending on the size of the pellet. This is the SS mitochondrial fraction, which can be stored on ice until the IMF fraction is isolated. If desired,

176   the cytosolic fraction, devoid of organelles, can be isolated by centrifuging the supernate at
177   100,000 x g in an ultracentrifuge (see **Table of Materials**) and thereafter discarding the pellet.
178
179   1.4.   IMF mitochondrial isolation
180
181   1.4.1.   Dilute the pellet from step 1.2.8 by 10-fold in Buffer 1 + ATP. Resuspend using a Teflon
182   pestle by gently mixing the pellet and buffer together until the sample is consistent.
183
184   1.4.2.   Homogenize the sample using an 8 mm twin-blade homogenizer (see **Table of Materials**)
185   at a power output of 9.8 Hz for 10 s.
186
187   1.4.3.   Centrifuge the sample at 800 x g for 10 min at 4 °C using a high-speed centrifuge.
188
189   1.4.4.   Discard the supernate and dilute the IMF mitochondrial pellet 10-fold using Buffer 2 and
190   resuspend using the Teflon pestle by gently mixing until it is homogeneous.
191
192   1.4.5.   Add 25 µL/g of tissue to 10 mg/mL nagarse protease (**Table 1**) and place the centrifugation
193   tube on its side on ice, gently mixing every minute by rocking the tube side to side.
194
195   NOTE: The addition of nagarse helps liberate IMF mitochondria by performing limited digestion
196   of the myofibrils.
197
198   1.4.6.   After 5 min, add 20 mL of Buffer 2 (**Table 1**) to dilute the nagarse protease to the point of
199   inactivity and stop the digestion.
200
201   1.4.7.   Immediately centrifuge the sample at 5,000 x g for 5 min at 4 °C using a high-speed
202   centrifuge.
203
204   1.4.8.   Discard the supernate and dilute the pellet 10-fold using Buffer 2 and resuspend using a
205   Teflon pestle by gently mixing.
206
207   1.4.9.   Centrifuge the sample at 800 x g for 15 min at 4 °C to pellet the fibrous material.
208
209   1.4.10. Pour the supernate in a new set of 50 mL plastic centrifuge tubes, careful not to disrupt
210   the pellet, which can be discarded.
211
212   1.4.11. Centrifuge the sample at 9,000 x g for 10 min at 4 °C as in step 1.4.7.
213
214   1.4.12. Discard the supernate, add 3.5 mL of Buffer 2 and gently resuspend the pellet using a
215   P1000 pipette.
216
217   1.4.13. Centrifuge the sample at 9,000 x g for 10 min at 4 °C as in step 1.4.7.
218

219 1.4.14. <mark>Discard the supernate and resuspend the pellet gently with roughly 180 µL of</mark>
220 <mark>resuspension medium using a P200 pipette.</mark>

221

222 NOTE: The volume of the resuspension medium can be altered depending on the size of the pellet.
223 This is the IMF mitochondrial fraction, which can be stored on ice until it is to be used in the
224 import assay.

225

226 **2.   Mitochondrial protein import**

227

228 2.1.   *In vitro* transcription

229

230 NOTE: These subsequent steps outline how to prepare a radiolabelled protein for import. The
231 import pathway under investigation may dictate the choice of target protein, for example, to
232 evaluate import into the mitochondrial matrix, ornithine carbamyl transferase, OCT, and malate
233 dehydrogenase, MDH, are commonly used, while Tom40 is commonly used as an indication of
234 import into the outer mitochondrial membrane. For the following steps, plasmid DNA encoding
235 the protein of choice is required. The transcription of plasmid DNA can be done prior to the
236 mitochondrial isolation on a separate day, and the mRNA collected from this experiment can be
237 stored and used in future translation and import experiments.

238

239 2.1.1. <mark>Linearize the DNA: Combine 40 µL of plasmid DNA (5 µg/µL), 5 µL of 10x enzyme buffer</mark>
240 <mark>and 5 µL of restriction enzyme and incubate at 37 °C for 30 min.</mark>

241

242 NOTE: The restriction enzymes utilized may vary based on the plasmid, which may require a
243 different enzyme buffer, and experimental conditions DNA can be used in any amount/volume,
244 as the template can be scaled up or down.

245

246 2.1.2. Purify and precipitate the linearized DNA using phenol and ethanol. Carry out all the
247 subsequent steps (2.1.3–2.1.15) in sterile 1.5 mL tubes, and use a tabletop centrifuge (see **Table**
248 **of Materials**) for the centrifugation steps.

249

250 2.1.3. Add the appropriate volume of sterile H_2O so that the final volume equals 400 µL. Then,
251 add 400 µL of phenol.

252

253 2.1.4. Mix vigorously by inversion for ~10 s, and centrifuge at 17,000 x *g* for 1 min at 4 °C.
254 Withdraw and save the upper phase.

255

256 2.1.5. Add 400 µL of phenol:chloroform:isoamylalcohol (25:24:1, v:v:v).

257

258 2.1.6. Mix vigorously by inversion for ~10 s, and centrifuge at 17,000 g for 1 min at 4 °C.
259 Withdraw and save the upper phase.

260

261 2.1.7. Add 400 µL of chloroform:isoamylalcohol (24:1, v:v).

262

263 2.1.8. Mix vigorously by inversion for ~10 s, and centrifuge at 17,000 g for 1 min at 4 °C.
264 Withdraw and save the upper phase.

265

266 2.1.9. Add 40 μL of 3M sodium acetate (pH 7.0), and add 1 mL of 95% ethanol.

267

268 2.1.10. Place sterile 1.5 mL tubes in -80 °C freezer on their side for 15 min.

269

270 2.1.11. Centrifuge the samples at 17,000 x g for 10 min at 4 °C.

271

272 2.1.12. Discard the supernate and wash the pellet with 300 μL of 80% ethanol.

273

274 2.1.13. Centrifuge the samples at 17,000 x g for 2 min at 4 °C.

275

276 2.1.14. Discard the supernate and air-dry the pellet. Once the pellet is dry, it should be clear.

277

278 2.1.15. Resuspend the pellet in 20 μL of sterile H_2O

279

280 2.1.16. Measure the DNA concentration using a spectrophotometer (see **Table of Materials**) and
281 ensure that the $A_{260:280}$ ratio is above 1.8.

282

283 2.1.17. Dilute the DNA to a concentration of 0.8 μg/μL in sterile H_2O.

284

285 2.1.18. Transcribe the DNA: Combine plasmid (0.8 μg/μL, 60.8 μL), sterile H_2O (8.4 μL),   10 mM
286 NTP (5.2 μL), 10 mM ATP (10.0 μL), 1 mM M-7-G (11.6 μL), the mix as mentioned step 2.1.19
287 (15.6 μL), RNAsin (5.2 μL) and an appropriate RNA polymerase (4.8 μL) to form "one reaction mix"
288 (total volume = 121.6 μL) and incubate for 90 min at the optimum temperature for the
289 polymerase (37 °C for T7 polymerase; 40 °C for SP6 polymerase)

290

291 2.1.19. To prepare the mix add 200 μL of 1M HEPES (pH 7.9), 100 μL of 1 M $MgAc_2$, 500 μL of 4
292 M KAc, 100 μL of 20 mM spermidine, 100 μL of 0.5 M DTT, and 200 μL of sterile H_2O.

293

294 NOTE: The reaction can be scaled up or down in volume as long as the provided proportions of
295 reagents are maintained. The RNA polymerase used is dictated by the bacterial promoter
296 sequence within the plasmid being used.

297

298 2.1.20. Then, purify and precipitate the mRNA using phenol and ethanol as in steps 2.1.2–2.1.15.

299

300 2.1.21. Bring the volume up to 400 μL with sterile H_2O (add 280 μL) and proceed as described in
301 steps 2.1.2–2.1.15. Resuspend the final pellet in 20 μL of sterile H_2O.

302

303 2.1.22. Measure the concentration of mRNA using a spectrophotometer, ensure that the $A_{260:280}$
304 ratio is ~2.0.

305

306 2.1.23. Dilute the mRNA to 2.8 μg/μL in sterile H_2O, and store at -20 °C in 50 μL aliquots.

307

308   2.2.   *In vitro* translation

309

310 NOTE: The translation of the desired DNA must be carried out on the same day of the import
311 experiment. This experiment requires the use of radiolabeled amino acids, and therefore the user
312 must have a permit for the use of radioisotopes, and all handling and disposal must be in
313 accordance with the institution's policies/requirements. Steps involving radioisotope safety
314 measures have been omitted or described briefly, as these will likely differ based on the
315 institution.

316

317 2.2.1. Prepare enough translation reaction mix (**Table 2**) to supply ~20 µL per sample of
318 mitochondria to be used in import experiment and include the same volume for a translation
319 lane.

320

321 2.2.2. Incubate the reaction for 30 min at 30 °C (time may vary with mRNA, 25–60 min).

322

323 2.2.3. Record the ^{35}S-methionine use as per the institution's radioactive safety requirements
324 and dispose of anything that has come into contact with the radioisotope per the institution's
325 guidelines.

326

327   2.3.   Protein import

328

329 NOTE: Freshly isolated mitochondria are required for the next steps. Refer to the mitochondrial
330 isolation protocol. Other isolation methods can be used as long as the mitochondria are viable
331 and functional. For the subsequent steps, the mitochondrial samples utilized are resuspended in
332 a resuspension buffer as described above (**Table 1**). The protein concentration of the
333 mitochondrial sample must also be measured prior to starting the import assay.

334

335 2.3.1. Roughly 15 min following the start of the translation reaction, aliquot 90 µg of
336 mitochondria into sterile 1.5 mL tubes and pre-incubate at 30 °C for ~10 min.

337

338 NOTE: Aliquot more mitochondria than what will actually be used in the experiment; only 75 µg
339 will be actually used. However, this is not a requirement.

340

341 2.3.2. In a fresh set of sterile 1.5 mL tubes, combine 75 µg of mitochondria with 18 µL of the
342 translation reaction and incubate at 30 °C for desired time.

343

344 2.3.3. Keep the remaining volume of the translation reaction on ice; this will be utilized later.

345

346 NOTE: Import is a time-dependent process, so it may be prudent to start with a time-course
347 experiment ranging in incubation times from 1–60 min. A typical reaction time is 30 min.

348

349   2.3.4.   During this time, prepare the sucrose cushion by combining 1 g of sucrose, 200 µL of 2.5
350   M KCl, 10 µL 1 M MgCl$_2$, 100 µL of 1 M HEPES (pH 7.4), and bring the volume to 5 mL using sterile
351   H$_2$O.

353   2.3.5.   Prepare a new set of sterile 1.5 mL tubes corresponding to each tube in the import
354   reaction. Aliquot 600 µL of the sucrose cushion into each tube and keep on ice until the import
355   reaction is over.

357   2.3.6.   To terminate the import reaction after the appropriate incubation time, remove the tube
358   from 30 °C, place it on ice and then carefully transfer the import reaction on top of the tube with
359   the sucrose cushion.

361   NOTE: This step needs to be done very slowly to ensure that the mitochondria are not
362   damaged/ruptured. The sucrose cushion helps slow down the migration of the mitochondria and
363   "cushions" its descent to the bottom of the tube during the subsequent centrifugation step.

365   2.3.7.   Centrifuge the samples + sucrose cushion at 17,000 x g for 15 min at 4 °C.

367   2.3.8.   Prepare the breaking buffer by combining 25 mL of 2.4 M sorbitol, 2 mL of 1 M HEPES (pH
368   7.4), and 72 mL of double-distilled H$_2$O.

370   2.3.9.   Prepare lysis buffer for SDS-PAGE gel electrophoresis: Combine 50 mL of heated glycerol,
371   11.5 g of SDS, 31.25 mL of 1 M Tris (pH 6.8) and bring the volume to 500 mL with double-distilled
372   H$_2$O.

374   2.3.10. For sample preparation, mix 475 µL of lysis buffer with 25 µL of β-mercaptoethanol to be
375   used in a later step.

377   NOTE: Lysis buffer can be made in advance and stored at room temperature; however, the
378   addition of β-mercaptoethanol is to be done fresh.

380   2.3.11. Using a P1000 pipette, carefully remove the supernate and do not disturb the pellet.
381   Dispose of the supernate in an appropriate radioactive waste container.

383   2.3.12. For import into the outer membrane, perform this using Tom40, resuspend the pellet in
384   50 µL of freshly prepared 0.1 M sodium carbonate (pH 11.5) and incubate on ice for 30 min.

386   2.3.13. Following the incubation, centrifuge the sample 14,000 x g for 5 min at 4 °C. This step is
387   only done for outer membrane import reactions.

389   2.3.14. To prepare the samples for SDS-PAGE electrophoresis, add 20 µL of breaking buffer, 20
390   µL of lysis buffer, and β-mercaptoethanol and resuspend well. Add 5 µL of sample dye.
391

2.3.15. Prepare the control/translation lane by combining 3 μL of the remaining translation reaction from step 2.3.3 with 37 μL of lysis buffer and 5 μL of sample dye. Mix well.

2.3.16. Boil the samples for 5 min at 95 °C and spin down gently at a low speed to avoid pelleting mitochondria.

2.3.17. Apply the samples to an SDS polyacrylamide gel.

NOTE: The gel percentage and subsequent run time will depend on which protein is being imported. For example, OCT is 37 kDa, and its mature processed band is slightly smaller; therefore, a 12% gel allows good separation of these bands.

2.3.18. Once electrophoresis is complete, remove the gel and place it in double-distilled H_2O.

2.3.19. Boil the gel in 5% TCA in a fume hood for 5 min, continuously stirring/moving the gel to avoid cracking.

NOTE: TCA helps solidify the gel for visualization; this can be done in a metal tray over a Bunsen burner. Use enough TCA to generously cover the gel, ~½ full.

2.3.20. Remove the gel and place it back in double-distilled H_2O. Agitate it on a rotating plate for 1 min at ~50 rpm.

2.3.21. Wash the gel in 10 mM Tris on a rotating plate for 5 min at ~50 rpm, again using enough to generously cover the gel, ~½ of the container.

2.3.22. Precipitate the protein by washing the gel in 1 M Salicyclic acid on a rotating plate for 30 min, again using enough Salicyclic acid to generously cover the gel, ~½ of the container being used.

2.3.23. Dehydrate the gel as described in steps 2.3.24–2.3.31.

NOTE: This can be done in a number of ways. A gel dryer (see **Table of Materials**) provides a time-efficient option (described below); however other commercially available methods/kits can be used that may be more cost-effective but more time-consuming. Gel drying kits can be utilized as an alternative that does not require the application of heat or suction but instead allows the gel to air-dry between cellophane sheets to prevent distortion.

2.3.24. Place a large sheet of blotting paper down on the porous bed of the gel dryer. Place one sheet of paper towel in the area where the gel will be applied.

2.3.25. Cut a 15 cm x 20 cm piece of blotting paper and place it on the paper towel.

435 2.3.26. Scoop out the gel from the container using a second piece of 15 cm x 20 cm blotting paper
436 and lay it down flat on top of the first piece.

438 2.3.27. Cut a slightly larger piece of plastic wrap, ~20 cm x 25 cm, and place it down on gel,
439 ensuring no creases or bubbles under the wrap.

441 NOTE: This may take a few attempts, but it is imperative that there are no trapped air pockets.

443 2.3.28. Gently lay down the plastic cover of the gel dryer over top of the wrap, again ensuring
444 that there are no bubbles or creases.

446 2.3.29. Turn on the pump/vacuum and ensure that the plastic cover has formed a seal. Test the
447 seal by lifting a corner of the plastic cover and wait for it to re-seal.

449 2.3.30. Close the gel dryer and run for 90 min. Set the temperature to start at 30 °C, gradually
450 reach 80 °C, and then return to 30 °C at the end of the run.

452 2.3.31. Once dehydrated, the gel will solidify and feel paper-thin. Wrap the gel in the plastic wrap
453 used in the drying process.

455 2.3.32. Place the dehydrated gel in a film cassette with a phosphorus film (see **Table of Materials**),
456 with the white side facing the gel. Exposure times vary but can be 24–48 h for visualization of
457 bands.

459 2.3.33. Visualize using autoradiography using any suitable imager that is capable of phosphorus
460 imaging.

462 **REPRESENTATIVE RESULTS:**
463 We have extensively illustrated that this protocol is a valid assay for determining the rate of
464 import into functional and intact isolated skeletal muscle mitochondria. In comparison to
465 untreated conditions, the import of typical precursor proteins such as malate dehydrogenase
466 (MDH) into the matrix is sensitive to membrane potential because it can be inhibited by
467 valinomycin, a respiratory chain uncoupler **(Figure 2A)**. Import is also impeded when
468 mitochondrial inner and outer membranes are solubilized in the presence of the detergent Triton
469 X-100. The import process is sensitive to the presence of external ATP, which serves to unfold
470 precursor proteins for translocation across the membranes, and is tightly controlled by the rate
471 of respiration and ATP provision (data not shown[20]). Distinct differences in the import are also
472 observed between intermyofibrillar and subsarcolemmal mitochondrial fractions, in part due to
473 variations in protein import machinery expression, as well as the respiratory rate between these
474 mitochondria **(Figure 2B)**.

476 Mitochondria in skeletal muscle are highly dynamic organelles that respond readily to changes in
477 energy demand. Mitochondrial content in the muscle increases following periods of chronic
478 exercise or in response to electrical stimulation-induced contractile activity (see for review[21]).

479 For example, 7 days of chronic contractile activity of rat skeletal muscle enhances import into the
480 OM and matrix by 1.6-fold and 1.4-fold, respectively[22] **(Figure 3A, 3B)**. These changes in
481 mitochondrial content are brought about, in part, by alterations in the capacity of the
482 mitochondrial protein import system. Indeed, a close relationship between the import rate of
483 precursor proteins and a good estimate of mitochondrial content, as measured by the complex
484 IV marker cytochrome oxidase under control or denervated conditions, can be illustrated[23]
485 **(Figure 3C)**.

487 The adaptability of the import system in muscle to alterations in contractile activity suggests that
488 exercise could be used as a treatment to resolve defects in the import pathway if identified.
489 During the investigation of mitochondrially-mediated apoptosis using Bax-Bak double knockout
490 animals, we noticed that the reduced mitochondrial content in the muscles of these experimental
491 animals was accompanied by a decrease in the import of precursor proteins into the matrix. We
492 then investigated the possibility that exercise could restore this import capacity. Indeed,
493 following 6 weeks of voluntary wheel run training, protein import was restored to control levels
494 in the knockout animals[24], illustrating the adaptive plasticity of the import pathway to rescue
495 mitochondrial content and function **(Figure 4)**.

497 **FIGURE AND TABLE LEGENDS:**
498 **Figure 1: Schematic of the workflow for isolating SS and IMF mitochondria from skeletal muscle**
499 **based on the previous work**[8]. This protocol allows the isolation of functional mitochondria based
500 on their geographic location within skeletal muscle. SS mitochondria are more rapidly and easily
501 liberated, while the IMF mitochondria require a further digestion step with protease to untangle
502 them from the myofibrils. Note that the isolation of these subfractions can be done in tandem.
503 An updated, similar procedure has recently been published[25].

505 **Figure 2: Protein import into the mitochondrial matrix**. (**A**) Normal rates of import for MDH are
506 shown in SS and IMF mitochondria (lanes 4 and 7) and the translation product of precursor MDH
507 (lane 1). The lower band represents the imported mature MDH. Addition of valinomycin inhibits
508 MDH protein import into the matrix of SS and IMF mitochondria, as this uncoupler dissipates the
509 membrane potential (lanes 2 and 5). Triton-X is a detergent that solubilizes the inner membrane,
510 thereby inhibiting MDH import into these subfractions (lanes 3 and 6). Protein import was carried
511 out for increasing time durations, 4 min, 7 min, 10 min, 15 min, and 30 min. These data illustrate
512 that import is a time-dependent process and also that SS and IMF mitochondria have different
513 rates or capacities for import (**B**). TL, translation lane; VAL, valinomycin; TRI, Triton-X; CTL, control;
514 SS, subsarcolemmal; IMF, intermyofibrillar. This figure was modified from Takahashi M & Hood
515 DA[20].

517 **Figure 3: Protein import is adaptable and tightly linked to estimates of mitochondrial content.**
518 Sprague Dawley rats were subjected to electrical stimulation to induce contractile activity, a
519 model of exercise training. (**A**) Tom40 import into the OM was 1.6-fold higher in muscle from
520 chronically stimulated animals compared to controls at any given time point. (**B**) OCT import into
521 the mitochondrial matrix was increased at every time point of incubation, and overall, this
522 resulted in a 1.4-fold increase in mitochondria from chronically stimulated muscle. (**C**) Import is

523   positively correlated with an index of mitochondrial content, as assessed by COX activity, $r = 0.69$.
524   These measurements were taken from animals that were subjected to denervation, which has
525   been shown to decrease mitochondrial content and the rate of import. Con, control; Den,
526   denervated; Stim, stimulated; TL, translation lane; * $p < 0.05$. Figures 3A and 3B were adapted
527   from Joseph A-M & Hood DA[22] and 3C from Singh B & Hood DA[23].
528
529   **Figure 4: Training rescues import defects *in vivo***. Bax/Bak double knockout animals exhibit
530   reduced protein import into the mitochondrial matrix by 37%. Six weeks of voluntary wheel
531   running rescued the import defect to control levels. WT, wildtype; DKO, double knockout; TL,
532   translation lane; * $p < 0.05$ main effect of genotype; ¶ $p < 0.05$ main effect of training. This figure
533   was adapted from Zhang Y et al.[24].
534
535   **Table 1: Buffers and resuspension media.**
536
537   **Table 2: Translation reaction mix**
538
539   **DISCUSSION:**
540   Mitochondria are uniquely dependent on the expression and coordination of both the nuclear
541   and mitochondrial genomes for their synthesis and expansion within cells. However, the nuclear
542   genome encodes the vast majority (99%) of the mitochondrial proteome, and this underscores
543   the importance of the protein import machinery in supporting mitochondrial biogenesis. Import
544   also serves as an important signaling event, as failure to import can promote the initiation of the
545   unfolded protein response and/or mitophagy[15,16,26]. Since import relies on the expression of
546   multiple functional channels and chaperones, an intact membrane potential, as well as the
547   availability of ATP, the evaluation of mitochondrial protein import can provide valuable insight
548   into the health and energy status of the organelle.
549
550   The evaluation of protein import capacity into mitochondria requires the isolation of the
551   organelle from the surrounding tissue using standard differential centrifugation techniques.
552   Import is evaluated in the same manner as one would evaluate the V_{max} of mitochondrial enzyme
553   activities, in a highly controlled manner dependent on time and substrate concentration and
554   independent of cellular cytosolic influences. The isolation method presented here, or similar
555   renditions of it, have been used for many years to evaluate mitochondrial respiration and enzyme
556   activities[8–11]. This technique is not without its limitations, as it disrupts the normal morphology
557   of mitochondria as they exist *in situ*[27], which in skeletal muscle is highly complex, ranging from
558   small spheroid structures, to a highly branched reticular network[3], depending on their
559   subsarcolemmal or intermyofibrillar location within the muscle cell. In addition, the use of the
560   protease nagarse has come under scrutiny[3,28]. An update to this method, using trypsin instead
561   of nagarse, has recently been published[25], and trypsin has been used by others to isolate muscle
562   mitochondria[29]. Indeed, any alternative isolation method that yields functionally intact
563   mitochondria can be used, including techniques that do not employ proteases[30] or those
564   designed for small biopsy-sized samples from human muscle[31]. The method presented here has
565   the advantage of isolating SS mitochondria quickly and easily, but a greater yield and purity of
566   mitochondria can be obtained through the isolation of the IMF subfraction. If done carefully, this

567   isolation protocol can result in functionally intact organelles with a high respiratory control ratio,
568   indicative of appropriate rates of state 3 and 4 respiration[8].

569

570   It is also imperative for the measurement of mitochondrial protein import that these organelles
571   maintain their membrane potential, as import across the inner membrane is dependent on this
572   electrophoretic proton motive force, which serves to attract positively charged precursor
573   sequences and help mediate the transport of the precursor into the negatively charged matrix
574   space.   Under such conditions, comparisons of mitochondrial function and import can be
575   evaluated between physiologically relevant experimental situations, such as exercise, aging, and
576   muscle disuse, for example. In this respect, previous work has shown that import is a highly
577   adaptable pathway that responds to altered states of muscle use and disuse and is sensitive to
578   inhibition via excessive ROS emission[10,22,23,32]. This plasticity is due, in part, to adaptive changes
579   in the expression of the protein import machinery components. Since this technique relies on
580   the isolation of mitochondria, any regulation on the import pathway that may stem from
581   cytosolic factors or inter-organelle crosstalk is removed from the interpretation of the
582   experiment. This is both a limitation and a strength of the technique, as conclusions can be made
583   of the capacity of the import pathway itself (similar to the V_{max} of enzyme activity), but transient
584   or external signals that may occur with the experimental model may be lost. To circumvent this,
585   it is possible to incubate the import reaction with a sample of the cytosolic fraction, isolated as
586   described above, in order to evaluate changes in the cytosolic environment that may influence
587   import rates, as done previously[22]. In addition, import machinery components are subject to
588   acute, post-translational modifications that can alter their functions. Recent work has shown
589   that phosphorylation of specific TOM import receptors can be linked to mitophagy[33]. Indeed, an
590   area of research that warrants more attention is the acute modulation of the import process via
591   intrinsic signaling pathways mediated by ROS or by covalent modification of import receptors
592   and chaperones, as documented in yeast and other lower organisms[34,35].

593

594   Protein import into mitochondria represents a gateway to adaptive organelle growth and is a
595   sensitive indicator of mitochondrial health. Understanding how this process is regulated can shed
596   light on the regulation of mitochondrial biogenesis, UPR signaling, and the initiation of mitophagy.
597   Mitochondrial protein import is not a widely studied process in mammalian experimental models,
598   and the development of the vast potential of research in this area could help us achieve a greater
599   understanding of diseases in which mitochondrial dysfunction is apparent or represent an
600   attractive therapeutic target to promote mitochondrial health.

601

608

609   **DISCLOSURES:**
610   No conflicts of interest, financial or otherwise, are declared by the authors.

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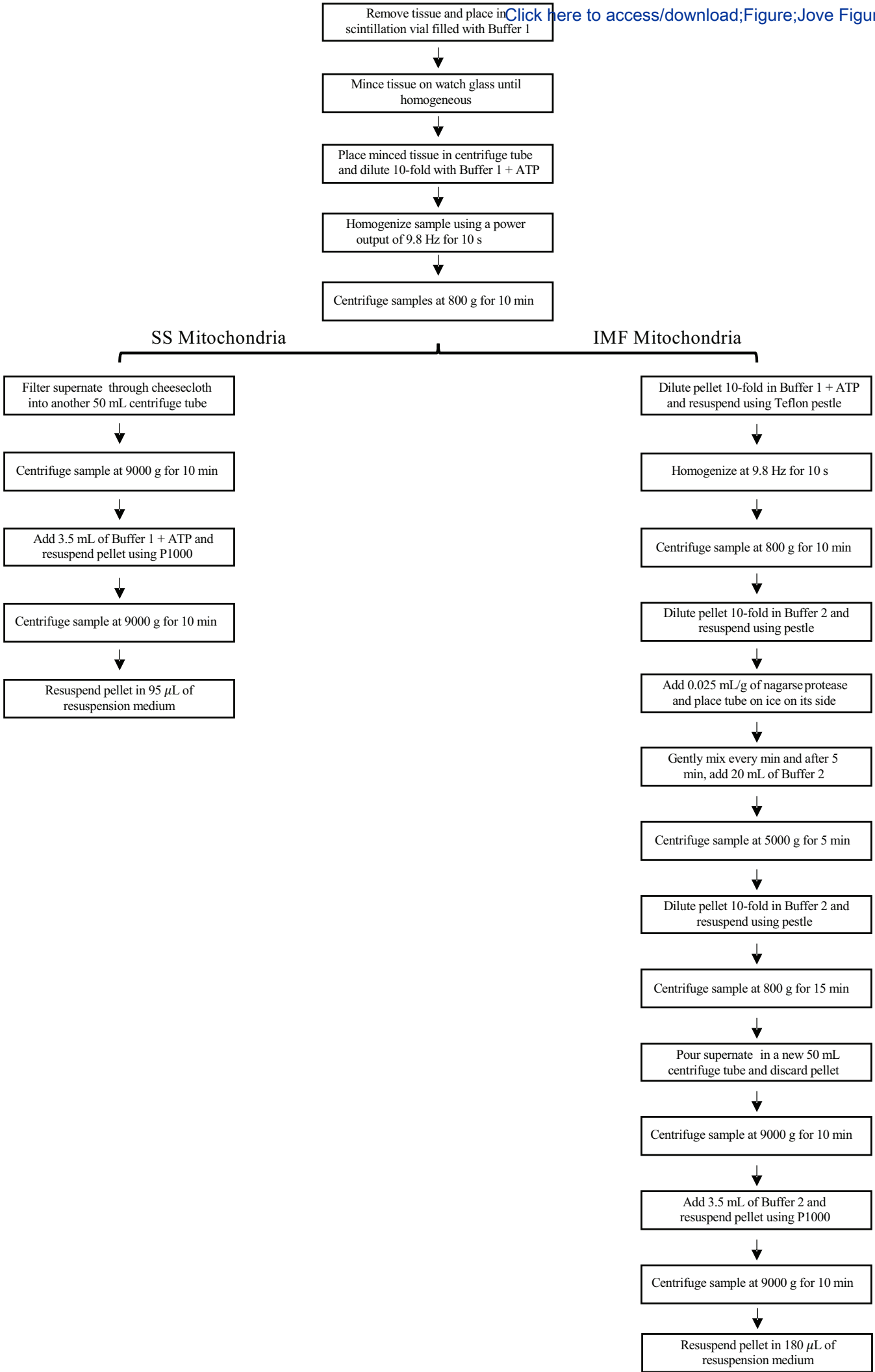686 107 (2016).
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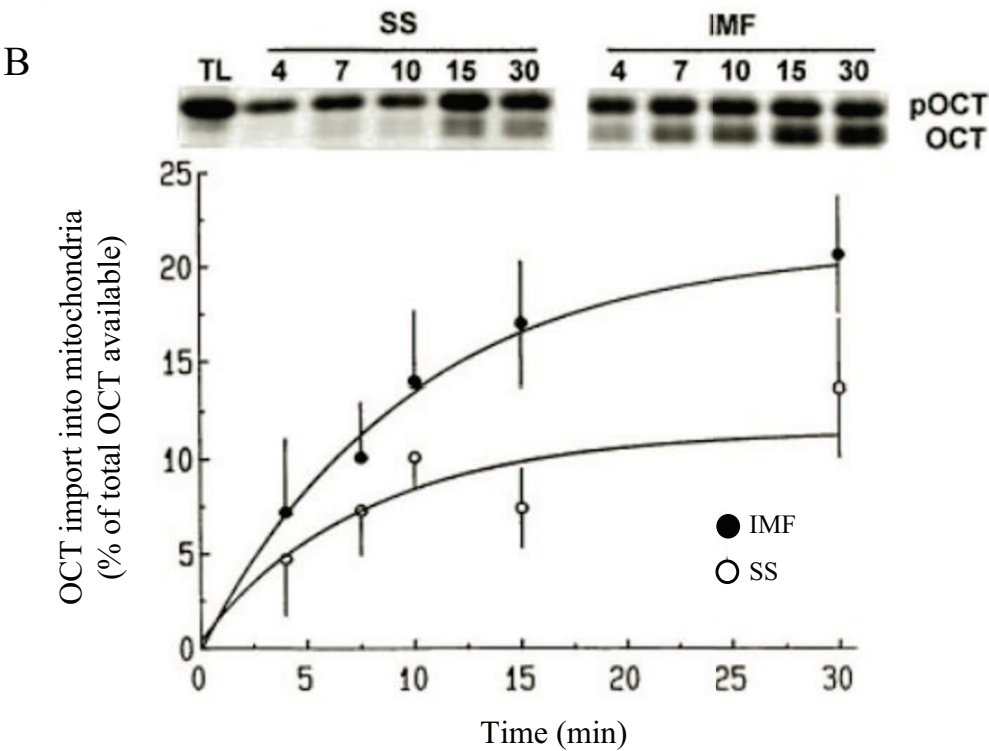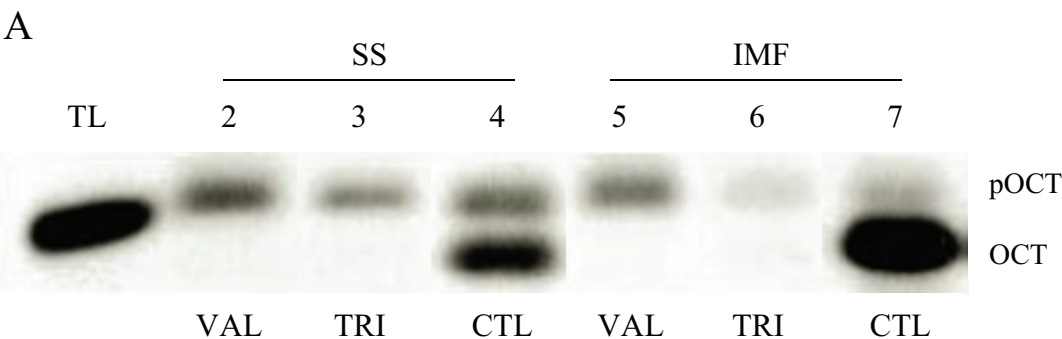689 Membrane Biology*. **253** (4), 309–318 (2020).
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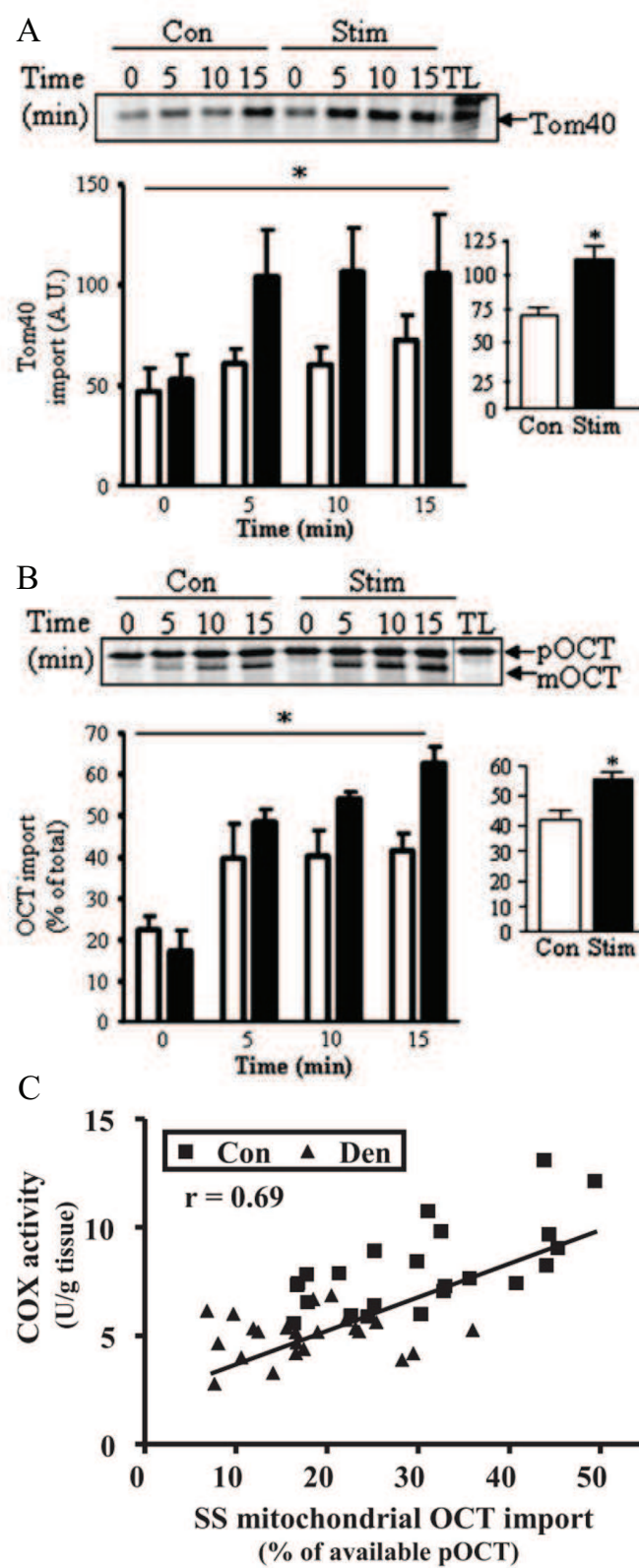692 (2020), 1–15 (2020).
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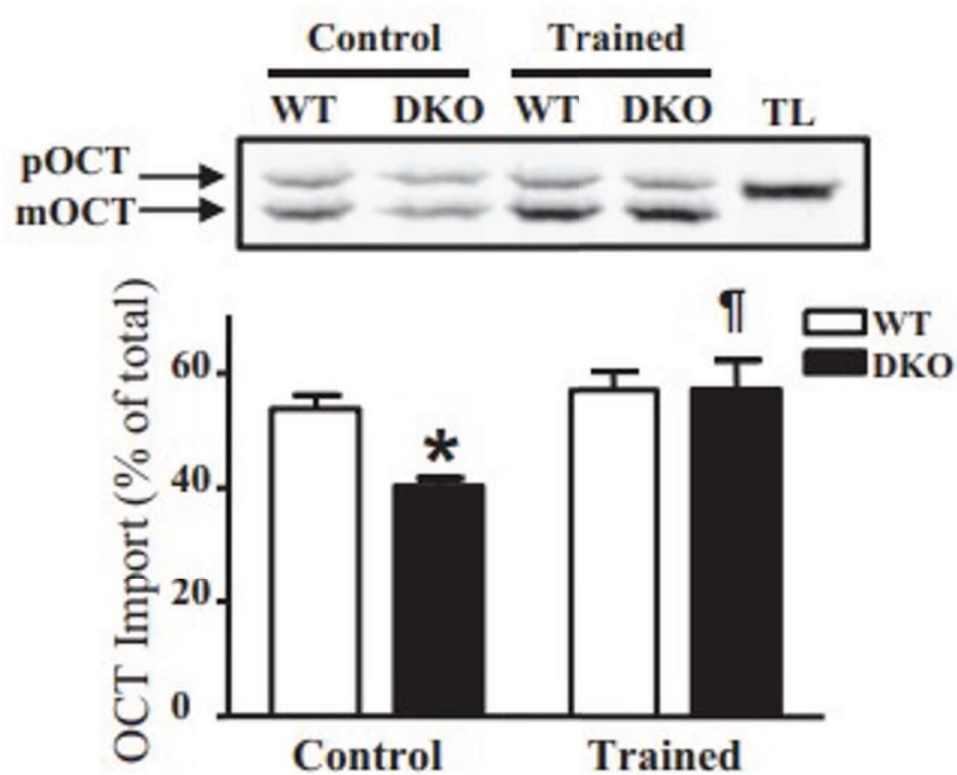704   cytosolic and mitochondria-bound kinases. *Cell Metabolism*. **18** (4), 578–587 (2013).

705

Figure

Remove tissue and place in scintillation vial filled with Buffer 1

↓

Mince tissue on watch glass until homogeneous

↓

Place minced tissue in centrifuge tube and dilute 10-fold with Buffer 1 + ATP

↓

Homogenize sample using a power output of 9.8 Hz for 10 s

↓

Centrifuge samples at 800 g for 10 min

SS Mitochondria

Filter supernate through cheesecloth into another 50 mL centrifuge tube

↓

Centrifuge sample at 9000 g for 10 min

↓

Add 3.5 mL of Buffer 1 + ATP and resuspend pellet using P1000

↓

Centrifuge sample at 9000 g for 10 min

↓

Resuspend pellet in 95 μL of resuspension medium

IMF Mitochondria

Dilute pellet 10-fold in Buffer 1 + ATP and resuspend using Teflon pestle

↓

Homogenize at 9.8 Hz for 10 s

↓

Centrifuge sample at 800 g for 10 min

↓

Dilute pellet 10-fold in Buffer 2 and resuspend using pestle

↓

Add 0.025 mL/g of nagarse protease and place tube on ice on its side

↓

Gently mix every min and after 5 min, add 20 mL of Buffer 2

↓

Centrifuge sample at 5000 g for 5 min

↓

Dilute pellet 10-fold in Buffer 2 and resuspend using pestle

↓

Centrifuge sample at 800 g for 15 min

↓

Pour supernate in a new 50 mL centrifuge tube and discard pellet

↓

Centrifuge sample at 9000 g for 10 min

↓

Add 3.5 mL of Buffer 2 and resuspend pellet using P1000

↓

Centrifuge sample at 9000 g for 10 min

↓

Resuspend pellet in 180 μL of resuspension medium

A

B

TABLE 1

Buffer 1:	Buffer 1 + ATP:	Buffer 2:
100 mM KCl	100 mM KCl	100 mM KCl
5 mM MgSO$_4$	5 mM MgSO$_4$	5 mM MgSO$_4$
5 mM EDTA	5 mM EDTA	5 mM EGTA
50 mM Tris	50 mM Tris	50 mM Tris
	1 mM ATP	1mM ATP

Buffer 1:	Buffer 1 + ATP:	Buffer 2:

Resuspension medium	Nagarse protease
100 mM KCl	10 mg/mL in Buffer 2
10 mM MOPS	NOTE: Make fresh and keep on ice
0.2% BSA	

Table

Table 2

	% of the reaction volume	1 Reaction mix
Reticulocyte Lysate	64.10%	11.8 µL
Amino acids (-methionine)	2.20%	0.4 µL
Sterile H_2O	21.60%	3.97 µL
[35]S-methionine	7.20%	1.33 µL
mRNA	5.40%	1.0 µL
Total Volume		18.5 µL

NOTE:

1) Add ^{35}S-methionine last;

2) Thaw the lysate slowly on ice, and limit freeze/thaw cycles to two. It is recommended that the lysate b

3) The volume of mRNA can be adjusted to optimize translational efficiency by altering the sterile H2O vc

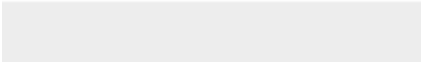e aliquoted upon arrival
olume accordingly.
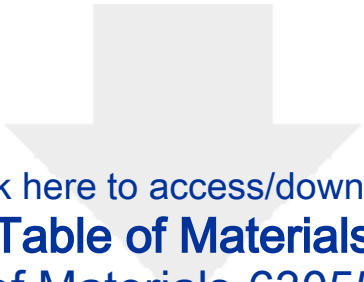
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Table of Materials

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