Metabolite extraction from suspension-cultured mammalian cells for global metabolite profiling

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Metabolite profiling of industrially important suspension-cultured mammalian cells is being increasingly used for rational improvement of bioprocesses. This requires the generation of global metabolite profiles that cover a broad range of metabolites and that are representative of the cells at the time of sampling. The protocol described here is a validated method for recovery of physiologically relevant amounts of key metabolites from suspension-cultured mammalian cells. The method is a two-step process consisting of initial quenching of the cells (to stop cellular metabolism and allow isolation of the cells) followed by extraction of the metabolites. The cells are quenched in 60% methanol supplemented with 0.85% (wt/vol) ammonium bicarbonate at −40 °C. Metabolites are then extracted from the quenched cells using two 100% methanol extractions followed by a single water extraction. Metabolite samples generated using this protocol are amenable to analysis by mass spectrometry–based techniques (e.g., gas chromatography–mass spectrometry, liquid chromatography–mass spectrometry), NMR spectroscopy and enzymatic assays.

INTRODUCTION

Metabolomics approaches have been used extensively in animal and plant tissues, yeast and bacteria, and therefore optimized sampling methods have been developed[1–5]. These methods are specific to the tissue type or cells being sampled and, as such, there is not a single sampling technique that can be used for generating samples for metabolite profiling. Animal and plant tissue samples are usually harvested and frozen in liquid nitrogen; next, the cells are lysed by either grinding or homogenizing before extraction[6–8]. This type of approach is not possible for cells cultured in suspensions in which they need to be separated from the medium, which would contaminate the intracellular metabolite profile. Therefore, metabolite sampling of yeast and bacterial cells is generally a two-step process consisting of quenching to stop metabolism and allow medium removal, followed by extraction to recover the metabolites from the cells[9]. It is also possible to quench without removing the medium (e.g., fast heating), but this has the disadvantage that metabolites that are present in both the medium and cells cannot be quantified separately (e.g., amino acids)[10–12]. Solvent-based quenching solutions are generally preferred because they can be cooled to extremely low temperatures (−40 °C), and the lower the temperature the slower the turnover rate of intracellular enzymes. However, the presence of methanol is detrimental to the cells and it has been reported that quenching of *Escherichia coli* cells in methanol can result in <60% loss of metabolites due to leakage[13]. This is a result of a combination of the high centrifuge speeds and extended centrifuge times required to pellet the cells, resulting in higher stresses on the cells and long exposure to methanol. The inclusion of additives in the methanol (e.g., agents that act as buffers and/or prevent osmotic shock) has been used successfully to substantially decrease this leakage[14–17]. The protocol for extraction of metabolites also requires optimization. This requires conditions in which the majority of the metabolites are soluble, stable and in an enzymatically inert environment. Naturally, alternate extraction methods are biased toward different classes of compounds because metabolites are preferentially recovered with specific methods. The selection of the appropriate extraction protocol should be based not only on the greatest amount of metabolites recovered but also on the reliability and reproducibility of the method. Various extraction methods have been recommended for recovery of metabolites from plants[18], fungi[19], bacteria[15], mammalian cells[20] and blood plasma[21].

Metabolite profiling is now increasingly being used in the area of biotechnology to improve the production of biopharmaceuticals[22]. Biopharmaceuticals are complex protein drugs (e.g., antibodies, growth regulatory factors, immunomodulators) that are used for the treatment of diseases that are not amenable to treatment with small-molecule drugs. The complex nature of these drugs means they require appropriate folding and post-translational modifications (PTMs) to function. Microbial expression platforms are unable to produce the required types of PTMs (e.g., glycosylation) naturally, and therefore these proteins are produced in mammalian cells. This presents a multistage process whereby genetically engineered cells are used to express substantial amounts of the desired recombinant protein drug, and in which the interaction of the cell with its culture environment (formulation of medium and potential feeds, environmental conditions) is part of a bioprocess. Optimization of the bioprocess is key to maximizing the yield of the desired protein with appropriate PTMs for effective functioning, and the manner in which the protein can be harvested for subsequent clinical use is also crucial. A key feature of the success of bioprocessing is the efficiency with which the cell culture medium supports cell growth (i.e., optimization of biomass) and appropriate PTMs. The development of optimal culture medium and feeds is linked to an understanding of the use of medium components. A direct readout of metabolite use and production would have great value, and we recognize that there is substantial scope for the use of metabolite profiling to improve current bioprocesses to optimize production of these biopharmaceuticals by mammalian cells. Therefore, robust and reproducible sampling methods are required to generate physiologically relevant intracellular samples. Commercial considerations favor cells that can be grown in

suspension because greater cell numbers can be achieved, and because they can be grown in large-scale fermentation conditions. For these reasons, Chinese hamster ovary (CHO) and mouse myeloma NS0 cells are, at present, the most industrially important cell lines as commercial cell 'factories' for production of valuable biopharmaceutical products. Metabolic profiling of these cell systems is of great importance early on in the development of a suitable process, which could subsequently scale up from laboratory level (i.e., 20 ml flasks) to the commercial production process (~20,000 liters). Profiling the metabolic needs of cells presents an early stage in the optimization of the bioprocess, but it can also be applied routinely to assess maintenance of stable cellular phenotype (using metabolism as the readout of phenotype). Several methods have been published for intracellular sampling of mammalian cells in adherent culture[23–25]; however, there were no existing simple sampling protocols for mammalian cells in suspension. To address this, we have developed a two-step protocol, based on yeast[9] and bacterial methods[14,15], with an initial quenching of cells to isolate them from medium before a subsequent extraction that recovers the greatest range and amount of metabolites (**Fig. 1** and **Table 1**)[20,26].

Development of the protocol

The protocol is a two-step process (quenching and extraction; **Fig. 1**) and the steps were optimized sequentially. Initially, we optimized and validated the quenching of the cells to assess metabolite yield, effectiveness of quenching, leakage of metabolites during the process, effects of washing to remove contaminating medium and effects on the downstream analysis of the samples[26]. Comparison of the yield of key labile metabolites from quenched and unquenched cells demonstrated that quenching was effective in halting cellular metabolism. We further verified the effectiveness from the adenylate energy charge (([ATP] + [ADP] / 2) / ([ATP] + [ADP] + [AMP])) and catabolic reduction charge ([NADH] / ([NAD$^+$] + [NADH])). These ratios provide insights into the state of the cells prior to metabolite extraction. We compared the values generated with published data for these metabolites to verify that the process generated a physiologically relevant metabolite profile. Supplementation of the 60% (vol/vol) methanol quenching solution with 0.85% (wt/vol) ammonium biocarbonate (AMBIC) was also shown to protect the cells from the damaging effects of the methanol (as demonstrated by the reduced leakage of intracellular metabolites into the quenching solution), thus improving metabolite yields by reducing membrane damage and subsequent leakage of metabolites. We achieved effective separation of the cell components from the medium using this methanol-based quenching method with limited contamination

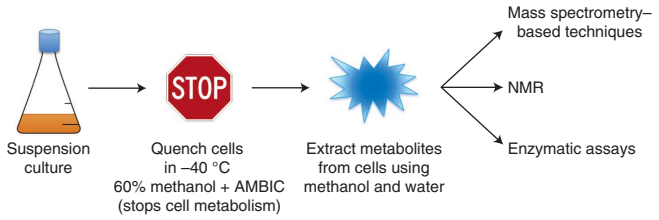

Figure 1 | Overview of protocol for metabolite extraction from suspension-cultured mammalian cells. Cells are initially quenched in quenching solution. Metabolites are then extracted from the quenched cells using two 100% methanol extractions followed by a single water extraction. Metabolites extracted using this protocol are amenable to analysis by mass spectrometry–based techniques (e.g., GC-MS, LC-MS), NMR and enzymatic assays.

Table 1 | Unique intracellular metabolites identified by GC-MS analysis using the procedures described in this protocol

1-hexadecanol	Lysine
1-methyl nicotinamide	Maleic acid
3-phosphoglyceric acid	Malic acid
4-aminobenzoic acid	Mannitol
6-phosphogluconic acid	Methionine
Adenosine 5′-monophosphate	Methionine sulfoxide
Alanine	Myo-inositol
Allothreonine	Myristic acid
Alpha-ketoglutaric acid	Nicotinamide
Asparagine	Norvaline
Aspartic acid	O-phosphocolamine
β-alanine	Oleic acid
β-glycerolphosphate	Ornithine
Cholesterol	Oxaloacetic acid
Citric acid	Palmitic acid
Citrulline	Pantothenic acid
Cyclohexanamine	Phosphoric acid
Cysteine	Porphine
Flavin adenine dinucleotide	Proline
Fumaric acid	Pyroglutamic acid
Glucose	Pyrophosphate
Glucose-6-phosphate	Pyruvic acid
Glutamic acid	Ribose
Glutamine	Sarcosine
Glyceric acid	Serine
Glycerol	Sorbitol
Glycerol-1-phosphate	Stearic acid
Glyceric acid	Succinic acid
Glycine	Threitol
Histidine	Threonine
Hydroxyproline	Tryptophan
Hypotaurine	Tyrosine
Isoleucine	Uracil
Lactic acid	Uridine 5′-monophosphate
Lauric acid	Valine
Leucine	

Table is adapted from reference 20.

from the medium (less than 1%). We decreased the medium contamination further (to less than 0.4%) by washing the cells in quenching solution prior to extraction. However, the increased exposure time to methanol increased leakage of specific intracellular metabolites (e.g., 60% of the ATP was lost from the cell fractions). Therefore, we recommend that the quenching be performed rapidly without any additional washes. Finally, we demonstrated that the presence of AMBIC in the quenching solution did not interfere with salt-sensitive analytical techniques such as gas chromatography–mass spectrometry (GC-MS).

We used a number of extraction methods to determine the range and amount of metabolites recovered from the quenched cells[20]. The extractions tested were methanol (100, 80 and 60%, vol/vol), 100% methanol followed by a water extraction, hot ethanol, acid (perchloric acid), alkali (KOH) and methanol/chloroform. We observed significant differences in the range of metabolites recovered with each extraction method. For example, solvent-based extraction methods result in the best recovery of fatty acids compared with aqueous-based methods because of their limited solubility in water. Conversely, some metabolites (e.g., citrulline) are only recovered by aqueous-based methods[20]. Extraction with two sequential 100% methanol extractions followed by a single water extraction (methanol/water) recovered the greatest range and amount of metabolites. We believe that this is a result of the combination of a solvent-based method with an aqueous-based method. The method used for the metabolite extraction can be changed if a specific class of metabolites is of interest but are not preferentially extracted using the methanol/water method. Use of a specific extraction method to improve the recovery of specific subsets of metabolites may limit the recovery of further subsets of metabolites. It is impossible to be prescriptive, but we recommend that researchers further optimize the protocols (e.g., by combinations of extraction processes) to ensure that they recover the desired profile of metabolites for their experimental situation. When choosing alternate extraction methods, it is essential that consideration also be given to the downstream analysis to ensure that the samples generated are suitable for the technique chosen for generation of the metabolite profiles (e.g., excess salt in samples interferes with GC-MS analysis). The protocol described here is based on quenching with 60% (vol/vol) methanol supplemented with 0.85% (wt/vol) AMBIC followed by methanol/water extraction, and represents a good global method.

Applications of the method

The protocol can be adapted for metabolite extraction from any suspension-cultured mammalian cell types. Metabolite samples generated using this protocol have been used successfully for generating intracellular metabolite profiles for CHO and NS0 cells using GC-MS and liquid chromatography–mass spectrometry (LC-MS). Metabolite pellets generated are also amenable to analysis by NMR. These samples have also been used for enzymatic assays (e.g., ATP, ADP, AMP, NAD^+, NADH and glucose-6-phosphate) for the determination of absolute intracellular concentrations for validation of the protocol against published data[20,26]. We have used metabolite profiling to assess, among other things, the effect of recombinant protein production and responses to a variety of perturbations in the culture conditions.

Experimental design

When designing metabolite-profiling experiments, it is essential to consider how the data are generated and reported to enable replication and comparison by others. As such, the Metabolomics Standards Initiative was published to provide guidance on minimum reporting standards[27]. As part of this, it is imperative that sufficient biological replicates be included to provide statistically significant results. The Metabolomics Standards Initiative recommends a minimum of three, and preferentially five, biological replicates and we routinely use six to ten biological replicates.

The exact experimental design used is dependent on the hypothesis being investigated. The protocol described here has been validated by assessment of recovery of key labile metabolites. If this protocol requires adaption (e.g., for use with different cell types), the recovery of labile metabolites (e.g., ATP, ADP, AMP, NAD^+, NADH and glucose-6-phosphate) can be checked by performing enzymatic assays on the recovered metabolite pellets to ensure that the quenching and extraction steps are working effectively before analysis is performed on expensive analytical equipment.

Comparison with other methods. Prior to the development of the protocol described here, no simple technologies had been developed for the generation of physiologically valid samples for intracellular metabolite profiling of suspension-cultured mammalian cells. A microstructure heat exchanger for quenching the metabolism of suspension CHO cells has been described, although the required specific equipment and validation of the quenching on the metabolite profile were not reported[28].

Subsequent to the development of this protocol, an alternative method was published describing an alternative quenching method using cold 0.9% (wt/vol) sodium chloride[29]. The authors of this paper attempted to repeat our method but found that they observed

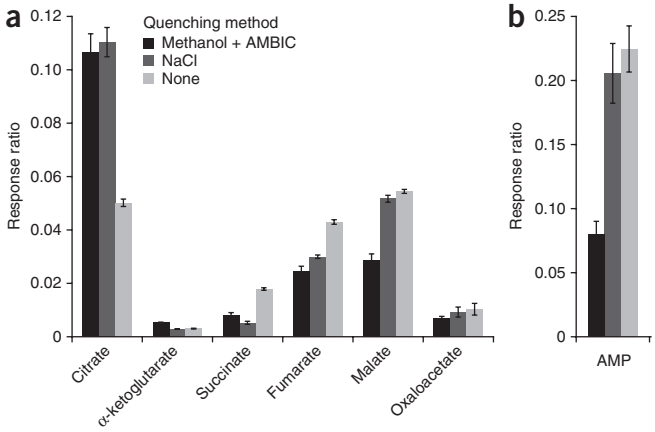

Figure 2 | Effects of different quenching methods on yields of key intracellular metabolites. Trichloroacetic acid (TCA) cycle intermediates are prone to leakage when the cell membrane is damaged and were used to assess cell membrane damage as a result of the quenching procedure. Adenosine monophosphate (AMP) has been shown to increase when quenching is ineffective[26] and can therefore be used as a measure of the effectiveness of the quenching procedure. Unquenched, exponentially growing CHO cells (day 5 of batch culture) were compared with cells quenched with either quenching solution at −40 °C (as described in **Fig. 3**) or with 0.9% (wt/vol) NaCl at 0.5 °C (as described by Dietmair *et al.*[29]). Metabolites were extracted from the cell pellets using the methanol/water protocol described in **Figure 4**. (**a**,**b**) The yields of TCA cycle intermediates (**a**) and AMP (**b**) were assessed. The data were generated by GC-MS; the response ratio corresponds to the ion counts divided by the internal standard (myristic acid). Values for each experiment represent the average of six biological replicates. Error bars show means ± s.e.m.

high cell leakage when quenching with methanol[29]. To compare the leakage observed with each of the protocols, we assesed the amount of intracellular trichloroacetic acid cycle intermediates (which are more prone to leakage than phosphorylated adenylates) (**Fig. 2a**). In each case, the amount of the intracellular trichloroacetic acid cycle intermediates detected was comparable between the two quenching methods. The one exception noted was malate, which was greater in the salt-quenched extracts. This result could arise from selective leakage of malate during methanol quenching or from inefficient quenching of the salt-quenched cells, resulting in enzymatic generation of malate. Overall, we could not detect any substantial leakage when using the methanol quenching method described here. The published results for other metabolites recovered using the salt-quenching method are generally comparable to the protocol described here, although the adenylate energy charge is typically lower (0.81) than that observed with the quenching method described here (0.92; ref. 26) and in data published for other types of mammalian cells (0.87–0.93; (refs. 30–34). In our comparison of the methods, we also detected increased amounts of AMP in extracts from NaCl-quenched cells, and the values we observed were similar to those seen in unquenched cells (**Fig. 2b**). The most likely explanation for this is that temperatures at or above 0 °C, while slowing cellular metabolism, are not sufficient to completely ablate all enzymatic activity. This is compounded by addition of a considerable volume of cells at 37 °C, which raises the temperature of the quenching solution further. The obvious advantage of solvent-based methods is the ability to cool them to −40 °C, which enables the temperature of the sample to be maintained below −20 °C even after addition of cells. This is particularly pertinent given the work of Wellerdiek *et al.*[35], who showed that even quenching at temperatures of −50 °C might not completely prevent metabolite interconversions. Rapid quenching is essential for the recovery of physiologically relevant amounts of labile metabolites (e.g., ATP, NAD^+ and glucose-6-phosphate). A recently published method by Volmer *et al.* for metabolite sampling of CHO cells using fast filtration reported no leakage, and ensured rapid and efficient quenching of the samples before extraction[36]. The adenylate energy charge was high (0.95), although the total yield of ATP was approximately 67% or 72% lower than the data reported for NaCl-quenched[29] or methanol + AMBIC–quenched[26] cells, respectively. Volmer *et al.*[36] suggested that this might be due to differences in the cell lines or culture conditions.

MATERIALS

REAGENTS

- Milli-Q water (Millipore)
- HPLC grade methanol ! CAUTION Wear gloves and safety glasses when handling methanol.
- Ammonium bicarbonate (AMBIC)
- Hydrochloric acid (12 M) ! CAUTION Wear gloves and safety glasses when handling.
- Dry ice ! CAUTION Wear gloves and safety glasses when handling dry ice.
- Liquid nitrogen ! CAUTION Wear gloves and safety glasses when handling liquid nitrogen.
- CD-CHO and CD-Hybridoma (Life Technologies)

EQUIPMENT

- Hemocytometer
- Conical tubes, 50 ml
- Microcentrifuge tubes, 1.5 ml
- Motorized pipetter
- Cryostat
- Low-temperature thermometer (−100 to + 40 °C)
- pH meter
- Refrigerated centrifuge capable of centrifuging 50-ml conical tubes. A centrifuge capable of chilling to −20 °C is recommended.
- Vacuum line for aspirating
- Microcentrifuge
- Vortex mixer
- Liquid nitrogen dewar
- Centrifugal evaporator
- 7890A GC System (Agilent Technologies)
- 5975C Inert XL MSD with triple-axis detector (Agilent Technologies)
- DB-5MS + DG column (250 µm × 30 m, 0.25 µm film thickness with 10 m Duraguard; Agilent Technologies)

REAGENT SETUP

Culturing of mammalian cells The protocol has been applied with great success to metabolite profiling of CHO and NS0 cell lines. We cultured cells in commercially available medium (CD-CHO and CD-hybridoma, respectively) and subcultured them every 3–4 d with a seeding density of 0.2×10^6 cells ml^{-1} with 100 r.p.m. orbital shaking. In our hands, this approach has proven to be robust for cells across a range of culture conditions (e.g., batch culture phase, passage number, medium and feeds). The protocol can easily be adapted to other suspensions or adherent mammalian cell types (e.g., human embryonic kidney (HEK293) or human embryonic retinal (PER.C6) cells), with appropriate validation.

Quenching solution Prepare the quenching solution (60% (vol/vol) methanol, 0.85% (wt/vol) AMBIC (pH 7.4)) by combining 600 ml of methanol, 100 ml of 8.5% (wt/vol) AMBIC (85 g l^{-1}) and 290 ml of Milli-Q water. Adjust the pH to 7.4 with 12 M hydrochloric acid and adjust the volume up to 1 liter with Milli-Q water. ▲ CRITICAL Fresh quenching solution must be prepared for each experiment because of pH drift.

PROCEDURE

Quenching of cells

1| Count the cells with a hemocytometer or comparable system and calculate the volume required for one sample (1×10^7 cells). See **Figure 3** for an illustration of the protocol for quenching cells.

▲ CRITICAL STEP Quenching of the cells is time sensitive, and therefore it is recommended that it be performed by two researchers working together.

▲ CRITICAL STEP The maximum sample volume is 8 ml for manipulation in 50-ml conical tubes. If the volume required is greater than 8 ml, it is possible to split the sample between two tubes and then recombine before extraction; however, this is not recommended.

2| Pipette five sample volumes of the quenching solution into 50-ml conical tubes.

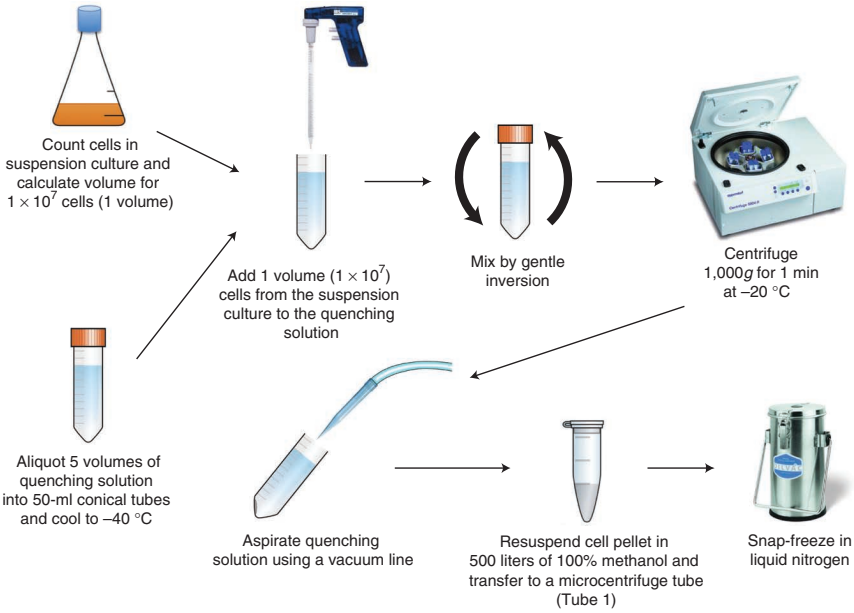

Figure 3 | Protocol for quenching of suspension-cultured mammalian cells. The cells (1 volume = 1×10^7 cells) are quenched in five volumes of quenching solution at −40 °C. The cells are then pelleted by centrifugation, and the quenching solution is removed by aspiration. The cell pellet is then resuspended in 100% methanol and snap-frozen in liquid nitrogen. The metabolites are then extracted from the quenched cells as described in **Figure 4**.

3| Cool the quenching solution to −40 °C using a cryostat. Alternatively, in the absence of a cryostat, this can also be achieved by incubating the tubes on dry ice (or in a dry ice/ethanol bath) and by monitoring the temperature with a low-temperature thermometer.
▲ **CRITICAL STEP** If you are cooling the quenching solution on dry ice, move the thermometer among all the tubes being cooled to ensure that they are all at the same temperature. If the thermometer is left in one tube, the solution will cool more slowly; therefore, any other tubes being cooled at the same time will be too cold when the cells are added.
▲ **CRITICAL STEP** We recommend that no more than four samples be prepared at a time (with two researchers; prepare only two samples if one researcher is working alone) to ensure that the quenching is done as rapidly as possible.

4| Pipette one sample volume of cells (1×10^7 cells) from the culture and dispense it into the center of the tube containing the quenching solution at −40 °C.
▲ **CRITICAL STEP** The temperature must be −40 °C when cells are added to ensure efficient quenching of cellular metabolism. Temperatures below −45 °C will result in instant freezing of the cells when they are added, resulting in damage to the cell membrane.
? TROUBLESHOOTING

5| Immediately mix the sample by gently inverting the tube.
▲ **CRITICAL STEP** Do not vortex the cells.

6| Pellet the cells by centrifugation at 1,000*g* for 1 min.

7| Remove the supernatant by aspiration using a vacuum line.
▲ **CRITICAL STEP** Keep the suction in place for a further 5 s after the supernatant has been aspirated to remove the supernatant retained by the sidewalls of the tube. This will reduce medium contamination.

8| Resuspend the cell pellet in 500 µl 100 % methanol (-80 C), transfer to a mirocentrifuge tube (Tube 1) and snap-freeze it in liquid nitrogen.

Extraction of metabolites from quenched cells
9| Thaw the quenched cells from Step 8 (Tube 1) and vortex them for 30 s. See **Figure 4** for an illustration of the protocol for the extraction of metabolites from quenched cells.

10| Pellet the cells by centrifugation at 800*g* for 1 min; transfer the supernatant to a fresh microcentrifuge tube (Tube 2) on dry ice.

11| Resuspend the cell pellet from Step 10 (Tube 1) in 500 µl of 100% methanol (cooled to −80 °C) and snap-freeze it in liquid nitrogen.
? TROUBLESHOOTING

12| Repeat Steps 9 and 10. Pool the supernatant into the microcentrifuge tube (Tube 2, from Step 10) on dry ice.

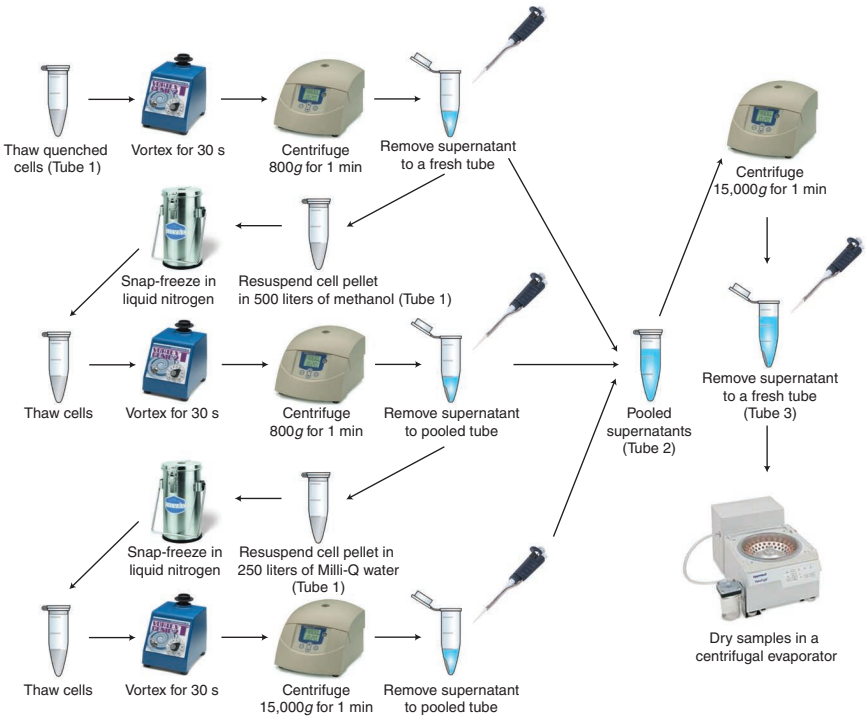

Figure 4 | Protocol for the extraction of metabolites from quenched cells. Metabolites are extracted from the quenched cells using two 100% methanol extractions followed by a single water extraction. The frozen-quenched cells (**Fig. 3**) are thawed, vortexed for 30 s and pelleted by centrifugation. The supernatant is removed to a fresh tube and the cell pellet resuspended in 100% methanol. The freeze-thaw cycle is repeated and the cells are then vortexed for 30 s and pelleted by centrifugation. The supernatant is removed and pooled with the previous methanol extract and the cell pellet is resuspended in Milli-Q water. The freeze-thaw cycle is repeated for the last time and the cells are then vortexed for 30 s and pelleted by centrifugation. The supernatant is removed and pooled with the previously pooled methanol extracts, and any remaining cell debris is removed by centrifugation. The supernatant is removed to a fresh tube and evaporated in a centrifugal evaporator to generate a dry metabolite pellet.

13| Resuspend the cell pellet (Tube 1) in 250 µl of ice-cold Milli-Q water and snap-freeze it in liquid nitrogen.
? TROUBLESHOOTING

14| Repeat Step 9 and pellet the cells by centrifugation at 15,000*g* for 1 min.

15| Remove the supernatant and pool with the methanol fractions in the tube on dry ice from Steps 10 and 12 (Tube 2).

16| Centrifuge the pooled supernatant fractions from Step 15 (Tube 2) at 15,000*g* for 1 min and transfer the supernatant to a fresh tube (Tube 3).

17| Dry the supernatant in Tube 3 using a centrifugal evaporator at 30 °C.

18| Subject the dried metabolite pellets in Tube 3 to analysis by mass spectrometry, NMR spectroscopy or enzymatic assays as described in **Box 1**.
? TROUBLESHOOTING

BOX 1 | METABOLITE ANALYSIS

The dried metabolite pellets are amenable to analysis by mass spectrometry, NMR spectroscopy and enzymatic assays. Precise conditions for any analysis method will depend on the availability of specific instruments. For GC-MS analysis we routinely use a 7890A GC System coupled to a 5975C Inert XL MSD with triple-axis detector and a DB-5MS + DG column (250 µm × 30 m, 0.25 µm film thickness with 10 m DuraGuard). Helium (1.2 ml min^{-1}) is used as the carrier gas; components are separated by isothermal chromatography for 1 min at 60 °C, followed by an increase to 325 °C at a rate of 10 °C min^{-1}, and then 10 min at 325 °C. This method generates more than 220 peaks and is described in more detail in our previous paper[20]. For NMR analysis, Tredwell *et al.*[37] have described a method that can be used with this protocol. Methods for enzymatic analysis of ATP, ADP, AMP, NAD^+, NADH and glucose-6-phosphate recovered using this protocol are based on commercially available kits and are described in detail in our previous paper[26].

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason
4	White material is observed when the cells are added to the quenching solution	The quenching solution is too cold and the cells are freezing on contact. Ensure that the quenching solution is at −40 °C when the cells are added
11, 13	Extracted cell pellets are difficult to resuspend	Centrifugation speed during extractions was too high
18	Low metabolite yield	Cells were exposed to the quenching solution for too long or excessively mixed after addition to the quenching solution (e.g., by vortexing), leading to cell membrane damage
	Medium contamination	The aspirator should be held in place for 5–10 s after the quenching solution has been aspirated to remove residual quenching solution from tube walls

● TIMING

Timing information is given for one set of samples. If two researchers are working in tandem, we recommend that no more than four samples be quenched and no more than eight samples be extracted at the same time. For a single researcher, we recommend that this be reduced to two and four samples for quenching and extraction, respectively.

Quenching

Step 1: 5 min per sample
Steps 2 and 3: 10 min
Steps 4–8: 2–3 min

Extraction

Steps 9–11: 3 min
Steps 12 and 13: 3 min
Steps 14–16: 5 min
Step 17: 4–5 h
Step 18: Variable

ANTICIPATED RESULTS

The efficiency of the quenching and extraction procedures was determined using specific metabolite assays to assess the yield of key labile metabolites. This is crucial because, as described in the TROUBLESHOOTING section, we found that several factors (e.g., the quenching solution being too cold or prolonged exposure to methanol) decreased the recovery of metabolites. Key metabolites that are subject to rapid turnover (i.e., ATP, NAD^+ and glucose-6-phosphate) were chosen to assess critically how rapidly cellular metabolism was halted. Comparison of the yield of these labile metabolites to unquenched cells (using the protocol described here) demonstrated that the quenching procedure rapidly and effectively halted cellular metabolism (**Fig. 5**). The intracellular concentrations of these metabolites were comparable to published values for other mammalian cell types[30–34,38–42]. Without quenching, the amount of metabolites recovered were significantly reduced (**Fig. 5**). The adenylate energy charge, defined by the equation ([ATP] + [ADP] / 2) / ([ATP] + [ADP] + [AMP]), and catabolic reduction charge, defined by the equation [NADH] / ([NAD^+] + [NADH]), can also be used to yield information regarding the relative well-being of the cells when metabolism was arrested.

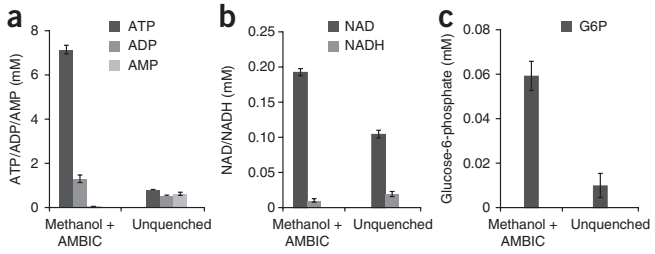

Figure 5 | Metabolite yields of key labile metabolites from quenched CHO cells compared with unquenched CHO cells. (**a**–**c**) Intracellular concentrations of ATP, ADP and AMP (**a**); NAD^+ and NADH (**b**); and glucose 6-phosphate (**c**). Values for each experiment represent the average of three biological replicates with two technical replicate readings of each. Error bars show means ± s.d. Modified, with permission, from Sellick *et al.*[26].

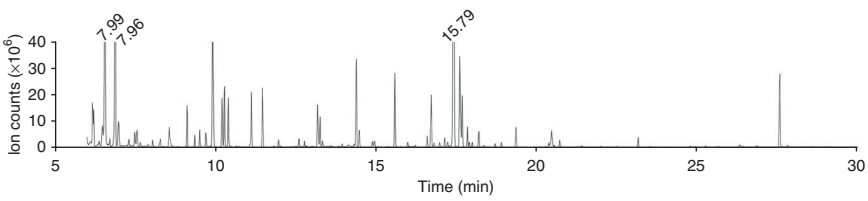

Figure 6 | Example of a raw GC-MS chromatogram of intracellular metabolites recovered from CHO cells in exponential growth phase using this protocol. The *y* axis has been manually set to 4×10^7 ion counts. Peaks greater than this set point are labeled with their numerical values.

The adenylate energy charge and catabolic reduction charge were determined to be 0.92 and 0.05, respectively, for CHO cells in exponential growth; this is comparable to published values for other mammalian cell types[30–34,38,43,44].

GC-MS analysis of intracellular metabolite samples that were generated using this protocol (**Fig. 6**) identified >220 unique features, and from these ~70 metabolites were definitively identified (**Table 1**). One limitation of GC-MS is that it separates metabolites using a heat gradient; therefore heat-labile metabolites such as certain adenine nucleotides (e.g., ATP, ADP, NAD, NADH) are not detected. However, we have shown that these can be detected in sensitive and complementary enzymatic analysis[26]. Mapping of the metabolites identified from these approaches onto metabolic pathway maps demonstrates that the metabolite profile generated using this protocol provides coverage of key metabolites from all the main metabolic pathways and regulatory points in cellular metabolism[20].

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AUTHOR CONTRIBUTIONS All authors discussed the steps of the protocol, its implications and applications. C.A.S. wrote the manuscript and R.H., G.M.S., R.G. and A.J.D. revised it.

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