Assessing hepatic metabolic changes during progressive colonization of germ-free mouse by 1H NMR spectroscopy

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Assessing Hepatic Metabolic Changes During Progressive Colonization of Germ-free Mouse by ^{1}H NMR Spectroscopy

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Abstract

It is well known that gut bacteria contribute significantly to the host homeostasis, providing a range of benefits such as immune protection and vitamin synthesis. They also supply the host with a considerable amount of nutrients, making this ecosystem an essential metabolic organ. In the context of increasing evidence of the link between the gut flora and the metabolic syndrome, understanding the metabolic interaction between the host and its gut microbiota is becoming an important challenge of modern biology.[1-4]

Colonization (also referred to as normalization process) designates the establishment of micro-organisms in a former germ-free animal. While it is a natural process occurring at birth, it is also used in adult germ-free animals to control the gut floral ecosystem and further determine its impact on the host metabolism. A common procedure to control the colonization process is to use the gavage method with a single or a mixture of micro-organisms. This method results in a very quick colonization and presents the disadvantage of being extremely stressful[5]. It is therefore useful to minimize the stress and to obtain a slower colonization process to observe gradually the impact of bacterial establishment on the host metabolism.

In this manuscript, we describe a procedure to assess the modification of hepatic metabolism during a gradual colonization process using a non-destructive metabolic profiling technique. We propose to monitor gut microbial colonization by assessing the gut microbial metabolic activity reflected by the urinary excretion of microbial co-metabolites by ^{1}H NMR-based metabolic profiling. This allows an appreciation of the stability of gut microbial activity beyond the stable establishment of the gut microbial ecosystem usually assessed by monitoring fecal bacteria by DGGE (denaturing gradient gel electrophoresis).[6] The colonization takes place in a conventional open environment and is initiated by a dirty litter soiled by conventional animals, which will serve as controls. Rodents being coprophagous animals, this ensures a homogenous colonization as previously described.[7]

Hepatic metabolic profiling is measured directly from an intact liver biopsy using ^{1}H High Resolution Magic Angle Spinning NMR spectroscopy. This semi-quantitative technique offers a quick way to assess, without damaging the cell structure, the major metabolites such as triglycerides, glucose and glycogen in order to further estimate the complex interaction between the colonization process and the hepatic metabolism[7-10]. This method can also be applied to any tissue biopsy[11,12].

Video Link

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Protocol

1. Colonization of germ-free animals and sample collection

1. Remove germ-free animals from isolators and house them in a conventional husbandry room in cages equipped with filter in front of the conventional animals which will serve as controls (Figure 1).
2. Mix half of the litter (3 days old) taken from the control conventional cage with the litter of the germ-free animals. Always keep 1/3 of the dirty conventional litter each time it is necessary to renew it in order to maintain a level of bacteria (keep it at least for 3 days).
3. Collect urine in a 1.5 mL microtube by handling the mouse over the tube and help micturition by gently massage the bowel. Snap-freeze immediately in liquid nitrogen. Store at least at -40°C until NMR analysis. A minimum volume of 20 µL is required for acquisition with a 5 mm NMR probe, but it is recommended to use 30 µL to improve quality of metabolic profiling.
4. Animals should be euthanized without use of any anesthetic in order to avoid confounding NMR resonances due to hepatic metabolism of anesthetic compounds (for example, use cervical dislocation followed by confirmation of death by exsanguination)

2. Recommendation for collection of liver biopsy

1. Do not use any product containing alcohol to avoid contamination. Wash tools using water only or saline solution.
2. Do not perforate gall bladder. In case of bile leak, wash tissue immediately with water or saline solution.

3. Collect liver biopsies (about 15-50 mg) from the left lobe as shown in Figure 2. For reproducible biopsies, collect consistently in the center of the left lobe avoiding the peripheral areas where tissue is thinner.
4. Snap freeze biopsies in liquid nitrogen immediately and store them at -80°C until NMR analysis.

3. ^{1}H NMR acquisition of urine microvolume

1. Prepare 0.2M sodium phosphate buffer solution in D2O (99.8 %), pH 7.4 containing 1 mM 3-(trimethylsilyl)propionic acid-d_4 (TSP).
2. Mix 30 µL of urine with 30 µL of sodium phosphate buffer.
3. Transfer 50 µL of mixed solution into 1.7 mm NMR capillary tube (Figure 3 (2)) using a 50 µL glass syringe equipped with a metal needle (OD 0.5 mm). Be careful to avoid bubbles.
4. Fit the capillary adaptor (Figure 3 (3)) on top of the capillary containing urine sample and place it into a 2.5 mm NMR microtube for 5 mm NMR probe (Figure 3 (1)). Use this combination of tubes as a regular 5 mm NMR tube for spectral acquisition.
5. Use the extraction rod (Figure 3 (4)) to remove capillary from 2.5 mm NMR tube by screwing the capillary adapter gently to pull it out.

4. ^{1}H HR MAS NMR of liver tissue biopsy: sample preparation

1. MAS rotor components and tools are described in Figure 4.
2. Insert biopsy (about 15-50 mg) into zirconium rotor (Figure 4 (1)) and fill the rest of the volume with pure D_2O for NMR lock. Be careful to not make any bubbles because this would alter the quality of subsequent shimming and quality of data acquisition.
3. Insert 50 µL Teflon spacer (Figure 4 (2)) using the cylindrical screw (Figure 4 (5)). Unscrew it and calibrate it using the depth gauge on the short side (Figure 4 (8)). At this step, it is important to pay a specific attention to the sample because part of it can leak through the spacer hole. If this is the case, then part of the biopsy is destroyed and sample weight is no longer reliable. It is thus necessary to start again from the beginning the preparation of the sample.
4. Place the thead pin (Figure 4 (3)) and screw it gently with the screwdriver (Figure 4 (6)). Dry out any residual water with a piece of tissue.
5. Place the cap (Figure 4 (4)) at the top of the rotor and insert it in the rotor packer (Figure 4 (6)). Press firmly until the cap is in place. There should not be any space left between the rotor and its cap.
6. Mark half of the bottom of the rotor using a black marker pen to allow optical spin rate detection.
7. Place the rotor inside the NMR spectrometer and start spinning at 5 kHz. Acquire ^{1}H NMR spectrum using CPMG pulse sequence[13] according to manufacturer's guidelines.
8. Use α anomeric glucose resonance at 5.22 ppm (doublet) to calibrate NMR spectra.
9. To unpack the rotor, proceed by removing the cap using the cap remover (Figure 4 (9)). Unscrew thead pin and remove Teflon spacer using the cylindrical screw. Wash thoroughly using water and detergent.

5. Representative Results

Gut microbial activity can be monitored using urinary metabolic profiling. A large number of urinary microbial co-metabolites identifiable by ^{1}H NMR have been described in the literature[7,14-17]. These microbial co-metabolites are particularly useful to monitor the colonization process as they provide a quick and noninvasive way to estimate when the newly established ecosystem is stable. Figure 5A clearly illustrates the appearance of gut microbial co-metabolites over the colonization process. This figure shows a urinary metabolic profile obtained by following the procedure described in Step 2 for an animal colonized 20 days using procedure described in Step 1. This animal did not excrete any indoxyl sulfate and very little amounts of phenylacetylglycine (PAG) and *p*-cresol sulfate at the germ-free state (day 0-blue). As colonization progresses, these 3 markers of protein metabolism by the gut microbiota increase considerably to reach an equilibrium at day 20 (red). This is particularly easy to monitor for a group of animals as illustrated on Figure 5B using the PAG resonance. This diagram was obtained by integrating the area under the resonances highlighted in gray in Figure 5A (δ 7.40-7.43), corresponding to a specific resonance (triplet) of PAG for a group of 7 animals.

^{1}H High Resolution Magic Angle Spinning (HR MAS) NMR spectroscopy is a non destructive technique that allows quick and reproducible acquisitions of metabolic profiles of any kind of biopsy[18]. In this protocol, we used this powerful technique to obtain a hepatic metabolic profile of 2 mice before (blue) and after (red) colonization (Figure 6). This figure illustrates well the information that can be derived from a MAS NMR-based metabolic profile. Numerous amino acids as well as metabolites derived from energetic metabolism such as glucose, glycogen, lactate, triglycerides, (*D*)-3-hydroxybutyrate and nicotinurate can be visualized. These profiles also contain information relevant to oxidative stress (i.e. ascorbic acid, glutathione), nucleotide metabolism (i.e. inosine, uridine) and methylamine metabolism (i.e. choline, Trimethylamine-N-oxide). In this example, it is very clear that the germ-free mouse displays almost no glycogen and very low amounts of glucose and triglycerides as was previously published[7].

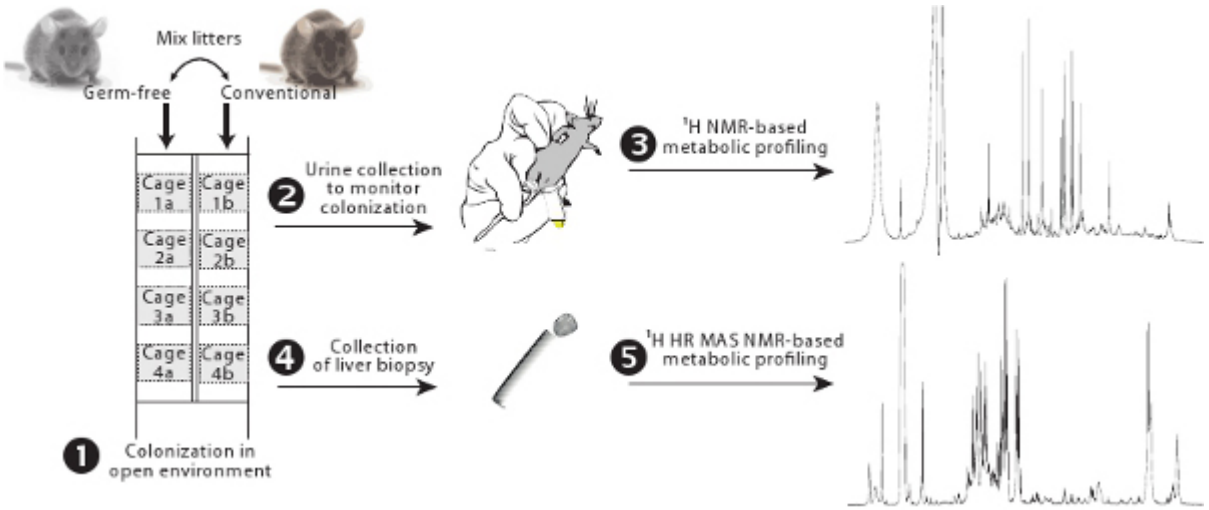

Figure 1. Overview of the colonization protocol. Germ-free and conventional animals are housed in cages equipped with filters side by side and their litters are exchanged to allow progressive colonization from the conventional gut microbiota (1). Gut microbial activity is monitored using ^{1}H NMR-based metabolic profiling (2-3). Hepatic metabolism is assessed by ^{1}H HR MAS NMR-based metabolic profiling (4-5).

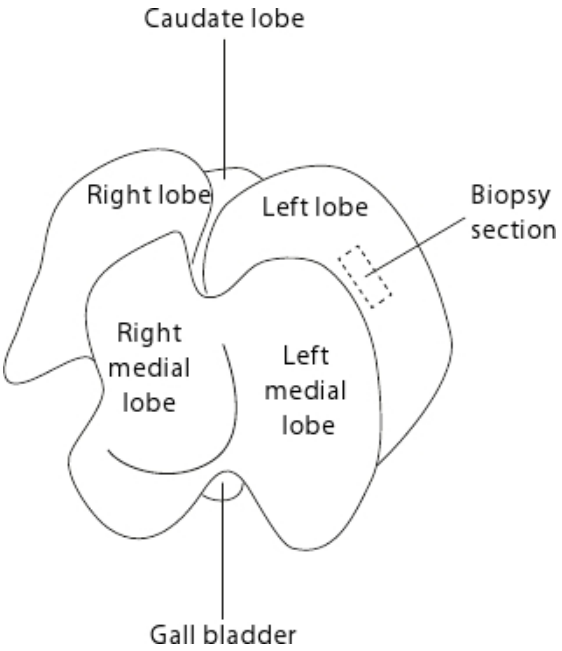

Figure 2. Mouse liver anatomy. The liver is displayed such as the flat side of the organ faces the table. For reproducible biopsies, it is advised to always collect samples from the center of the left lobe as indicated by the dashed rectangle.

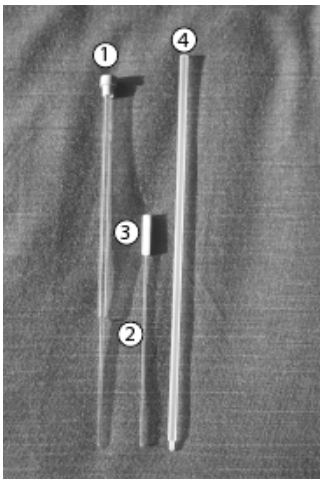

Figure 3. 1.7 mm NMR capillary kit to work with microvolumes. *Key*: 1: 2.5 mm NMR microtube, 2: 1.7 mm NMR capillary tube, 3: Capillary adapter, 4: Extraction rod.

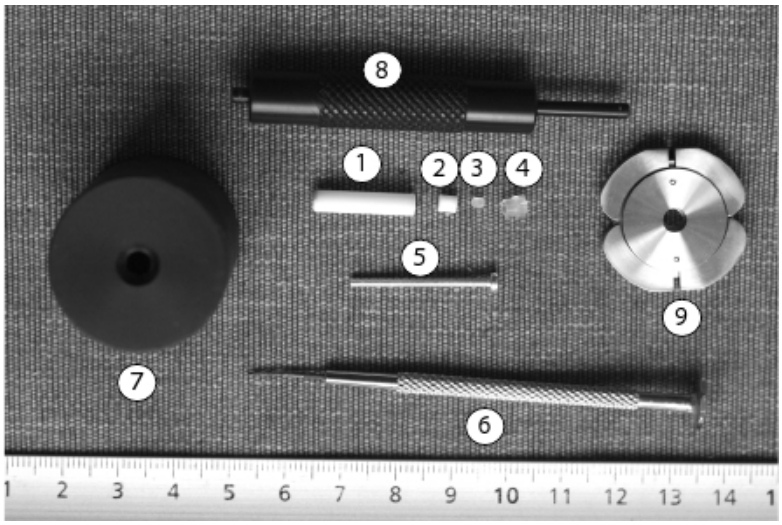

Figure 4. MAS rotor equipment. *Key*: 1: MAS rotor, 2: 50 µL Teflon spacer, 3: Thead pin, 4: cap, 5: cylindrical screw, 6: screwdriver, 7: rotor packer, 8: depth gauge.

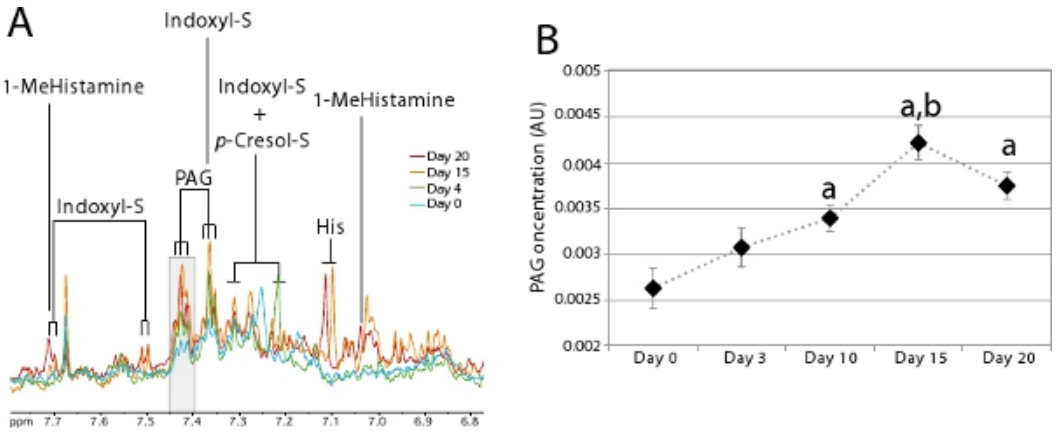

Figure 5. Evolution of urinary metabolic profiles during colonization.

A. Zoom on the aromatic region of the spectra between 6.8-7.8 ppm where microbial co-metabolites can be visualized. ^{1}H NMR spectra were derived from a single individual at day 0 (blue), 4 (green), 15 (orange) and 20 (red) post-colonization. The grey zone corresponds to the area that was integrated to make the diagram in B. Key: 1-MeHistamine: 1-methylhistamine; Indoxyl-S: Indoxyl sulfate; His: Histidine; p-Cresol-S: p-Cresol sulfate; PAG: Phenylacetylglycine.
B. Average PAG concentration during colonization (n=7). Student's t-test was used to compare the difference in PAG concentration at various time-points: a: $p<0.05$ compared to day 0; b: $p<0.01$ compared to day 10.

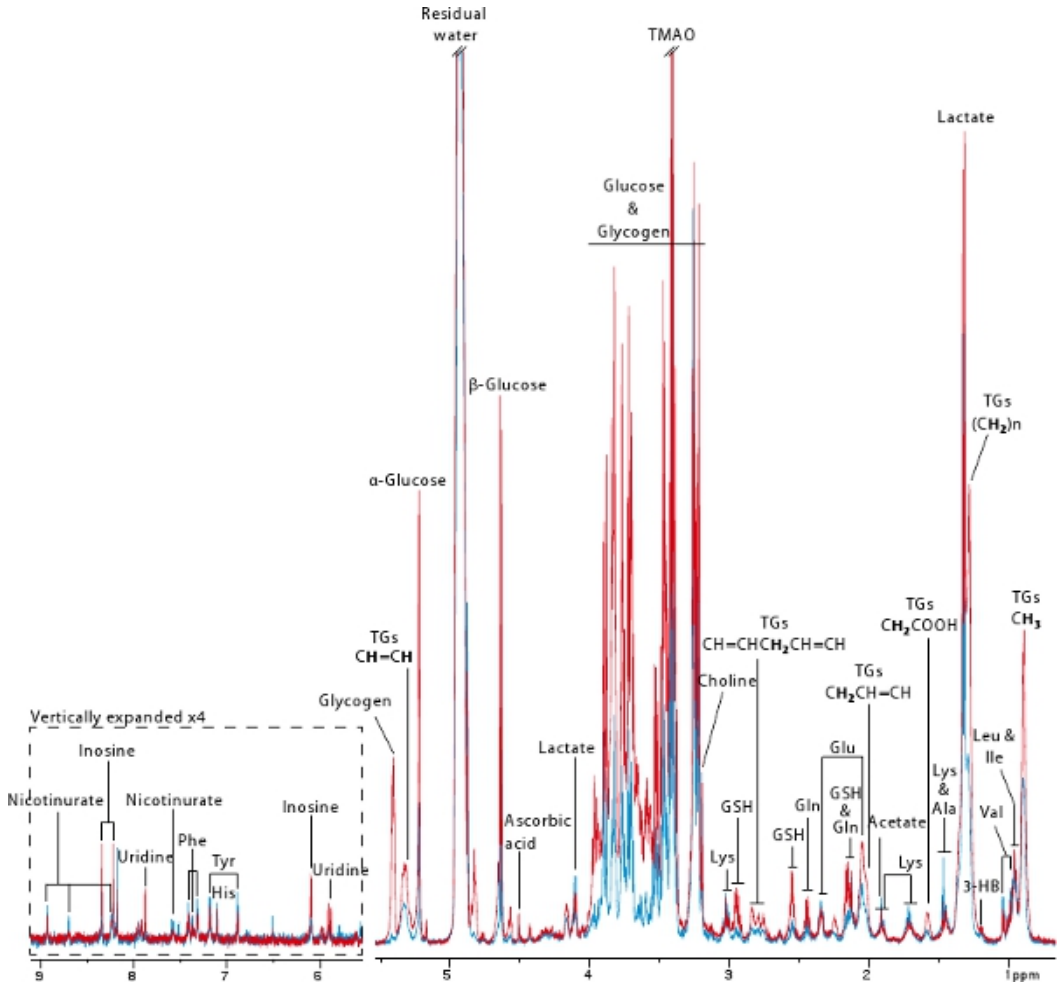

Figure 6. Typical 600 MHz ^{1}H HR MAS NMR spectra of liver biopsies derived from germ-free (blue) and ex-germ-free (red) mice. Bold protons are responsible for the triglyceride resonance. *Key*: 3-HB: 3-hydroxybutyrate, GSH: reduced glutathione, TGs: Triglycerides, TMAO: Trimethylamine-N-oxide.

Discussion

In this protocol, we described a progressive colonization procedure in an open environment to further investigate the impact of gut microbiota on hepatic metabolism assessed by ^{1}H HR MAS NMR profiling of intact biopsy. Various methods of colonization have been described in the literature. The most common methods to colonize animals with a defined microbiota are oral gavage or contaminated drinking water[19,20]. Fecal inoculation can also be used as previously described[21]. The colonization method presented here is derived from a "normalisation" method of germ-free animals described by Koopman JP et al. in 1986[22]. In this publication, the authors placed a living conventional animal into the isolator among the germ-free animals. However, it is not always possible to keep animals in isolators, especially if they need to be manipulated during the colonization process (this is particularly difficult if sample collection is required). An alternative is thus to house ex-germ-free animals in a conventional open environment in presence of litter soiled by conventional animals who will be used as controls. In this way, animal manipulation for the purpose of colonization is minimal and this results in lower stress compared to oral gavage. This method also allows a progressive colonization of the gut which is closer to a natural colonization process and offers a homogenous colonization of animals sharing the same cage as demonstrated by DGGE (Denaturing Gradient Gel Electrophoresis) assessment of microbial DNA profiles (available as supplemental material in Claus et al.[7]).

Monitoring the colonization process by urinary metabolic profiling is a noninvasive, easy, quick and effective way to detect when the microbial activity becomes stable. As it is not necessary to manipulate animals every day for such purpose, as shown in Figure 5B, the stress level is kept at its minimum. It is noteworthy to mention that even if the colonization is initiated by a litter soiled by conventional control animals, it is necessary to keep an equal number of those control animals which will allow one to estimate the mixed effects of stress and ageing on hepatic metabolism. Other techniques based on mass spectrometry (MS) such as GC-MS (Gas Chromatography) or LC-MS (Liquid Chromatography) can also be used to determine urinary microbial co-metabolites as well as to obtain a metabolic profile of liquid samples (i.e. urine, plasma, tissue extracts), but they cannot be applied on intact tissue biopsy. GC-MS has been successfully applied to targeted analysis of stable volatile fatty acids[23]. This technique requires a derivatization step which introduces biases that have to be carefully considered during data analysis[24]. LC-MS can be particularly useful to improve the detection of microbial co-metabolites in targeted profiling[25]. Although untargeted LC-MS metabolic profiling substantially improves the detection sensitivity of low concentration metabolites, identification can be difficult and a large number of detected metabolites may remain unassigned[26]. Therefore, most of the untargeted metabonomic studies have been performed using one-dimensional ^{1}H NMR-based platforms. An interesting discussion of the various analytical methods available for metabolic profiling purposes has been recently published by Ryan et al. [27].

Hepatic metabolism was assessed by non destructive ^{1}H HR MAS NMR spectroscopy. This method was chosen because it does not necessitate an extraction step which destroys the tissue and results in the oxidation of highly reactive compounds such as glutathione. ^{1}H NMR-based metabolic profiling also presents the advantage of offering an untargeted metabolic profile of the biopsy. It thus allows the observation of a large

range of metabolites covering energetic, amino acid, nucleotide, methylamine and oxidative stress related pathways. The only restriction is the limit of detection which varies according to a compound's molecular structure. Indeed, the detection limit is determined by the chemical (i.e. metabolite) concentration as well as the number of protons giving the resonance peak and their chemical environment. Identification of metabolite resonances can also be difficult based on ^{1}H HR MAS NMR spectra alone and it is thus advised to perform some extra 2D NMR experiments to confirm assignments (i.e. J-resolved, COSY, TOCSY, HSQC, HMBC experiments[28-30)31,32]. This ^{1}H HR MAS NMR technique is commonly used for metabonomic studies, in which case the use of multivariate statistics (also called pattern recognition methods) is necessary[33]. The ^{1}H NMR-based metabolic profiling methods described in this protocol have been extensively applied to various biological conditions and are not limited to the analysis of urine and liver samples[34-36]. General sample preparation protocols for NMR-based metabonomics have been reviewed by Beckonert *et al.*[18,37].

Disclosures

We have nothing to disclose.

Acknowledgements

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