**Raman spectroscopy for real-time and *in* *situ* monitoring of mechanochemical milling reactions**

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**Abstract:**

We describe the protocol for real-time and *in situ* monitoring of mechanochemical milling reactions conducted on vibratory ball mills by Raman spectroscopy. Recently, solid-state milling reactions became an attractive and sustainable alternative for preparing of almost all classes of compounds and materials. Raman spectroscopy monitoring complements already established *in situ* monitoring based on powder X-rays diffraction, which requires synchrotron X-ray source and therefore has limited accessibility to a broader research community. The here described Raman spectroscopy monitoring setup exploits affordable and modular nature of the currently available instrumentation, and consists of a portable small spectrometer, a laser source, and a Raman probe. As such, it is readily implemented in a laboratory environment. Translucent plastic reaction jars enable interaction of the laser beam with the solid sample residing inside the closed reaction jar and collection of Raman–scattered photons while the ball mill is in operation. Acquired Raman spectra are analysed using commercial or open-source software for data analysis (e.g. Matlab, Octave, Python, R). Plotting the Raman spectra versus time enables qualitative analysis of reaction paths, as well as phase identification. We demonstrate extraction of a reaction profile from changes in intensities of selected Raman bands. Alternatively, a more rigorous approach should include the use of multivariate analysis.

**INTRODUCTION**

Mechanochemical milling reactions belong to the field of mechanochemistry which encompasses various aspects of reactivity achieved by exertion of mechanical force, via milling, shearing and grinding[[1]](#endnote-1),[[2]](#endnote-2), as well as polymer cleavage under sonication mechanical stress[[3]](#endnote-3), and compression and stretching of individual molecules[[4]](#endnote-4). Milling, grinding, and shearing of solids have been used throughout human history for comminution and mixing of foods and materials in general[[5]](#endnote-5). Recently, the immense potential of mechanical action as a means for material synthesis in the solid state has been recognized[[6]](#endnote-6) as an alternative to traditional solution-based synthesis offering faster, atom-efficient, environmentally benign reactions and processes that also, often, require minimal post-synthetic work-up. Thus, by now, mechanochemistry found numerous applications in organic[[7]](#endnote-7),[[8]](#endnote-8), inorganic[[9]](#endnote-9),[[10]](#endnote-10), organometallic[[11]](#endnote-11),[[12]](#endnote-12), supramolecular[[13]](#endnote-13),[[14]](#endnote-14), coordination compounds and metal-organic frameworks[[15]](#endnote-15), as well as in catalysis[[16]](#endnote-16),[[17]](#endnote-17), and preparation of active pharmaceutical ingredients (APIs)[[18]](#endnote-18),[[19]](#endnote-19). Mechanochemistry goes hand-in-hand with the principles of "Green Chemistry", especially when considering sustainability as one of the major UN goals[[20]](#endnote-20). IUPAC recently recognized the importance of mechanochemical procedures naming them as one of the "emerging technologies in Chemistry with the potential to make our planet more sustainable"[[21]](#endnote-21).

Nowadays, milling reactions are most often carried in shaker or planetary ball mills, attritors, and extruders[[22]](#endnote-22), overcoming the limitations of a mortar and pestle. The introduction of mechanical mills offers better reproducibility and efficiency of mechanochemical processing where reactions are performed in a closed, often non–transparent reaction jars, rotating or oscillating rapidly. In this *Protocol*, we focus on shaker mills where the jar oscillates in the horizontal or vertical plane with frequencies ranging from 20 to 50 Hz. In such an environment, obtaining information about the sample inside of the jar during the mill operation remained an insurmountable challenge, and limited, at first, to temperature[[23]](#endnote-23),[[24]](#endnote-24) and pressure monitoring[[25]](#endnote-25). While such insights were valuable, they cannot provide direct information about structural or chemical changes during milling. All this has led to a wide perception of milling as a "black–box" method for material synthesis.

A milling reaction is performed in a jar where solid reactants are put together with the milling media (most usually milling balls). Such an approach is known as neat grinding (NG), or dry milling. However, it has become a common practice to use additives in substoichiometric amounts which may drastically influence the reactivity of solids1,6. In mechanochemical terminology, milling reactions conditions are most often referred to as liquid–assisted grinding (LAG)[[26]](#endnote-26), in the case the additive is a liquid, ion– and liquid-assisted grinding (ILAG)[[27]](#endnote-27), when simple ionic compounds are used along with liquids, and polymer–assisted grinding (POLAG)[[28]](#endnote-28) when a polymer such as polyethylene glycol is used as an additive. The newest addition to this toolbox are ionic liquid additives, and the procedure is referred to as ionic liquid–assisted grinding (IL-AG)[[29]](#endnote-29).

The course of a chemical reaction is monitored by the changes in the chemical composition with time. A reaction mixture can be sampled at regular time intervals and analysed later at any point in the laboratory. Such *ex situ* approach has been applied to the milling reactions, and samples were characterized by powder X-ray diffraction (PXRD)[[30]](#endnote-30),[[31]](#endnote-31), Raman[[32]](#endnote-32),[[33]](#endnote-33),[[34]](#endnote-34) and nuclear magnetic resonance (NMR) spectroscopy[[35]](#endnote-35), as well as high–performance liquid chromatography (HPLC)[[36]](#endnote-36),[[37]](#endnote-37). It, nonetheless, required periodically stopping the milling process and opening the jar for sampling of the reaction mixture. Since the jar is usually closed, opening it disturbs the established equilibrium and exposes the reaction mixture to air, which, in turn, may result in chemical reactivity different from an uninterrupted reaction[[38]](#endnote-38). Also, a chemical reaction may continue even after cessation of milling[[39]](#endnote-39),[[40]](#endnote-40), and the resulting ex situ analysis needs not reflect the true course of the chemical and physical processes taking place during milling. To alleviate this problems with periodic interruptions multiple experiments may be performed with each experiment having the milling process running for a successively longer time36,37,[[41]](#endnote-41). By gradually prolonging milling time and analysing reaction mixture composition, it could be possible to construct the reaction profile for an uninterrupted reaction. However, because of the stochastic nature of nucleation[[42]](#endnote-42), and since every experiment is independent, the repeated experiments may not follow exactly the same reaction path. Moreover, even for a modest reaction time sampling, this approach requires laborious work, numerous experiments, a significant amount of reactants, while still facing the mentioned issues related to a belated ex situ analysis. Therefore, information from monitoring of uninterrupted milling is essential.

**Real-time and *in situ* monitoring of milling reactions**

Real-time and *in situ* temperature and pressure monitoring24,25,[[43]](#endnote-43),[[44]](#endnote-44),[[45]](#endnote-45) of milling reactions were achieved by implementing sensors on, or within the reaction jar. However, these methods can only indirectly track changes in the chemical composition of the reaction mixture. Thus, it is of no surprise, that significant progress in understanding the milling reaction environment came with the development of the first in situ method for uninterrupted monitoring, which was based on synchrotron powder X–ray diffraction (PXRD)[[46]](#endnote-46),[[47]](#endnote-47). This method relies on high–energy and high–flux synchrotron X–rays source (Energy range: 60–90 keV) which are able to penetrate through the reaction jar during milling. Scattered X–rays are detected on a 2D area detector, and raw diffraction patterns are plotted in real–time enabling direct observation of the evolution of crystalline phases during the milling reaction. It was soon recognised that X-ray diffraction is limited in characterising any amorphous phases, which are often present during milling[[48]](#endnote-48).

The next step forward came with the introduction og real-time and *in situ* monitoring method based on Raman spectroscopy[[49]](#endnote-49),[[50]](#endnote-50). The rapid development of affordable, small, and portable Raman spectroscopy equipment established it as one of the main techniques in process analytical technologies (PATs) and became widely used in chemical and biopharmaceutical industry[[51]](#endnote-51),[[52]](#endnote-52). There, in-line approach enabled real-time tracking of chemical processes, from monitoring chemical reactions and crystallization[[53]](#endnote-53),[[54]](#endnote-54), to the optimisation of process parameters in manufacturing[[55]](#endnote-55). Furthermore, Raman spectroscopy is applicable not only to monitoring of crystalline phases but also to slurries, and liquids, while being regularly used in analytical laboratories for qualitative and quantitative analysis[[56]](#endnote-56). The basis for implementation of this methodology for milling reaction monitoring is the non-contact nature of Raman spectroscopy. A contactless Raman probe is suitable for positioning in the vicinity of the oscillating reaction jar49. A focused laser beam is then introduced in the jar that need to be made of a transparent material such as the plastic poly(methyl 2–methylpropenoate) (PMMA)49 or a sapphire crystal[[57]](#endnote-57). Finally, Raman scattered photons are collected again by the same probe and transferred to the spectrometer.

Raman and PXRD methods thus are complementary, and experimental setups permit their simultaneous use in tandem[[58]](#endnote-58),[[59]](#endnote-59),[[60]](#endnote-60),[[61]](#endnote-61), and even coupling each of them with temperature monitoring[[62]](#endnote-62),[[63]](#endnote-63),[[64]](#endnote-64),, or all three together[[65]](#endnote-65). While PXRD detects changes of bulk crystalline phases, chemical transformations at the molecular level, including bond breaking and formation, as well as rearrangement of supramolecular interactions are readily observed by Raman spectroscopy. The *in situ* PXRD proved as a powerful method in the characterization of reaction mixture composition and extraction of reaction profiles using quantitative Rietveld analysis of time–resolved powder diffraction patterns48. However, milled reaction mixtures may experience partial or full amorphisation, and previously structurally uncharacterized phases may be formed59. This limits the use of Rietveld analysis, and full reaction profiles may become unobtainable. While quantification in the presence of an amorphous fraction is possible with the use of an internal scattering standard48, this still does not provide information on the chemical nature of the amorphous fraction but rather only its amount. Another disadvantage of *in situ* PXRD method is that it requires access to a high-energy synchrotron source, putting a severe limitation to its availability to a general user. On the other hand, Raman spectroscopy, aside from being a readily implementable laboratory technique, enables the extraction of reaction profiles in cases where partially amorphous and poorly crystalline phases are present, and where the reaction mixture may also contain structurally uncharacterised phases59. As a drawback, Raman spectroscopy is limited in some cases by a high fluorescent signal from the sample, which could partially or fully cover the sample’s Raman bands, and, in effect, disable *in situ* monitoring by Raman spectroscopy. In such a case, reducing the power of the incident laser beam, or switching to a lower-energy laser source excitation wavelength could be beneficial. Another disadvantage arises from the necessity of using translucent reaction jars. Although many materials are suitable for milling in PMMA, it may degrade upon contact with some standard industrial solvents like acetone, or chloroform, which may be used as liquid additives. Even prolonged exposure to ubiquitous alcohols, like methanol, ethanol, 1-propanol, etc. can lead to a shorter lifespan of the jar and help induce scratches and damage. These put a restrain to the range of solvents and their amounts that can be efficiently used in LAG and ILAG experiments for *in situ* Raman spectroscopy monitoring.

Both methodologies, PXRD and Raman, are applicable for horizontally or vertically operating ball mills demonstrating robustness, and not limiting users to a specific mill manufacturer58,59. Worth mentioning are also recent advances made by introducing X–ray absorption spectroscopy[[66]](#endnote-66) and solid-state NMR[[67]](#endnote-67) for *in situ* reaction monitoring, broadening the possibilities for uninterrupted reaction monitoring of milling reactions.

*In situ* monitoring revealed fast and selective[[68]](#endnote-68) milling reactions that could proceed through multiple reaction pathways59,[[69]](#endnote-69). It was also successfully applied for the elucidation of competitive ball milling reactions[[70]](#endnote-70),[[71]](#endnote-71),[[72]](#endnote-72). The importance of these methods is further demonstrated with direct observation of metastable intermediates, enabling their isolation and characterization by timely stopping the milling in repeated experiments for their targeted isolation for the purposes of characterisation42,[[73]](#endnote-73),[[74]](#endnote-74). The influence of additives on the reactivity of milling reactions could also be studied *in situ[[75]](#endnote-75)*. Liquid additives may selectively favour the formation of only one polymorphic form, or induce a polymorphic transformation[[76]](#endnote-76). Besides steering the reaction towards a particular outcome, liquids can even serve as catalysts in organic reactions[[77]](#endnote-77). Next to liquids, substoichiometric amounts of salts were also observed to drastically increasing the rate of formation of porous materials starting from metal oxides46. However, when it comes to the understanding of why and how additives affect chemical reactivity, little is still known. The reasons are likely both kinetic and thermodynamic since the nature of interactions76,[[78]](#endnote-78), as well as solids particle sizes36, plays an important role. Thermodynamically, interactions between additives and solid reactants, which differ depending on an additive, change the energy landscape (the potential energy surface), possibly resulting in selective stabilization or destabilization of a particular solid form. On the other hand, kinetically, additives may aid in nucleation or have a catalytic effect, evident by the change of reaction rates and reaction pathways. Next to additives, essential parameters influencing milling reactions include also the milling frequency[[79]](#endnote-79),[[80]](#endnote-80), as well as size, mass, number, and type of balls used[[81]](#endnote-81),[[82]](#endnote-82),[[83]](#endnote-83). Up to now, *in situ* monitoring encompassed and was successfully applied to reactions of all major classes and types of compounds, from porous[[84]](#endnote-84), coordination[[85]](#endnote-85), supramolecular[[86]](#endnote-86), organometallic[[87]](#endnote-87), organic[[88]](#endnote-88), and purely inorganic[[89]](#endnote-89) materials.

Table 1. Advantages and disadvantages of Raman spectroscopy for *in situ* monitoring of milling reactions.

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| --- | --- |
| **Advantages** | **Disadvantages** |
| **Information on chemical composition and changes in functional groups** | **Photoluminiscence** |
| **Contactless-method** | **Requires transparent jars** |
| **Qualitative and quantitative information** | **Sensitivity to mill geometry can lead to reproducibility issues when using different mills** |
| **Sensitive to isotopes (H/D exchange etc.)** | **Some thermo-labile materials can be easily destructed** |
| **Affordable** |  |
| **Laboratory method** |  |
| **Fast and high time-resolution** |  |
| **Possible estimation of reaction mixture temperature** |  |
|  |  |
|  |  |
|  |  |

**Scope of milling reactions and monitoring studies**

In this Protocol, we demonstrate real-time *in situ* Raman spectroscopy monitoring of milling reactions on solid-state cocrystal formation between nicotinamide (na) and salicylic acid (sal) (Scheme 1). The product of milling of the stoichiometric 1:1 mixture of solid na and sal is the 1:1 cocrystal (na:sal) (Extended Figure xx – PXRD and Raman) first reported by Berry *et al*[[90]](#endnote-90). Both na and sal are active pharmaceutical ingredients (APIs) and can be found on the World Health Organization's list of essential medicines. While na is a form of vitamin B3 and is used in vitamin B3 complex medications, sal is a constituent of the over-the-counter antacid drug Pepto-Bismol[[91]](#endnote-91) and an active form of acetylsalicylic acid. Cocrystal drugs are expected to increasingly emerge on the market due to them offering an elegant way to modify and improve physical and chemical properties[[92]](#endnote-92),[[93]](#endnote-93) of APIs in formulations, as evidenced by the recent commercialization of the sacubitril-valsartan cocrystal drug used for the treatment of chronic heart failure[[94]](#endnote-94).

Cocrystal formation between na and sal is monitored by following changes of Raman band intensities caused by disruption and formation of new hydrogen bonding patterns. Intermolecular interactions stabilizing na and sal, as well as in the nascent na:sal cocrystal are dominated by strong hydrogen bonds. Upon the cocrystal formation, rearrangement of hydrogen bonding patterns bonds will result in the appearance and disappearance of certain bands in the Raman spectra of the reaction mixture. Raman spectroscopy is most sensitive when the molecular structures are changing and may be less sensitive when the chemical reaction is the result of more subtle rearrangements in supramolecular interactions. For example, in the case of polymorphic transformations, the polymorphs may readily exhibit a striking resemblance in their Raman spectra. In such cases, distinguishing between phases becomes challenging and may limit the use of Raman spectroscopy in monitoring the reaction course. However, some Raman spectrometers and probes include the spectral range below 200 cm−1 that corresponds to phononic transitions, i.e., lattice vibrations of the whole crystal. Raman bands in this region (30–200 cm−1) are greatly influenced by a change in the crystal structure and even if the remainder of the Raman spectra of the two polymorphs is similar, the phononic part will most likely exhibit enough differences[[95]](#endnote-95), to enable tracking the reaction course and extracting the reaction profile.

In our case, depletion of sal can be followed by changes in the intensity of the Raman bands at 778, 1324, and 1636 cm−1, while na has a distinguished band at 1042 cm−1 (Figure 1a). On the other hand, the formation of na:sal cocrystal is evident by the increase of intensity of the band at 795 cm−1 (Figure 1a). As mentioned, the phonon part of Raman spectra changes upon the cocrystal formation because the crystal structure of the na:sal cocrystal is significantly different from the crystal structures of pure na and sal (Figure 1b) It is thus evident that the reaction of cocrystal formation occurs directly from reactants and without the formation of an intermediate phase. From our *in situ* experience, many milling multicomponent reactions do result in the formation of intermediate phases. Sometimes, these intermediates can be isolated as pure and characterised, but this is not possible when they appear as transient short-lived phases in the mixture with reactants or products. In such cases, if intermediates do not have distinct Raman bands, they may even be unnoticed. Combining Raman spectroscopy with another complementary technique can then be essential in detecting the intermediate phase.

The most simple reaction profile is obtained by plotting intensities of selected peaks at their maximum versus time (Figure 2). While the intensity of the Raman peak is proportional to the amount of the chemical species, it should be noted that the exact quantification of the solid reaction mixture can, in this case, only be done through a careful calibration. Nevertheless, a quantitative assessment of the reaction profile is possible by performing a least-squares procedure (LS). The drawback here is that LS requires Raman spectra of all reaction components, which should be collected under the same conditions as the *in situ* reaction monitoring has been conducted. The representative Raman spectrum of each solid should be a spectrum obtained by averaging Raman spectra acquired during milling of each pure solid, typically for several minutes. LS procedure thus implies some *a priori* knowledge of the system under study. If we are unable to collect the representative Raman spectrum of all reaction components, as may likely be the case with transient intermediates, then some of the multivariate data analysis tools may be employed, such as multivariate curve resolution (MCR)[[96]](#endnote-96), to estimate the Raman spectrum of the intermediate phases59,87,[[97]](#endnote-97). However, in order for MCR to yield a meaningful estimation, the spectrum of the intermediate should be uncorrelated with spectra of other components, which is also the limitation of MCR since the spectrum of the intermediate will almost always, to a certain degree, be correlated with the spectra of reactants and products.

While we showcase here a simple neat grinding cocrystallization as an example, the scope of *in situ* Raman reaction monitoring of milling reactions extends beyond supramolecular reactions13,14, and encompass a whole range, from organic7,8, inorganic9,10, and organometallic11,12 reactions, to the synthesis of nanoparticles, metal–organic frameworks15, porous materials[[98]](#endnote-98), perovskites[[99]](#endnote-99), and even enzyme catalysis16,17,[[100]](#endnote-100). Since the Raman spectroscopy is a technique related to molecular vibrations, it is particularly convenient for H/D exchange and kinetic isotope effect studies60, and the use of reactants enriched with other specific isotopes of, e.g., carbon or oxygen[[101]](#endnote-101). For many of these, in situ Raman spectroscopy could be utilized to reveal novel milling phenomena and improve our understanding of solid-state chemical reactivity.

**General considerations on Raman system setup for reaction monitoring and on performing milling reactions**

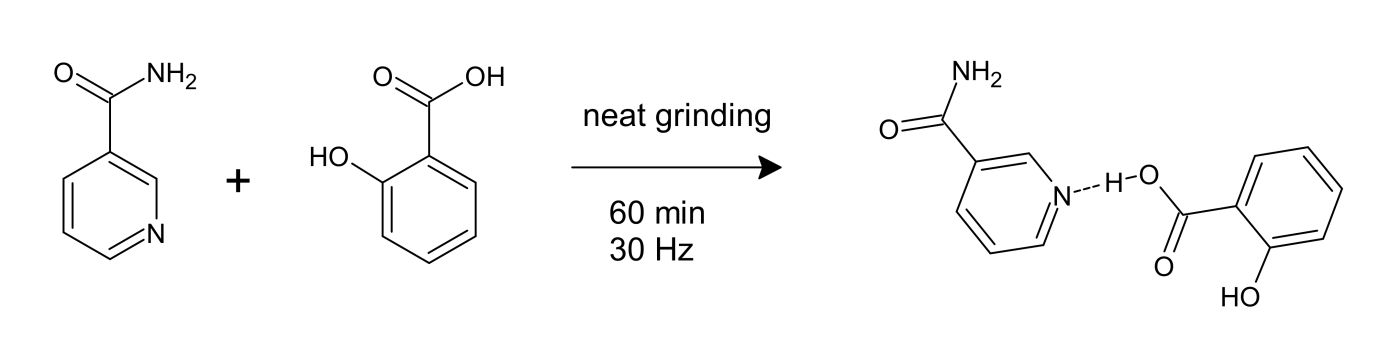
Here, we will highlight the general features of the Raman system for mechanochemical milling reaction monitoring. Details of our Raman system setup are provided under the Equipment setup section. Currently, many manufacturers offer affordable and portable Raman systems that come in various models, price ranges, and for specialized uses. In a nutshell, a Raman system involves a diode laser source, a spectrometer, and a contactless Raman probe connected to a computer. The choice of the detector in the spectrometer will depend on the characteristics of the laser source, notably, the laser excitation wavelength. Nowadays, manufacturers offer coupled pre-defined spectrometers and laser sources. For Raman spectroscopy, the most often used is the red laser with the excitation wavelength of 785 nm, but 532 or 638 nm are not uncommon. However, higher energy laser is more likely to induce sample fluorescence, which creates problems with the detection of weaker Raman signals. A possible solution is to consider a lower energy laser source with the excitation wavelength in the infrared. The spectrometer is usually preconfigured depending on the laser excitation wavelength to maximize the signal over a specific spectral range. Spectrometers often have chilled CCD detector, enabling higher sensitivity and reduces noise, even for long integration times. Many manufacturers offer custom configured spectrometers, and additional care should be taken when considering parameters like the width of the entrance slit, which influences resolution. From our experience, a 785 nm laser excitation source coupled with the spectrometer having a spectral range of at least 50–3300 cm−1, a 50 μm entrance slit, and an optical resolution of 2–4 cm−1 works well for various chemical systems. However, we emphasize again the possibility to custom configure the spectrometer for the user’s specific needs.

Preferably, the Raman system should be physically detached from the mill since the mill can produce a significant amount of vibrations while operating. For example, the Raman system should be assembled on a separate table (Figure xx). If the milling arm oscillates in the horizontal plane, the main goal here is to be able to position the laser beam to enter the jar from below perpendicular to the horizontal plane. In our experience, most of the milled material resides at the bottom of the jar enabling the best Raman signal from the sample. The probe distance from the jar is determined with the probe working distance, and the probe should be placed such that it does not interfere with the regular mill operation. The choice of the probe is voluntary, and some probe has a focused laser beam while others are focus-free and cover a larger area laser spot size. We use B&W-Tek BAC102 probe with a focal point in a working distance of 5.5 cm and 85 μm laser spot diameter at the focal point. Notably, the probe should be mounted on a stand, ideally, equipped with a micro-screw (or precise laboratory scissor jack for upwards movement), that will allow for precise tuning of the probe’s distance from the jar (Figure xx). Precise tuning of the probe distance is necessary to obtain the best signal from the sample (for details see Protocol section).

It must be noted that the collected Raman signal contains contributions of the milled sample and the reaction jar (PMMA). Sometimes PMMA will dominate the Raman spectra covering and overlapping with the signal from the reactants and products. In the most simple implementation, only the region of the spectra without any contribution from the PMMA, is used for reaction monitoring. Such an application is ex limiting, especially when considering that the Raman spectrum of PMMA is rich in bands over the entire region up to 1750 cm−1 (Figure xx). Thus, to get the maximum out of Raman spectroscopy monitoring, it is essential to subtract the PMMA contribution from the Raman spectra. Fortunately, PMMA subtraction is readily achieved if we can identify a single Raman band belonging only to the PMMA that is not overlapping with the signal from reactants and products. We can then use regression of Raman bands in the monitored spectrum and the PMMA reference spectrum to obtain the scale factor with which we multiply the reference spectrum and subtract it from *in situ* collected spectra, leaving only the signals from reactants and products. In the Procedure section, this step is explained in details and implemented in the provided example code.

Since mechanochemical reactions may be sensitive to temperature, ideally, the ball mill and the Raman monitoring setup should be positioned in an air-conditioned room. This is of particular interest when conducting a kinetics study. The mill heats up during normal operation and the collisions of the milling media also lead to an increase in temperature of the milling assembly[[102]](#endnote-102). From our experience, it can make a significant difference between reaction rates when comparing reactions started from a cool or a warmed-up mill, which was, for example, just in operation. Cooling of the reaction jar is determined by the air flow around the jar which may be influenced by the cooling fan of the mill itself. Some mill manufacturers orient their ventilators to blow the hot air from the inside of the mill over the reaction jar. This ventilator may be turned on at any time during mill operation effectively blowing hot air over the jar and speeding up the reaction in the jar. Obviously, this may be detrimental to kinetic measurements. These details are difficult to spot, are easily overlooked and are therefore rarely reported, but are highly relevant in monitoring experiments, especially when replicating reaction monitoring for the purpose of kinetic studies. One way to minimize the mentioned effects is by reproducing the experiment under as similar conditions as possible. Consistency is crucial in LAG, ILAG, POLAG, or when using other additives. For example, if an additive is added together with multiple reactants, then it should always be added in the same half of the jar with the milling balls, or with one (and always the same) reactant. Special care should be taken when adding a liquid additive since liquids with high vapour pressure can evaporate fast. Liquids are best added last to the reaction jar, and two halves of the jar should be closed fast and with care. Detailed instruction on how performing mechanochemical reactions emphasizing the liquid additives can be found in ref. [[103]](#endnote-103).

The general scheme of the protocol for *in situ* Raman spectroscopy monitoring of milling reactions is given in Figure xx.



Scheme 1. The selected reaction of mechanochemical cocrystals formation between nicotinamide and salicylic acid.



Figure 1. 2D time-resolved Raman spectra of neat grinding cocrystals formation between nicotinamide and salicylic acid. (**a**) Part of the spectra in the range 230 – 1840 cm−1, and (**b**) phonon part of the spectra ranging 50 – 230 cm−1. The onset of the reaction is after two minutes into milling, and gradual transformation to the cocrystal form lasts for about 35 minutes.



Figure 2. Reaction profile obtained by plotting intensities at the peak maximum versus time for neat grinding of na and sal.



Figure 3. Experimental setup. (**a**) The Raman modular system consists of a Raman probe, spectrometer (black), and a laser source (blue). (**b**) The reaction jar is mounted on the mill hand and the probe is positioned beneath the jar. (**c**) Probe position from the side. (**d**) Probe head position from above. (**e**) PMMA jars and some typical milling balls made from stainless steel and zirconium(IV) oxide.

**MATERIALS**

REAGENTS

**!Caution**

Use personal protective equipment while handling and weighing solids. Avoid dust formation and inhalation as well as breathing vapour, mist or gas of solids.

* Pyridine-3-carboxamide is also known as nicotinamide (CAS no. 98–92–0, Alfa Aesar purity: 99%). **!Caution** Nicotinamide causes severe eye irritation.
* 2-Hydroxybenzoic acid also known as salicylic acid (CAS no. 69–72–7, Kemika purity: 99%). **!Caution** Salicylic acid causes mild eye and skin irritation, and it is a potential teratogen.
* Ethanol (CAS no. 64–17–5, Gram-mol purity: 96%). **!Caution** Ethanol is highly flammable and toxic. Keep away from heat, sparks, open flames, and hot surfaces. Avoid breathing dust, fume, gas, mist, or vapours of ethanol. Work in properly ventilated areas.
* Benzoic acid (CAS no.65–85–0, Alfa Aesar purity: 99%) **!Caution** Benzoic acid causes skin irritation and severe eye damage.

EQUIPMENT

* InSolido IST 636 mill (radial oscillation in the horizontal plane with the fixed amplitude of 17.5 mm, frequency range 3–36 Hz, https://www.insolidotech.com/ist636.html )
* In-house made PMMA milling jars with the internal volume of 14 mL (Figure 3e and SI Figure xx)
* OceanOptics (now OceanInsight) HighRes Maya2000Pro Spectrometer with spectral resolution of 1 cm−1 and slit of 25 mm (<https://www.oceaninsight.com/products/spectrometers/high-sensitivity/custom-configured-maya2000-pro--series/>, Figure xx )
* PD-LD (now Necsel) BlueBox laser source class IV, 785 nm excitation wavelength, power range 10–500 mW (<https://www.ushio.com/files/specifications/necsel-blue-compact-luxxmaster-raman-boxx.pdf>, Figure xx)
* B&W-Tek fiber–optic Raman BAC102 probe (https://bwtek.com/products/bac102/)
* In-house made micro screw stand for precise positioning of the Raman probe (Figure xx)
* In-house made Raman probe stabilizer (Figure xx)
* Manual laboratory scissor jack stand (Figure xx)
* Silicon wafer (Figure xx)
* Stainless steel balls (type 440C), diameter 7mm (Figure xx)
* PC-compatible computer
* Appropriate software for data acquisition (OceanView 2.0, RamanBoxx) and analysis (e.g. MATLAB, GNU Octave, Anaconda). OceanView is commercial software and can be downloaded for a free-trial from OceanInsight web page (<https://www.oceaninsight.com/support/software-downloads/>). RamanBoxx is a PD-LD (now Necsel) software for operating the laser source, and it is a part of the instrument. MATLAB is commercial software from MathWorks (<https://www.mathworks.com/products.html?s_tid=gn_ps>). GNU Octave is free software in many aspects compatible with MATLAB, available at <https://www.gnu.org/software/octave/download.html>. Anaconda is a data science platform which incorporates languages like Python and R for data analysis and visualization. The individual edition is available at no cost from <https://www.anaconda.com/products/individual>.

EQUIPMENT SETUP

**Raman setup** Here we present the details of Raman setup in our laboratory. General aspects of the instrumentation are given in the introduction part of the Protocol. While we will focus here on the experimental setup we have the most experience with, we have successfully implemented also parts from other producers, such as the 785 nm Raman laser source from the OceanOptics or OceanOptics Raman probe. We are sure that the same protocol will be applicable for setups entirely made from other producers if the the energy of the laser and the focal distance are suitable for data collection.

Before connecting the B&W–Tek probe and Ocean Insight spectrometer to the PC, make sure to install all necessary drivers following the manufacturer’s instructions. Connect the laser source and spectrometer with the PC via a USB (Universal Serial Bus) cable. Connect the laser source to the power outlet (laser is now turned on, but its optical shutter is closed). CAUTION! Every time the laser source is turned to the power outlet allow it to stabilize for at least one hour. In this way, fluctuations in the laser power and excitation wavelength are minimized. Raman probe has a bifurcated fiber-optic cable, with one end connected to the excitation source (laser), and the other end to the spectrometer (Figure 3a). Mount the probe to the stabilizer on top of the micro screw stand which is securely fixed to the scissor jack (from now on, stand) (Figure xx). Mount the empty reaction jar on the mill arm to roughly position the probe beneath the jar (Figure xx). To adjust the probe position open the laser optical shutter and visually inspect where the laser beam hits the jar (it is not important to set the distance from the jar accurately at this stage), and adjust the probe, together with the stand so that the laser beam is hitting the middle of the jar. **CAUTION!** A laser beam can cause eye damage. Use safety goggles and never look directly at the laser beam.

Adjustment and control of laser and spectrometer parameters can be achieved in three ways: i) by using default software from manufacturers, ii) through commercial software like MATLAB, and iii) by using free, open-source project *seabreeze* written in Python for OceanOptics spectrometers (<https://github.com/ap--/python-seabreeze>). MATLAB or Python are programming environments where simultaneous control over the laser source and spectrometer is achieved by using programming commands. Moreover, it is possible to code in pre- and post-processing of data, which may take place in real-time during milling experiments. While such an approach provides a lot of advantages and customization, it is also non-trivial and requires some programming skills since a user must write the whole program and adjust it to its needs. If using MATLAB, an additional toolbox is required (Instrument Control Toolbox). At the same time, OceanInsight provides drivers and test examples of how to set up communication with the spectrometer in the MATLAB environment (<https://www.mathworks.com/hardware-support/ocean-optics-spectrometers.html>). Alternatively, readily available software from instrument manufacturers allows straightforward control over the laser source and spectrometer, but may impose limits for customization and real–time analysis to already pre-coded routines in the provided software. Here, we demonstrate the use RamanBoxx Control Panel v1.3 software for laser source control (Figure 4), and for controlling and acquiring spectra on the spectrometer, OceanInsights’ software OceanView v2.0 (Figure 5). First, calibrate the wavenumber axis with the standard (silicon wafer, Figure xx). Then, adjust the Raman probe distance by gradually changing the distance until the Raman signal of the benzoic acid (our arbitrary standard) is of the highest intensity on average (Figure xx). With this at hand, proceed to collect the Raman spectra *in situ*.

Mill protection cover is made from plastic, and it is transparent. Therefore, it should be covered with a dark fabric during *in situ* monitoring to reduce the possibility of non-scattered photons reaching the detector (Figure 6b). General features of our setup can be transferred to other mills, especially those oscillating the jar in the horizontal plane.

**Data analysis and visualization** Perform analysis of acquired spectra in any software for data analysis, like Anaconda, which incorporates a graphical interface for Python and R. Similar solutions are MATLAB or Octave, which we use to demonstrate data analysis in this Protocol. A result of monitoring a milling reaction is a set of Raman spectra, which OceanView outputs as separate textual ASCII files. These files can then be imported into Octave and plotted as a two-dimensional figure of time-resolved spectra (Figures 1) from which a simple reaction profile is derived (Figure 2). Imported Raman spectra in Octave are assembled in a spectral matrix where individual Raman spectra are represented as column vectors. Dimensions of spectral matrix are *n*×*p* where *n* is number of spectral points (wavenumbers) and *p* is total number of spectra. Collected *in situ* Raman spectra contain the contribution from PMMA, reactants and products. First step is to scale and subtract the PMMA Raman spectrum from *in situ* spectra. Afterwards, the baseline is subtracted from the spectra using simple asymmetric least squares algorithm. Finally, to alleviate for the effects of non-uniform amount of material in the laser beam during the spectrum acquisition, corrected spectra are normalized by dividing each spectral point with the *l*1 norm. At this point, the simple reaction profile is obtained by plotting the intensities of the selected Raman band maxima versus time. All steps of data analysis are explained in details in Procedure section, and we provide the example Octave script with which user can easily perform all the above tasks on the data presented in this Protocol.

**PROCEDURE**

**Stage I: Connecting the laser source and a spectrometer to PC**

**▲CRITICAL** The main foal of this stage is to establish a communication between the laser source, spectrometer, and PC.

1| Open previously installed Raman Boxx Control Panel v1.3 (Figure 4)

2| Set *COM PORT* to an appropriate port number and click *Set* button. To find the correct port number go to *Device Manager* in Windows, click *Ports (COM & LPT)* icon, and check which port number is assigned to *Silicon Labs CP210x USB to UART Bridge*.

**? TROUBLESHOOTING**

3| In RamanBoxx Control Panel, click *Start* button to connect to the laser (Figure 4). Communication between PC and the laser is now established.

4| Open OceanView v2.0.6 (Figure 5).

5| On the welcome screen, click *Spectroscopy Application Wizard*.

6| A new window pops up. Here, click on *Raman*.

7| Check *Active Acquisition* box and click *Next*.

8| A new window pops up. Click *Next*.

9| Click *Store Background* icon in the upper left corner and set the *Wavelength* to 785 nm. Click *Apply* (Figure xx)*.* Background spectrum corresponds to the spectrum when the laser beam is off. It represents all the non–Raman scattered photons that falls on the detector, as well as the noise without the presence of the laser light. It is stored and automatically subtracted from every subsequently collected spectrum.

**? TROUBLESHOOTING**

10| Click *Finish*. We have now established the communication between the spectrometer and OceanView, as well as defined Raman spectroscopy mode and the excitation wavelength of the laser.

■ **PAUSE POINT**

**Stage II: Preparation for monitoring – Calibration**

**▲CRITICAL** The main goal of this stage isthecalibration of the wavenumber axis. Due to the laser power source fluctuations or manufacturing we could experience a shift in the wavenumber axis because the excitation wavelength is not exactly 785 nm. For this reason, we use some reference with well-defined Raman bands whose maxima are exactly known. Here, we will use silicon, which has a Raman band with maxima at 520.5 cm−1.

11| First calibrate the wavenumber axis (x–axis) using the silicon wafer as a reference. Put the black cap with the small hole in the middle on the Raman probe head and a silicon wafer on top of it (Figure 6a).

12| In OceanView, under *Acquisition Group Window* set *Scans to Average* to 10, and *Integration Time* to 500 ms (Figure xx).

**! CAUTION** These two parameters define the time needed to acquire one Raman spectrum, thus also define the time–resolution in monitoring experiments. Time to acquire one Raman spectrum is equal to a product of *Scans to Average* and *Integration Time* (in ms). In specific case here, it will be 5000 ms, or 5 s. Every 5 s, we will acquire one Raman spectrum, and if we would keep this settings for *in situ* monitoring, then 5 s would be monitoring experiment's time resolution. Also, since *Integration Time* directly reffers to the time the spectrometer is collecting one spectrum, longer times will result in more collected photons, thus increasing the intensity of the signal and intensity of the whole spectrum. For materials that exhibit weak Raman signals, a longer *Integration Time* could be needed. Take note that too long *Integration Time* could completely saturate the detector. Increasing *Scans to Average* will result in better signal–to–noise ratio. It should born in mind that increasing both parameters also changes the time–resolution in monitoring experiments so, ideally, both parameters should be as lower as possible to minimize the time for collection of consecutive Raman spectra during monitoring. Values of these parameters are pertained to each reaction and only way of optimization is by trial–and–error through several exploratory experiments. From our experience, values for *Integration Time* can vary from 100 ms to 1500 ms, and *Scans to Average* usually from 1 to 60. On average, we usually try to achieve the time resolution in range of 5 to 15 seconds, and we use values from 500 to 1250 ms for *Integration Time* while adjusting value for *Scans to Average* accordingly.

13| After a new spectrum appears on the screen, press the *Update background data for this acquisition* icon (small bulb icon) on the toolbar (Figure xx).

**▲CRITICAL** **STEP** Background spectrum is the spectrum that contains all signal that the spectrometer detector collects when the laser’s optical shutter is closed and no laser beam hits the jar. It is internally and automatically subtracted from every collected spectrum when the laser’s optical shutter is open and laser hits the jar. This result in Raman spectrum containing only signals from PMMA, reactants, products and signal noise. Background spectrum is also known as dark spectrum (in absence of excitation light). Parameters which influence background spectrum are *Scans to Average* and *Integration Time*. Thus, it is of paramount importance to collect a new background spectrum every time after changing values of *Scans to Average* and *Integration Time* parameters.

14| In RamanBoxx Control Panel under *Power Setting*, set the laser power to 350 mW. Press *Set* and wait until the window pops up with the message *Power setting OPM set*. To open the laser’s optical shutter, check the *On* in *Laser* box (Figure xx).

**! CAUTION** The laser source is a class IV laser. Take all necessary safety precautions, wear appropriate safety goggles and never attempt looking directly into the laser beam. Make sure to let the laser power stabilize. This usually takes only couple of seconds, however, if the laser is just plugged in the power outlet and turned on, then make sure to leave it for longer periods. Power range for the laser is 10–500 mW. Since the probe is focusing the beam, some thermally labile materials could burn. Intensity of Raman–scattered photons is proportional to the excitation light intensity so to achieve the best signal, laser power should be as high as possible. If there is no fluorescence the power could be set close to maximum. If, however, milled material exhibit strong fluorescence, lower the power of the laser and adjust also *Scans to Average* and *Integration Time* parameters, for example, lower *Integration Time* but increase *Scans to Average*.

15| Spectrum is instantly plotted on the screen after the acquisition since the spectrometer is automatically and continuously acquiring spectra (we set this in step 7). To manually set the x–axis and y–axis range, press *Manually set numeric ranges* icon on the secondary toolbar (Figure xx). A new window will pop up. Here, set *X Minimum* to 510 and *X Maximum* to 530. Read the values for intensities of the peak and adjust the Y–axis range accordingly. Then press *Apply* and close the window. Now, only a selected range of the Raman spectrum will be plotted on the screen. Silicon is used as a reference in the calibration of Raman instruments and has a well–defined Raman band with maxima at 520.5 cm−1 (Figure xx).

**? TROUBLESHOOTING**

16| By double–clicking on the plotted screen, a line will appear on the screen together with the X– and Y–axis values in the bottom (Figure xx). Use this line to read the wavenumber value at the maxima of Raman band. If this value is offset from 520.5 cm−1, we need to adjust the X–axis by slightly changing the excitation wavelength. Navigate to *Window* in upper left corner and open *Schematic*. Here, double–click on the *RamanShift* and change the value of *Laser wavelength* slightly. There is some probability that laser excitation wavelength will be slightly offset from exactly 785 nm. From our experience, it is usually in the range 784.7 – 785.3 nm. During calibration, change wavelength manually in increments of 0.1 nm. Click *Apply* and then click on *View* tab next to the *Schematic Window* tab to view the Raman spectrum of the silicon wafer (Figure xx). Changing the excitation wavelength changes the Raman shift of the spectrum and appears as the Raman band shifted. Continue changing the *Laser wavelength* until the maximum of the silicon peak falls at approximately 520.5 cm−1. Setting the laser wavelength to have this silicon band precisely at 520.5 cm−1is usually not possible and having it in the range 520–521 cm−1 is sufficient, considering that is within the resolution of the spectrometer.

**? TROUBLESHOOTING**

**▲CRITICAL** **STEP** Offset in laser wavelength is related to the laser source, but once calibrated it can be stable for days. However, it is recommended to perform calibration every day, and it is mandatory every time the laser source is unplugged from the power outlet.

17| Close the laser’s optical shutter by ticking *Off* in the *Laser* box in Raman Boxx Control Panel. We have now calibrated the wavenumber axis.

■ **PAUSE POINT**

**Stage III: Preparation for monitoring – Raman probe distance adjustment**

**▲CRITICAL** Main goal of this stage is to adjust the Raman probe distance from the jar. Insolido IST636 mill is preconfigured for *in situ* monitoring. For Raman monitoring, the most important modification is the ability of a Raman probe to approach the milling jar from the bottom (see Figure 3). The probe should be placed about 1 cm beneath the bottom of the jar since a laser beam focal point is at this distance from the probe head. The aim is to place the focal point on the inner wall of the reaction jar where most of the material falls during milling. Finding the optimal probe distance from the jar affects the quality of acquired Raman spectra, and it is recommended to adjust it from time to time since mill or the probe stand can slightly move due to the vibrations caused by milling. Emphasis here is that signal–to–noise ratio for acquired spectra will depend on how well the probe distance is adjusted, rather then on the spectrometer manufacturer’s value which represents the upper limit, or, to put it in another words, signal–to–noise ratio will depend on how much material on which the laser photons can scatter, will effectivelly be in the beam during a spectrum acquisition. From our experience, this is the most important step of the protocol that can make significant difference on quality of acquired data. To find the optimal distance, use a reference milling material, which exhibits low fluorescence. In our laboratory, we preferably use benzoic acid (ba). Adjust the distance in small steps while milling ba and observe how it influence the Raman spectrum. The optimal distance will result with the highest intensity signal for ba on average.

18| Mount the empty jar on the mill arm (Figure 3d).

**▲CRITICAL STEP** Mounting the jar should be done with extra care because it ensures that jar does not fall out during the milling which can damage the probe or the mill. The jar locking mechanism varies from mill to mill, and it is assumed a user is familiar with this basic mill operation. On IST 636, a jar is mounted between two clamps, one of which is movable by rotating the side screw (Figure xx). We put the jar in grooves on sides of the clamps, and by rotating the screw we move one clamp towards the other, tightly locking the jar. To ensure the rotating screw will not unclock under vibrations caused by milling, we tighten the secondary screw in counter clockwise direction (Figure xx).

19| In OceanView, set *Scans to Average* to 15 and *Integration Time* to 1000 ms (Figure 7).

20| Click *Update background data for this acquisition* (Figure xx).

**? TROUBLESHOOTING**

21| In Raman Boxx Control Panel under *Power Setting* set the laser power to 450 mW and click *Set*.

22| Open the laser’s optical shutter by checking the *On* in *Laser* box.

22| After the Raman spectrum of the jar appears on the screen, click on *Convert active spectrum to overly* icon on the secondary toolbar. This will display the acquired Raman spectrum on the screen permanently.

23| In Raman Boxx Control Panel close the laser’s optical shutter by checking the *Off* in *Laser* box, and remove the jar.

**! CAUTION** To remove the jar follow the same steps for mounting the jar in reverse order. First, unscrew the secondary screw. Then rotate the main screw in counter direction and distance the clamps enough to be able to gently remove the jar from the mill arm.

24| Weigh 244.2 mg (2 mmol) of benzoic acid (ba) in one half of the empty reaction jar, and put two stainless steel milling balls with 7 mm diameter (1.4 g is the mass of each ball) in another half of the jar.

**! CAUTION** Sometimesthe jar, or spatula can become electrically charged. A good practice is to discharge the static electricity from the jar or spatula before weighing.

25| Gently snap the two halves of the jar together to close the jar (Figure xx).

26| Mount the jar on the mill arm (Figure xx).

**! CAUTION** Before start of milling, make sure to mount an empty jar on the second mill arm as a counter weight. Double check that security screws are fasten to ensure jar will not fall out during milling.

27| Set milling frequency to 30 Hz, and time to 0. On an IST636 mill this sets milling time to unlimited.

**▲CRITICAL** Frequency is the important parameter in milling reactions since it directly relates to the number of impacts, velocity of the milling ball(s), and the impact energy, leading to the different rate of heating of the jar due to the friction. Horizontally operating mills usually operate in range 20–36 Hz.

28| Start milling by pressing the Start button on the mill (Figure xx).

29| In OceanView, click *Update background data for this acquisition* and collect another background spectrum.

**? TROUBLESHOOTING**

31| In Raman Boxx Control Panel open the laser’s optical shutter by checking the *On* in *Laser* box.

32| Start adjusting the Raman probe distance from the jar by rotating the micro screw (Figure 3b). Wait for the new spectrum to appear on the screen after each small screw rotation. To differentiate between PMMA signal and ba you can compare the Raman spectrum of the empty jar (which is permanently on the screen, step 22) to the currently acquired spectrum.

**▲CRITICAL** **STEP** Since the probe has a laser focal point the intensity of the Raman signal will vary mostly due to the position of focal point inside the jar. A good strategy to find an optimal distance is to first place the focal point in the wall of the jar by increasing the distance of the probe from the jar. There, the Raman signal from ba will be minimal or non–existant, and the PMMA signal will dominate the spectrum. Then, start reducing the distance from probe to the jar in increments. As the distance between the probe and jar is reduced, the focal point will move towards the inner jar wall, and the intensity of the Raman signal of ba will increase. With the focal point going further into the jar interior, the Raman signal of ba will again start to diminish. Once this min-max signal range is identified, the optimal distance is attained by going back and forth until, on the average, the highest intensity Raman signal of ba is achieved.

33| In Raman Boxx Control Panel close the laser’s optical shutter by checking the *Off* in *Laser* box.

34| Stop the mill.

35| Remove the jar with ba. You can store it and use it for future probe distance adjustments.

■ **PAUSE POINT**

**Stage IV: Sample preparation and monitoring of the experiment**

**▲CRITICAL** In a neat grinding reaction, reactants should be weighed in separate halves of the jar and should not be ground before weighing or before the onset of milling to avoid the potential start of reaction before milling is initiated. On the other hand, when performing LAG or ILAG reactions, ideally, reactants should be weighed together in one half of the reaction jar, and the liquid added to the other. However, if reactants can react when brought into contact without mechanical activation, then they should be weighed in separate halves. In such a case, always add liquid and milling balls to one half, preferably with the same reactant in every experiment. Use of liquids with high vapour pressure may result in their partial evaporation during sample preparation. To reduce this, add the liquid quickly and just before closing the jar. Additionally, use of PMMA jars can limit the type of a liquid used in LAG or ILAG reactions, since some organic solvents like acetone, chloroform, *etc*., may react with the PMMA or damage it. Use of a jar made from different material, like sapphire can mitigate this problem.

36| Weigh 122.1 mg (1 mmol) of nicotinamide and 138.1 mg (1 mmol) of salicylic acid, both in the same half of the reaction jar and add two stainless steel milling balls (Figure xx).

37| Snap the two halves together carefully to avoid contact of reactants with milling balls (Figure xx).

**▲CRITICAL** **STEP** In a case of LAG, ILAG, or POLAG reaction, when snapping the two halves together, it is important to ensure the liquid stays in one side of the jar with the balls and the reaction mixture on the other side. This way, we can avoid onset of reaction before milling is initiated. In presence of a liquid, take your time to carefully mount the jar on the mill arm.

38| Carefully mount the jar to the mill arm (Figure xx).

**▲CRITICAL** **STEP** Ensure the reactants stay on the one half of the jar, while balls on the other half to prevent mixing and possible onset of the reaction before the start of milling.

39| In OceanView, set *Scans to Average* to 15 and *Integration Time* to 1000 ms (Figure xx).

40| Click *Update background data for this acquisition* icon and collect the new background spectrum (Figure xx).

**? TROUBLESHOOTING**

**▲CRITICAL** **STEP** Make sure to update the background spectrum every time you change *Scans to Average* and *Integration Time* parameters, and before each monitoring experiment.

41| Click on the *Configure graph saving* icon on the secondary toolbar (Figure xx). A new window, *Algorithm Parameters Controls*, will pop up.

42| Select the File Format to be ASCII (plain) and browse to the target directory where you want to save the data. Then choose the name for the file and the file suffix. In the Save options tick *Save every scan* and *Stop after this amount of time*, and set time duration of the experiment to 60 minutes (Figure xx).

**▲CRITICAL** When you choose *Save every scan*, OceanView continuously collects and outputs every spectrum as a single two-column ASCII file. However, this prevents plotting the spectra in real–time on the screen. Suppose you want to have a real–time feature, then tick *Stop after this many scans* instead of *Stop after this amount of time*. Now, every time you click on the *Save graph to files* icon the currently collected spectrum will be saved, written to a file, and plotted on the screen.

43| Set the milling frequency to 30 Hz and milling time to 60.5 min (Figure xx).

44| In Raman Boxx Control Panel open the laser’s optical shutter by checking the *On* in *Laser* box..

**▲CRITICAL** **STEP** The most common mistake is to start milling with laser’s optical shutter closed.

45| Start milling by pressing the *Start* button on the mill (Figure xx).

46| Start saving spectra by clicking *Save graph to files* icon on the secondary toolbar. After 60 minutes, OceanView will stop saving spectra and you can access it in a directory.

47| In Raman Boxx Control Panel close the laser’s optical shutter by checking the *Off* in *Laser* box.

48| Replace the reaction jar with an empty jar. Mount it in the same position as the reaction jar (Figure xx).

49| In OceanView, set *Scans to Average* to 30 (Figure xx) and update the background spectrum (Figure xx).

50| Open *Algorithm Parameters Controls* by clicking on the *Configure graph saving* icon on the main toolbar. Untick *Stop after this amount of time* and tick *Stop after this many scans* thus setting the number of scans to 1. Change *BaseName* to Jar\_spectrum.

51| Click *Apply* and then click *Exit*.

52| In Raman Boxx Control Panel open the laser’s optical shutter by checking the *On* in *Laser* box.

53| Start milling.

54| Save Raman spectrum of the empty jar by clicking *Save graph to files* icon on the toolbar (Figure xx).

55| When the graph is saved close the laser’s optical shutter by checking the *On* in *Laser* box.

56| Stop milling and remove the jar.

■ **PAUSE POINT**

**Stage V: milling jars cleaning**

57| After the end of the experiment, collect the product from the milling jar using a rounded spatula to minimize damage to the PMMA jar.

**! CAUTION** Milling is used also for particle size reduction, and often, resulting with nanoparticle sized product. Collecting the product from the jar should be performed inside fume hood, and you should always wear a personal safety equipment, lab coat, googles, gloves, and face mask, if necessary with appropriate filter.

58| Wash the jar with soap and water, and rinse with ethanol. If necessary, clean PMMA jars by soaking them in a solution of 20 % (vol/vol) of hydrochloric acid in ethanol. After soaking, rinse the jar with deionized water and ethanol and leave to dry on a bench or in an oven at 60 °C. The acid-ethanol bath can be reused.

**! CAUTION** Ethanol may induce the crazing and decrease the lifespan of the jars. Be careful if using sonication bath to soak and wash PMMA jars since in some cases PMMA jars can break during sonication. If, after washing the jar has visible cracking spots, or if unable to wash the product completely, dispose the jar and use a new one for monitoring experiment. Jars with cracking and crazing spots are more likely to break during milling under mechanical pressure and damage Raman probe or other equipment.

59| Wash milling balls with soap and water, and scrub if necessary. Rinse with ethanol and leave to dry on a bench, or in an oven at 60 °C.

■ **PAUSE POINT**

**Stage VI: data analysis and visualization**

**▲CRITICAL** Main goal of this stage is demonstrating simple data analysis and visualization of collected data from the monitoring experiment able to perform the user with minimum knowledge in data analysis. Specifically, main steps in analysis and visualization consist of choosing the spectral range of interest, removing the Raman signal contribution from PMMA, correcting the baseline, and normalizing the spectra prior to the visualization (Scheme xx). In most cases, advanced multivariate or correlation analysis are performed on normalized data. The simplest reaction profile is derived by plotting intensities at the maximum of selected Raman bands versus time (Figure xx). To perform all these actions, use an option already described in EQUIPMENT SETUP. Here, we will demonstrate the use of GNU Octave, an open-source software for scientific calculations and visualizations. We use GNU Octave installed on a Windows 10 operating system.

60| Download and install GNU Octave on your computer. Installation files can be downloaded from the webpage: <https://www.gnu.org/software/octave/#install>

61| Open the GNU Octave on your computer (Figure 8).

62| Download the GNU Octave scripts from Supplementary Information and save them locally to your computer.

63| GNU Octave can access the files that are on its internal path. In order to use downloaded scripts, first put them on the GNU Octave path. Add folder with the scripts on GNU Octave path by writing the “addpath” command inside command window and press *Enter*: addpath('replace\with\path\to\the\folder\with\the\scripts').

**! CAUTION** Write the string with the path to the folder with the scripts correctly to avoid syntax error, for example: 'C:\User\Download\Scripts'.

**? TROUBLESHOOTING**

64| For basic analysis and visualization, use the script named *Example\_script*. Download the example data and modify the script step by step. The script is aimed to be general and easily modified, which still requires a basic knowledge of scripting. It can be used as a starting point for data analysis and visualization, even if Raman spectra are acquired using other spectrometers. The only prerequisite is that Raman spectra are outputted as ASCII textual files containing two columns, first with wavenumbers, and second with signal intensities.

65| To open the script in GNU Octave, click on the *Editor* tab, and then on *Open an existing file in editor* icon (Figure xx). Browse to the folder with the script and open it.

66| Within the script, in DEFINE PATH TO WORKING DIRECTORY part, change the value of variable *cwd* with the string of the path to the downloaded example data. Command *cd(cwd)* will switch the default current working directory for the directory containing example data.

**? TROUBLESHOOTING**

67| In part CREATE A MATRIX OF RAMAN SPECTRA we first create the data structure containing names of files from *cwd* directory. We then use *for* loop to import each monitoring spectrum and append it to a matrix named *SpectraAll*.

**! CAUTION** After importingspectra the best practice is to plot them and inspect visually. Sometimes a faulty pixel can appear as a positive or negative spike with varying intensities. These spikes always occur at the same position unless they are caused by some events like background radiation (cosmic rays) which, in that case, causes randomly distributed spikes. Spikes can even be due to the poor subtraction of background spectrum. Function *Cosmic* used in the example script corrects for some of these spikes, whose values are identified by simply plotting the spectra without the values for wavenumbers (for example, write in command line *figure,plot(SpectraAll)* and press enter.) In that case, on x–axis are given only ordinal number of points of a spectrum and it can be easily identified at which point is a spike. Function *Cosmic* takes three parameters as inputs: spectra, and two points corresponding to the spikes. It replaces the value at those points with an average value of four adjacent points, specifically value at *x*i is replaced with (*x*i−1 + *x*i−2 + *x*i+1 + *x*i+2) / 4. This means, that first two, as well as last two points in the spectrum are not valid input points and will lead to an error.

68| In part SUBTRACT THE RAMAN CONTRIBUTION OF THE JAR replace only part of the string named *replace-with-path-to-the-folder-with-the-Raman-data* within parentheses and leave *\Jar* as is. Folder with the data you downloaded contains the folder named Jar with a .txt file of the jar Raman spectrum.

**▲CRITICAL** Our aim here is to compare the Raman spectrum of the empty jar with the monitoring spectrum and use the Raman band that belongs only to PMMA to scale the empty jar spectrum. After scaling, simple subtraction of scaled empty jar spectrum from monitoring spectrum results in Raman spectrum without PMMA contribution.

69| Change parameter value of *first\_point* to 1492 and *last\_point* to 1555.

**▲CRITICAL** **STEP** These valuesrefer to the first and the last point of a base of a Raman band belonging to PMMA at 1745 cm−1 (Figure xx). For subtraction of jar contribution from experimental spectra any band of PMMA can be used if it does not overlap with a Raman signal coming from the reaction mixture. Care should be taken as the reaction mixture is changing over time and a non-overlapping PMMA band may overlap with sample bands later during the experiment. Before determination of scaling factor, both bands are baseline corrected using asymmetric least squares (details are given in step 72). We determine the scaling factor by least–squares procedure from **a**s = **b**, where **a** is column vector of intensities of empty jar Raman band, **b** is column vector of intensities of monitoring spectrum Raman band, and s is scaling factor. In the script, s is named scale, **a** is *jarCorr*, and **b** is *spectrumCorr*. We perform scaling and subtraction inside *for* loop for each monitoring spectrum, and store the resulting spectra in a matrix named *Spectra*.

70| In part CREATE COLUMN VECTOR OF TIMES, change *Scans* to 15, and set *Integration\_time* to 1. These values should correspond to the values for *Scans to Average* and *Integration Time* used for monitoring experiment. We create variable *Time* which is column vector whose values represent a time elapsed from the beginning of monitoring until acquiring each Raman spectrum.

71| In part DEFINE SPECTRAL AND TEMPORAL RANGE OF THE EXPERIMENT, change the values of next parameters: *first\_wnmb* to 90, *last\_wnmb* to 1650, *from\_t0* to 1, and *to\_t* to *size(Spectra, 2)*. With first two variables we consider the spectra in range 90–1650 point, which corresponds to wavenumbers in range 200–1700 cm−1. This range also refers to the first dimension of matrix *Spectra*. Variables *last\_wnmb* and *from\_t0* refers to second dimension of matrix *Spectra* and determine the time component, i.e. we chose from which to which spectrum we will plot the data.

**▲CRITICAL** Identification of parts of the spectra to exclude is easily done by visual inspection of spectra. See step 67.

72| Do not change any parameter value within part BASELINE CORRECTION since they contain optimal values for these parameters for our specific example case. After baseline correction using function ALSbaseline, baseline–corrected spectra are normalized by dividing each spectral point with spectrum l1 norm (known also as Taxicab or Manhattan norm), and stored in matrix named *N1sp*.

**▲CRITICAL** Function *ALSbaseline* uses asymmetric least squares[[104]](#endnote-104) for finding the optimal baseline. Given the vector ***y*** = {*y1*,*y2*,…,*yi*} whose values are spectral intensities at *i*-th wavenumber, we can define a smoothing series ***z*** = {*z1*, *z2*, …,*zi*} that will be as close as possible to ***y***, and it will represent baseline. In literature this is also expressed as ***z***being faithful to ***y***. Then, we can create the penalized least squares function *F* and minimize it:

where *λ* is parameter controlling the balance between smoothness and fitness, *Δ* is a second-order differential operator, *Δ*2*z*i = (*zi* − *zi*−1) − (*zi*−1 − *zi*−2) = *zi* − 2*zi*−1 + *zi*−2, *i* ∊ [1, 2, 3, …, *m*], and ***w*** is vector of weights of fitness. Without ***w***, the equation would correspond to the Whittaker smoother[[105]](#endnote-105). However, differences (*yi* − *zi*) where spectrum has a peak should always be a positive since spectrum has positive–valued peaks, and we need account for that by introducing asymmetry in the weights with another parameter *p*, such that *wi* = *p* if *yi* > *zi*, and *wi* = 1 – *p* otherwise. In the example script, user inputs values for two parameters, namely *Smoothing\_factor* and *asymmetry\_factor*, where former corresponds to *λ* and latter to *p*. Simply put, the value of *Smoothing\_factor* influences the overall smoothness of the baseline, while the *asymmetry\_factor* influences the ability of baseline to go “under” the peak. Typical range for the *Smoothing\_factor* is 500–10000 where lower values favour more flexible and pronounced baseline, while for higher values baseline tends to be a straight line. On the other hand, typical range of value for *asymmetry\_factor* is 0.003– 0.0001, and higher value will tend to tuck the baseline more under the peaks. Trying to find the optimal values is matter of trial–and–error approach, and the best practise is to first find approximate value for *Smoothing\_factor* and then tune the *asymmetry\_factor*. After subtraction it is possible some values will become slightly negative. For this reason we can add an absolute value of the least negative one as a constant to each spectral point to obtain non-negative spectral values.

**! CAUTION** Spikes with positive values will have no effect on baseline correction, but negative ones will drastically affect the baseline estimation. Thus, it is of paramount importance to exclude all negatively valued spikes before baseline correction. Spikes can be excluded using function *Cosmic* as described in step 67.

73| In part VISUALIZATION, if you leave the default values for *x1*, *x2*, *t1*, and *t2*, than you will obtain two figures, one with the phonon part of the Raman spectra, and other with the rest of the spectra. These parameters will have the same effect as already described parameters in step 71. However, in step 71 we define the spectral and temporal range of spectra on which further analysis (baseline correction, normalization) takes place. If you wish just to plot different parts of spectra use rather *x1*, *x2*, *t1*, and *t2*, since they only define spectral and temporal range on figures.

74| Save and run the script by clicking *Save file and Run* icon (Figure xx). Figures with 2D time-resolved Raman spectra and a reaction profile derived from their maximum peak intensities of peaks at 778, 795, and 1042 cm−1 will pop up in a new window.

**▲CRITICAL** Octave does not support live scripts like MATLAB, and you can run the script only after you save it.



Figure 4. RamanBoxx Control Panel.



Figure 5. OceanView software.



Figure 6. (**a**) The silicon wafer used for calibration is positioned on top of the hollow black cap approximately 1 cm from the probe head. (**b**) Transparent safety cover on the mill is covered with black cloth to reduce outside light being collected by the probe.



Figure 7. *Integration Time* and *Scans to Average* parameters can be changed in the *Acquisition Group Window* panel.



Figure 8. GUI for GNU Octave. In the upper left corner, File Browser defines a path to the current working directory. All variables are stored bellow in workspace. Switching from the Command window to the editor is easily done by clicking the appropriate tab at the bottom.

**TIMING**

Steps 1 – 10: 2 min; 11 – 17: 5 min; 18 – 35: 30 min; 36 – 56: 75 min; 57 – 59: 20 min; 60 – 74: 20 min;

**▲TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 1.**

**TABLE 1** | Troubleshooting table.

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Problem** | **Possible reason** | **Solution** |
| 2 | Cannot locate the *Silicon Labs CP210x USB to UART Bridge* under *Ports (COM & LPT)*. | The driver is not installed, or it is installed incorrectly. | Re-install the drivers that came with the laser, or download the driver from the internet. |
| 9 | Collecting background spectrum results in a spectrum on the screen with visible peaks, humps, or valleys. | The background spectrum is not properly collected and subtracted. | Click again on the Store Background icon, and wait until the resulting spectrum on the screen become a “flat” featureless spectrum. |
| 15 | Cannot find *Manually set numeric ranges* icon. | This icon is not on the secondary toolbar. | In the left corner on the main toolbar, click on icon *Switch to OceanView advance mode*. Now you should be able to find *Manually set numeric ranges* icon on the secondary toolbar. |
| 16 | Silicon Raman peak maximum is not located at 520.5 cm−1. | Variations in laser source excitation wavelength can come from the unstable power input, or manufacturing imperfections. | Change the excitation value from default 785 nm. If 784.9 nm results in shifting of the peak maximum further away from 520.5 cm−1, then go in the opposite direction, and try 785.1 nm. Increase, or decrease the set wavelength in increments of 0.1 nm until peak maximum is approximately at 520.5 cm−1 (at least between 520 and 521 cm−1). |
| 20, 29, 40 | After collecting the background spectrum, the resulting spectrum on the screen is still the same as it was before changing parameters and updating background spectrum. | The background spectrum is not properly collected. | Click again on *Update background data for this acquisition* icon and wait until the acquisition of the spectrum is complete. Repeat this once or twice until the collected spectrum is a flat line with just noise. |
| 63 | During execution of the script the error occurs at the line corresponding to the *cwd* parameter. | Syntax error. | Write the string with the path correctly. Check if “/” was used instead of “\”, and check that string is within parentheses. |
| 67 | Current working directory does not contain Raman spectrum files. | Path to the directory with files is wrong. | Check that the path given to varibale *cwd* is path to the directory with the files, and not the path to the directory with scripts used in step 63. |
|  |  |  |  |
| 63 | During excetu |  |  |
|  |  |  |  |

**ANTICIPATED RESULTS**

The *in situ* Raman spectroscopy allows for laboratory monitoring of mechanochemical milling reactions where it is suitable to track the whole range of chemical species as well as crystal phase transformations resulting from rearrangements of supramolecular bonds. Time-resolved Raman spectra enable fast and qualitative identification of the reaction course and possibly identification of intermediates. The simplest semi-quantitative reaction profiles are obtained by plotting intensities at the maximum of selected Raman bands belonging to reactants or products. If however, Raman spectra for pure phases of reactants, intermediates and products can be collected, the reaction profile may be extracted with the use of restrained least-squares leading to quantitative reaction profiles. Even when spectrum for each reaction mixture component is not accessible, as may often be the case with intermediate phases that could be short lived and cannot be isolated as bench-stable compounds, more advanced techniques, like multivariate curve resolution,**Error! Bookmark not defined.** can be used to estimate the species spectrum, followed by extraction of reaction profiles again by restrained least-squares. Even a qualitative insight into the course of a milling reaction, allows for optimization of reaction conditions, and a systematic study of the influence of various additives on reaction mechanisms and rates. Furthermore, Raman spectroscopy enables the use of isotopically labelled atoms and molecules which should permit exploration of reaction mechanism at the molecular level.

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**AUTHOR CONTRIBUTIONS**

The methodology was originally designed and adapted by IH and KU and further developed by SL. SL performed experiments and script preparation. SL wrote the manuscript with input from IH and KU.

**COMPETING FINANCIAL INTERESTS**

The authors declare the following competing financial interest(s): Ivan Halasz and Krunoslav Užarević are share-holders in InSolido Technologies (Croatia).

**Data Availability**

The dataset generated during the current study are available in the Supplementary Information.

**Code availability**

All the in-house written scripts used for analysis are available in the Supplementary Information.

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