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APPLICATION NOTE

Homogeneous Matrix Deposition on Dried Agar for MALDI Imaging Mass Spectrometry of Microbial Cultures

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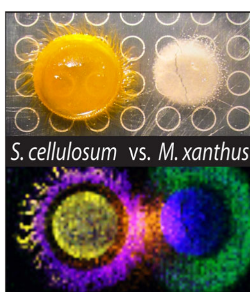

Abstract. Matrix deposition on agar-based microbial colonies for MALDI imaging mass spectrometry is often complicated by the complex media on which microbes are grown. This Application Note demonstrates how consecutive short spray pulses of a matrix solution can form an evenly closed matrix layer on dried agar. Compared with sieving dry matrix onto wet agar, this method supports analyte cocrystallization, which results in significantly more signals, higher signal-to-noise ratios, and improved ionization efficiency. The even matrix layer improves spot-to-spot precision of measured *m/z* values when using TOF mass spectrometers. With this technique, we established reproducible imaging mass spectrometry of myxobacterial cultures on nutrient-rich cultivation media, which was not possible with the sieving technique.

Keywords: Microbial IMS, MALDI, Matrix application, Natural products, Myxobacteria

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Introduction

Over the past years, imaging mass spectrometry (IMS) has become a popular method for the analysis of small molecules and biomarkers [1–4]. It is not surprising that the principle of MALDI-based IMS has been adapted to visualize the spatial distribution of metabolites produced by microbes growing on agar [5]. In particular, agar pieces with either single microbial cultures or co-cultivations of different strains can be analyzed by MALDI-IMS in order to create two-dimensional distributions of small molecules [6]. Such a technique offers the potential to study the conditions under which small molecules are produced and their natural roles [7, 8].

In addition to its highly hydrophilic nature, cultivation agar contains a substantial amount of complex media components and salts, all of which are essential for proper growth and overall metabolism of the microbes. Applying a suitable matrix layer on such samples can be a difficult task, which is further complicated by the components of the microbial cells and their extracellular matter. Consequently, several sample preparation techniques may be used, mainly based on methods established for tissue imaging [9]. Following the approach of applying dry matrix on a tissue sample [10, 11], sieving matrix powder onto wet agar samples has become a common sample preparation technique for microbial IMS in our lab [12]. This sieving method has been proven to be useful in most studies and certainly benefits from its simple and straight-forward procedure [12]. Vergeiner et al. have recently shown a modification to this approach by spraying a matrix suspension onto wet agar, which resulted in a more homogeneous matrix coverage and better sensitivity for a specific metabolite [13]. As an alternative to this dry matrix approach, Debois et al. sprayed a matrix solution on dried agar samples [14]. Since using the sieving technique for myxobacterial cultures did not result in proper detection of known compounds, we set out to establish a method that works for myxobacteria and, as such, may be useful for other microbial systems where it is difficult to find molecules of interest. We followed the idea of spraying a nebulized matrix solution onto dried agar and showed that the method is suitable to form a crystalline matrix layer on dried agar and myxobacterial colonies. The resulting matrix layer led to improved S/N ratios alongside with the detection of significantly more signals in MALDI-TOF analysis compared with dry matrix application.

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Experimental

Sieving Technique

Matrix was ground with a mortar utilizing *n*-pentane and thoroughly dried in a desiccator at reduced pressure. The matrix was applied to wet agar by sieving through a 53 μm stainless steel sieve until the surface was covered with matrix powder as previously described [12]. A second round of matrix was applied after 1 h of drying at 40°C followed by a final drying step of 2 h at 40°C.

Spray Technique

The sample was dried at 40°C for 3 h and transferred to the enclosed spray chamber of a Bruker ImagePrep device (Bruker Daltonics, Billerica, MA, USA). The plate was covered with matrix by spraying 2.5 mL of a matrix solution in 60 consecutive cycles in a nitrogen atmosphere. Each cycle comprised three steps: 2 s spraying, 10 s incubation time, and 40 s of active drying using nitrogen gas. After 30 cycles, the sample was turned by 180° to avoid inhomogeneous matrix deposition. Matrix solutions were of 20 mg/mL dissolved in ACN/MeOH/H_2O (70:25:5, v/v/v) with a 1:1 mixture of CHCA and DHB being the matrix of choice.

MALDI-MS

Samples were dried in vacuo for 30 min prior to measuring with an Autoflex MALDI-TOF mass spectrometer equipped with a Nd:YAG smartbeam2 laser (Bruker Daltonics) [15]. The laser modulator was set to "ultra" mode, which results in a laser focus of 90 μm diameter. For each raster spot of 350×350 μm, 100 shots (20 shots random walk) at a frequency of 500 Hz were acquired in reflectron positive mode. All data were analyzed using FlexImaging 4.1 b116 (Bruker Daltonics).

Results and Discussion

Matrix Application on Agar

Initial attempts to use the sieving method for myxobacterial cultures yielded no or inconsistent detection of known secondary metabolites in subsequent MALDI-TOF analyses. Moreover, the sieving method led to unwanted effects, such as rapid matrix absorption by some common agars and agglomeration of matrix powder on cells (Supplemental Figure S9). Both, the lack of reproducible detection of growth-related signals and the problems with proper matrix deposition prompted us to explore an alternative matrix application method. We showed that consecutive cycles of spraying a matrix solution can form a uniform matrix layer even on dried agar. This approach is similar to electrospraying or pneumatic spraying on tissue sections [16, 17] and has been applied to agar-based samples by Debois and coworkers [14]. Here we specifically compare the spraying method to the sieving method and thereby show the improvement for the analysis of myxobacterial compounds. The key principle of this process is the avoidance of moist conditions over prolonged time to minimize macerating of the agar. One way to achieve this is the repetition of numerous short spray pulses of a fine matrix aerosol using volatile organic solvents. Initial tests using water-soluble 2,5-dihydroxy benzoic acid (DHB) showed that a volatile solvent with low surface tension such as acetonitrile is suitable for spray-depositioning a matrix layer on dried agar. A high organic solvent content of 95% supports the partial drying of the sprayed matrix even before the aerosol droplets reach the surface. Each spray cycle comprises of a short spray pulse followed by a waiting period to give the matrix time to settle and interact with the surface. Subsequent active drying by flushing the spray chamber with nitrogen finishes each cycle. Spray cycles were repeated up to 60 times to obtain an evenly closed matrix layer on the agar.

Figure 1 depicts the visual differences in matrix deposition when using the sieving technique or the spray technique on microbial samples. Sieving the matrix onto wet agar and subsequent drying results in a granular matrix powder forming an inhomogeneous coverage (Figure 1, left column). The matrix may partly dissolve or agglomerate during drying of the sample and thus create even larger uncovered areas (Figure 1c), an effect that varies with different cultivation media and microbial strains. This seems to be a general problem that can be avoided by drying down the sample first. At the same time, the spray method yields a more homogeneous matrix deposition on both the agar surface and the microbial cell surface (Figure 1, right column).

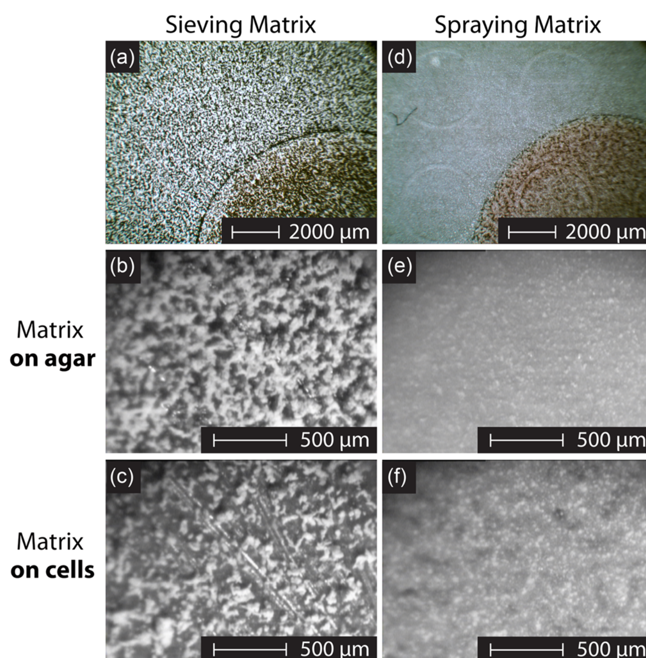

Figure 1. Comparison of matrix layer preparations on complex media agar derived by two different matrix application methods: (1) sieving on wet agar and (2) spraying on dry agar. The lower magnification pictures show both the agar and the *Sorangium cellulosum* cell spot (**a**), (**d**). The higher magnification pictures show matrix on either agar (**b**), (**e**) or cell spot (**c**), (**f**). Both methods used a 1:1 mixture of 2,5-DHB and α-CHCA

There is, however, for both methods, an obvious difference between an agar surface and a cell-covered surface, especially when a dense colony was dried down, an effect likely related to lipids and extracellular matter, which do have an influence on matrix crystallization. Nonetheless, the sprayed matrix is less affected than the sieved matrix and thus suitable for spatially high resolved imaging with laser foci of 50 μm as shown in Supplemental Figure S5. As inferred from Figure 1c, such small laser foci could easily come across matrix-free regions when the standard sieving technique is used. This is different from dry matrix applications on tissue samples, where the dry method allows high resolution imaging [18]. Other matrix application methods such as precoating of the target as shown by Grove et al. [19] is supposedly not suitable for agar-based samples owing to the thickness and density of the dried agar compared with a tissue section (i.e., the laser will not penetrate the agar sample). It should be noted that a direct comparison of tissue sections and agar samples is problematic owing to highly different properties of both sample types. We did not examine vapor deposition for matrix application onto dried agar but assume it to work as well [20]. In general, utilizing automated spray devices seems to be a suitable and versatile method for homogeneous matrix coatings on dried agar samples.

MALDI-TOF Analysis

Sprayed samples showed significantly more signals compared with sieved samples. Conversely, three *m/z* values are detected only for the sieved sample but not for the sprayed one (Supplemental Figure S2). This is in line with results from Gemperline et al. [21] and Goodwin et al. [11], which showed that different matrix application methods lead to different signals. In addition to the overall increased number of signals for sprayed samples, ionization was possible with lower laser energies compared with sieved samples (Supplemental Figures S3 and S4). Both findings indicate that the spraying process facilitates the cocrystallization of matrix and analytes [16, 17], an important step that is likely less pronounced when dry matrix is sieved. The small amount of sprayed organic solvent may further support surface microextraction of small molecules. Such a process of microextraction and recrystallization is repeated for the initial spray cycles until the matrix layer is fully closed. Consecutive spray cycles promote the recrystallization of already deposited matrix and thereby further incorporate extracted analytes, eventually leading to higher sensitivity and better S/N ratios.

For example, pellasoren, a known compound produced by *Sorangium cellulosum* So ce38 is hardly detected on the sieved sample but gives good signals and clear distributions on the sprayed sample (Figure 2, left column) [22]. For a second compound, microsclerodermin M [23], the even matrix layer guarantees a highly reproducible peak shape and *m/z* value comparing 10 spectra taken from various high abundant positions all over the colony (Figure 2f, black traces). In contrast, the uneven surface of the sieved sample causes drastic *m/z* deviations and peak shape distortions (Figure 2f, red traces),

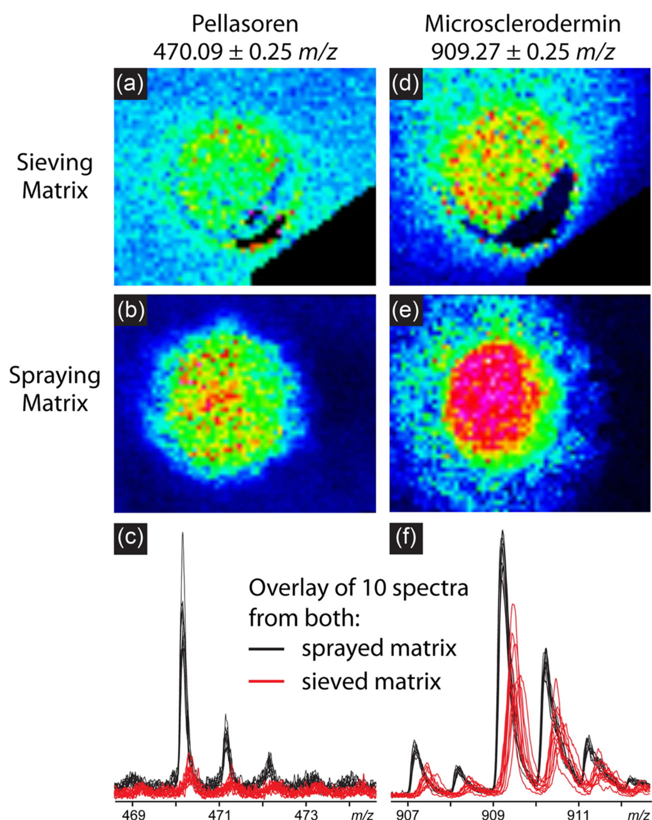

Figure 2. Differences in imaging results for both matrix application methods, highlighted using two known secondary metabolites from *S. cellulosum*. The colony is identical to the central area seen in the pictures. (**a**)–(**c**) The distribution of pellasoren can be clearly linked to the colony owing to a ≥5-fold increase in signal intensity when using the spray method compared with the sieving method. (**d**)–(**f**) In case of microsclerodermin M, the perfect spot-to-spot signal reproducibility observed for sprayed matrix leads to a highly defined distribution with a clear hot spot on the colony. The overlayed spectra were randomly picked from high abundant positions all over the colony

which eventually result in less defined imaging results for TOF-based IMS data comparing the sprayed and the sieved sample (Figure 2d and e).

Conclusion

We were able to show that spraying a matrix solution onto dried agar-based samples is suitable for establishing a homogeneous, evenly closed matrix layer. Macerating of the agar sample was prevented by repeating short cycles of spraying and drying. Each recurring spray cycle supported cocrystallization of analytes and matrix, which eventually led to improved ionization efficiency, an increased number of observed signals, and better S/N ratios. Microbial IMS of myxobacterial samples was significantly improved when using the spray method compared with the sieve method. The spray method is thus a valuable alternative to the sieving method whereas automated spray systems open up

the possibility for versatile sample preparation protocols which may help to further improve the breadth of microbial IMS applications.

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References

1. Svatoš, A.: Mass spectrometric imaging of small molecules. Trends Biotechnol. **28**, 425–434 (2010)
2. Watrous, J.D., Alexandrov, T., Dorrestein, P.C.: The evolving field of imaging mass spectrometry and its impact on future biological research. J. Mass Spectrom. **46**, 209–222 (2011)
3. Bjarnholt, N., Li, B., D'Alvise, J., Janfelt, C.: Mass spectrometry imaging of plant metabolites—principles and possibilities. Nat. Prod. Rep. **31**, 818–837 (2014)
4. Spengler, B.: Mass spectrometry imaging of biomolecular information. Anal. Chem. **87**, 64–82 (2015)
5. Yang, Y.-L., Xu, Y., Straight, P., Dorrestein, P.C.: Translating metabolic exchange with imaging mass spectrometry. Nat. Chem. Biol. **5**, 885–887 (2009)
6. Esquenazi, E., Yang, Y.-L., Watrous, J., Gerwick, W.H., Dorrestein, P.C.: Imaging mass spectrometry of natural products. Nat. Prod. Rep. **26**, 1521–1534 (2009)
7. Shih, C.-J., Chen, P.-Y., Liaw, C.-C., Lai, Y.-M., Yang, Y.-L.: Bringing microbial interactions to light using imaging mass spectrometry. Nat. Prod. Rep. **31**, 739–755 (2014)
8. Watrous, J.D., Dorrestein, P.C.: Imaging mass spectrometry in microbiology. Nat. Rev. Microbiol. **9**, 683–694 (2011)
9. Goodwin, R.J.: Sample preparation for mass spectrometry imaging: small mistakes can lead to big consequences. J. Proteome **75**, 4893–4911 (2012)
10. Puolitaival, S.M., Burnum, K.E., Cornett, D.S., Caprioli, R.M.: Solvent-free matrix dry-coating for MALDI imaging of phospholipids. J. Am. Soc. Mass Spectrom. **19**, 882–886 (2008)
11. Goodwin, R.J., MacIntyre, L., Watson, D.G., Scullion, S.P., Pitt, A.R.: A solvent-free matrix application method for matrix-assisted laser desorption/ionization imaging of small molecules. Rapid Commun. Mass Spectrom. **24**, 1682–1686 (2010)
12. Yang, J.Y., Phelan, V.V., Simkovsky, R., Watrous, J.D., Trial, R.M., Fleming, T.C., Wenter, R., Moore, B.S., Golden, S.S., Pogliano, K., Dorrestein, P.C.: Primer on agar-based microbial imaging mass spectrometry. J. Bacteriol. **194**, 6023–6028 (2012)
13. Vergeiner, S., Schafferer, L., Haas, H., Müller, T.: Improved MALDI-TOF microbial mass spectrometry imaging by application of a dispersed solid matrix. J. Am. Soc. Mass Spectrom. **25**, 1498–1501 (2014)
14. Debois, D., Ongena, M., Cawoy, H., De Pauw, E.: MALDI-FTICR MS imaging as a powerful tool to identify paenibacillus antibiotics involved in the inhibition of plant pathogens. J. Am. Soc. Mass Spectrom. **24**, 1202–1213 (2013)
15. Holle, A., Haase, A., Kayser, M., Höhndorf, J.: Optimizing UV laser focus profiles for improved MALDI performance. J. Mass Spectrom. **41**, 705–716 (2006)
16. Schwartz, S.A., Reyzer, M.L., Caprioli, R.M.: Direct tissue analysis using matrix-assisted laser desorption/ionization mass spectrometry: practical aspects of sample preparation. J. Mass Spectrom. **38**, 699–708 (2003)
17. Bouschen, W., Schulz, O., Eikely, D., Spengler, B.: Matrix vapor deposition/recrystallization and dedicated spray preparation for high-resolution scanning microprobe matrix-assisted laser desorption/ionization imaging mass spectrometry (SMALDI-MS) of tissue and single cells. Rapid Commun. Mass Spectrom. **24**, 355–364 (2010)
18. Trimpin, S., Inutan, E.D., Herath, T.N., McEwen, C.N.: Matrix-assisted laser desorption/ionization mass spectrometry method for selectively producing either singly or multiply charged molecular ions. Anal. Chem. **82**, 11–15 (2010)
19. Grove, K.J., Frappier, S.L., Caprioli, R.M.: Matrix pre-coated MALDI MS targets for small molecule imaging in tissues. J. Am. Soc. Mass Spectrom. **22**, 192–195 (2011)
20. Hankin, J.A., Barkley, R.M., Murphy, R.C.: Sublimation as a method of matrix application for mass spectrometric imaging. J. Am. Soc. Mass Spectrom. **18**, 1646–1652 (2007)
21. Gemperline, E., Rawson, S., Li, L.: Optimization and comparison of multiple MALDI matrix application methods for small molecule mass spectrometric imaging. Anal. Biochem. **86**, 10030–10035 (2014)
22. Jahns, C., Hoffmann, T., Müller, S., Gerth, K., Washausen, P., Höfle, G., Reichenbach, H., Kalesse, M., Müller, R.: Pellasoren: structure elucidation, biosynthesis, and total synthesis of a cytotoxic secondary metabolite from Sorangium cellulosum. Angew. Chem. Int. Ed. Engl. **51**, 5239–5243 (2012)
23. Hoffmann, T., Müller, S., Nadmid, S., Garcia, R., Müller, R.: Microsclerodermins from terrestrial myxobacteria: an intriguing biosynthesis likely connected to a sponge symbiont. J. Am. Chem. Soc. **135**, 16904–16911 (2013)