Submicron 3-D mass spectrometry imaging reveals an asymmetric molecular distribution on chemotaxing cells

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Abstract
Background: Dictyostelium discoideum is a ~10 µm diameter unicellular eukaryote that lives on soil surfaces. When starved, D. discoideum cells aggregate into streams of cells in a process called chemotaxis. In this report, we studied D. discoideum cells during chemotaxis using 3D - mass spectrometry imaging (3D-MSI).

Methods: The 3D-MSI consisted of the sequential generation of 2D molecular maps using burst alignment coupled to delayed extraction time-of flight secondary ion mass spectrometry (TOF-SIMS) combined with a soft sputtering beam to access the different layers.

Results: Molecular maps with sub-cellular high spatial resolution (~300 nm) indicated the presence of ions at m/z = 221 and 236 at the front and sides, but reduced levels at the back, of cells moving toward of aggregation streams. The 3D-MSI also detected an ion at m/z = 240 at the edges and back, but reduced levels at the front, of aggregating cells. Other ions showed an even distribution across the cells.

Conclusions: Together, these results demonstrate the utility of sub-micron MSI to study eukaryotic chemotaxis.

Keywords
Dictyostelium, chemoattraction, mass spectrometry imaging, aggregation, molecular imaging

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**Author roles:**
- **Castellanos A:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing
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**Competing interests:** No competing interests were disclosed.

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Introduction
Mass spectrometry techniques are immensely useful for the identification and characterization of molecular components in biological samples.\(^1\)\(^-\)\(^5.\) For instance, mass spectrometry imaging (MSI) permits the characterization of molecular components with high sensitivity and without the need for labels or pre-selection of molecules of interest.\(^6\) Using MSI, molecules can be detected and localized simultaneously.\(^7\) The spatial resolution of MSI is ultimately determined by the dimensions of the desorption probe (from tens of nanometers to hundreds of micrometers).\(^8\)\(^-\)\(^10\) One such technique, Time of Flight Secondary Ion Mass Spectrometry (TOF-SIMS), particularly excels in the ability to provide good spatial resolution. Recent developments of surface probes for the analysis of biological samples have been based on the search for higher molecular desorption yields; for example, the introduction of cluster and nanoparticle probes for surface interrogation of biological surfaces with enhanced secondary ion yield and reduced damage cross section has permitted the detection of a broad range of chemical classes.\(^11\)\(^-\)\(^15\) In addition, the combination of high spatial resolution cluster ion probes with reduced damage sputtering sources permits a 3D characterization of biological samples.

*Dictyostelium discoideum* are ~10 μm diameter motile eukaryotic cells that during starvation aggregate into dendritic streams to form a fruiting body as a mechanism to disperse spores to start new colonies. During aggregation, delayed pulses of cyclic adenosine monophosphate (cAMP) activate signal transduction pathways that cause cells to move toward the source of cAMP.\(^16\) The cells form dendritic aggregation streams, and because when the streams start forming, the single cells outside aggregation streams are consistently observed moving toward the streams, one can assign a front and back to a cell near a stream. Although there is some understanding of the distribution of selected cytoskeleton and signal transduction components in the moving cells, little is known about the general distribution of molecules in these cells.

In this report, we examine the distribution of chemical species associated with *Dictyostelium discoideum* chemotaxis using a dual beam interrogation probe based on a 25 keV Bi\(^{3+}\) imaging probe combined with a 20keV Ar\(_{1500}\)\(^+\) sputtering beam in spectral, imaging, and delayed-extraction imaging TOF-SIMS modes for high spatial resolution. We observe an asymmetric distribution of three prominent molecules or molecule fragments in the aggregating cells, illustrating the advantages of 3D-MSI.

Methods
Sample preparation
Aggregating *Dictyostelium discoideum* Ax2 cells were prepared for mass spectrometry surface analysis as described previously.\(^17\) Briefly, mid-log cells (1-2 \(\times\) 10\(^6\) cells/ml) grown in shaking culture at 21°C in Formedium HL-5 were collected by centrifugation at 1,500 \(\times\) g for 4 minutes, followed by three rounds of resuspension in deionized water, and collection by centrifugation at 1,500 \(\times\) g for 4 minutes as described previously.\(^18\) Following the final collection step, cells were resuspended in deionized water to a final concentration of 5 \(\times\) 10\(^6\) cells/ml. To initiate chemotaxis on a surface, 80 µl droplets of the cells were spotted onto 1×1 cm gold-coated silicon chips (Sigma Aldrich). After allowing cells to settle for 30 minutes, 40 µl of the overlaying deionized water was removed and the Au/Si chips were placed in a humid box at 21°C for 17 hours. After verifying the existence of aggregation streams, Au/Si chips were gently drained, freeze-dried and stored over a CaCl\(_2\) desiccant at room temperature prior to mass spectrometry analysis. This procedure was repeated for all the replicates (n = 3), and high reproducibility of stream formation, surface coverage, and cell distribution was observed.

Mass spectrometry
Mass spectrometry experiments were performed utilizing a TOF SIMS\(^3\) instrument (ION-TOF, Münster, Germany) retrofitted with a liquid metal ion gun analytical beam for high spatial resolution (25 keV Bi\(^{3+}\)), an argon cluster sputtering gun (20 keV Ar\(_{1500}\)\(^+\)), and an electron flood gun to reduce surface charging during mass spectrometry analysis. The TOF-SIMS instrument was operated in spectral (“high current bunched”, HCBU), imaging (“burst alignment”, BA) and delayed-extraction imaging (BA-DE) modes as described previously.\(^19\)-\(^21\) The tradeoff between the three modes is the mass resolving power, spatial resolution and secondary ion collection efficiency.\(^22\),\(^23\) Two dimensional maps (2D-MSI) were collected by rastering the primary 25 keV Bi\(^{3+}\) beam over the field of view of interest. In spectral HCBU mode, mass spectra were collected in positive and negative mode with a typical spatial resolution of 1.2 μm, a mass resolving power of \(m/\Delta m\approx\sim\)5,000 at \(m/z = 591\) (Au\(_{4}\)\(^+\)) and total ion dose \(~\sim\)1-4×10\(^{12}\) ions/cm\(^2\). Tridimensional maps were collected by sequentially collecting two dimensional maps using the BA imaging (or BA-DE) modes of the 25 keV Bi\(^{3+}\) imaging probe followed by a sputtering cycle using the 20 keV Ar\(_{1500}\)\(^+\) sputtering beam. The BA and the BA-DE imaging modes provided high spatial resolution (~300 nm) and nominal (m/Δm≈ ~200) and high (m/Δm ≈ ~4,000) mass resolutions at m/z= 197, respectively. The main differences between BA-DE and BA were the higher mass resolution and longer acquisition times (~4x) for BA-DE relative to BA. The typical total ion doses were 3×10\(^{12}\) ions/cm\(^2\) for the 25 keV Bi\(^{3+}\) imaging probe and 5×10\(^{12}\) ions/cm\(^2\) for the 20 keV Ar\(_{1500}\)\(^+\) sputtering beam during BA and BA-DE analysis. The tridimensional experiments comprised 300-500 two dimensional scans. Replicate measurements (n = 3) were performed on independent chips.
Data analysis

TOF-SIMS data processing was performed using SurfaceLab 6 software (ION-TOF, Münster, Germany). Two dimensional maps were processed from the BA and the BA-DE imaging modes at the level of unit and 0.1 m/z, respectively. Except when noted, two dimensional maps were not binned. Peak signals were exported in the BIF3D format, binned 5X and imported into ZCORRECTORGUI. The Total Counts Threshold Value was set to 75 before initializing data.

Results

Visual inspection of *D. discoideum* cells developed on the Au/Si chips showed that cells were aggregating (Figure 1A and B). Closer inspection showed a recurrent trend of single cells moving towards a recently formed stream. Cell integrity

![Figure 1](image_url)

**Figure 1.** Optical (A, 4500×4500 μm and B, 120×120 μm field of view) and secondary ion total images (C, 120×120 μm field of view) of aggregating *D. discoideum* cells on a Au/Si substrate. Data are representative of 3 independent experiments.
and morphology was preserved during the drying process, burst cells were not observed, and sample reproducibility was very high. A good correlation was observed between the optical and the TOF-SIMS images using HCBU, BA and BA-DE modes (see for example Figure 1B and C). Summed spectra of the TOF-SIMS images in HCBU mode were used to detect characteristic molecular ion signals from the single cells and from the streams in the process of aggregation. The $m/z$ = 0-230 region of the spectra showed ions at $m/z$ of 139.00, 160.98, 166.03, 184.07, 224.10 detected on the cells, and the characteristic substrate signal of Au+ ion at 196.97 detected in regions where no cells were present (Figure 2A). The $m/z$ = 500-950 region showed ions at $m/z$ of 518.30, 520.32, 740.50, 742.51, and 908.58 detected on the cells, as well as characteristic Au/Si chip substrate gold cluster ions at $m/z$ 590.90 ($\text{Au}_4^+$), 787.87 ($\text{Au}_6^+$), and 418.94 ($\text{Au}_2\text{C}_2\text{H}^+$) detected in regions where no cells were present (Figure 2B). These observations are in good agreement with our previous FT-ICR SIMS analysis of $D. \text{discoideum}$ cells developed on Au/Si chips. There were no obvious differences in the HCBU mode $m/z$ signals from single cells and from streams, and the ~1.2 $\mu$m resolution $m/z$ signals showed an apparently homogenous distribution on the ~10 $\mu$m diameter cells and the streams of cells.

Higher spatial resolution imaging (~300 nm resolution) was performed over smaller fields of view (40×40 μm; dashed squares in Figure 1B and C) using BA and BA-DE modes to study the distribution of molecular components in the cells and in the streams. The analysis has the added benefit that multiple 2-dimensional scans can be added, thus increasing the signal to noise and increasing the probability of observing lower abundance components. There appeared to be asymmetric distributions on cells and streams of ions at $m/z$ 221, 236 and 240, while all the other signals were assigned to either Au/Si chip substrate peaks or components evenly distributed across the cells and the streams (Figures 3, 4, and S1). The signals at $m/z = 221$ and 236 were concentrated at the leading edge and sides of cells moving toward streams and at the borders/edges of streams, while the signal at $m/z = 240$ was concentrated at the edges of cells and the sides of streams (Figures 3, 4, and S1).

**Discussion**

In this report, we observed asymmetric distributions of molecular components at $m/z = 221$, 236 and 240 on chemotaxing cells using 3D-MSI. We also observed several other molecular components that showed an even distribution on cells. Beam effects related to the incident angle of the primary beam during TOF-SIMS analysis (e.g., shadow effects) were not the cause of the asymmetric distributions, since they are expected for all the observed secondary ion signals and not a subset of them.

A challenge during 3D-MSI is the high diversity of biological molecules responsible for the ion signals, and complementary mass spectrometry tools would be needed for candidate assignments. One possibility is to perform tandem mass spectrometry for structural identification; however, due to the low secondary ion yields observed at the $m/z$ of interest using TOF-SIMS, this approach is almost unpractical at the spatial resolution level required despite recent advances in MS/MS SIMS instrumentation. Alternatively, ultrahigh resolution mass spectrometry might permit assignments based on accurate mass measurements.
Figure 3. A) Optical image of aggregating *D. discoideum* cells (40×40 μm field of view). B) TOF-SIMS BA mode total secondary ion image of the same field. C-I) TOF-SIMS BA mode images of this field at m/z C) 591 (Au$_3^+$ substrate, light grey), D) 184 (Blue), E) 518 (Blue), F) 740 (Blue), G) 221 (Red), H) 236 (Red), and I) 240 (Red). J-L) Overlays of 591 (Au$_3^+$ substrate, light grey), 184 (Blue), and J) 221 (Red), K) 236 (Red), and L) 240 (Red). Data are representative of 3 independent experiments.
Ethical approval

*Dictyostelium discoideum* is a unicellular invertebrate eukaryotic microbe, so no human subjects or vertebrate animal ethical approval was needed for these studies. *Dictyostelium discoideum* is a BSL-1 organism, and all work with live cells was done under a Texas A&M Institutional Biosafety Committee-approved BSL-1 protocol (IBC2018-155, with the most recent re-approval on March 22, 2022).

Data availability

Underlying data

Figshare: 20150124 DictyPT2 S4 Bi3+ SM+FGON 0.21pA 3scans FOV 200 007.itm, https://doi.org/10.6084/m9.figshare.20346711.v1.27

Figshare: 20150124 DictyPT2 S4 Bi3+ SM+FGON 0.21pA 3scans FOV 200 007_0.ita, https://doi.org/10.6084/m9.figshare.20346705.v1.28

Figshare: 20151023 ND7 5 3D FI-DE+ 0.04pA Ar1500 0.48nA FG ON 015_0.ita, https://doi.org/10.6084/m9.figshare.20346696.v1.29

Figshare: 20140814 Dicty Stream #2 ROI E6 Bi3 Primary + mode 019 0.11pA PI.itm, https://doi.org/10.6084/m9.figshare.20346690.v1.30

Figshare: 20140814 Dicty Stream #2 ROI E6 Bi3 Primary + mode 020 0.11pA PII_0.ita, https://doi.org/10.6084/m9.figshare.20346726.v1.31

Figshare: 20140814 Dicty Stream #2 ROI E6 Bi3 Primary + mode 020 0.11pA PII.itm, https://doi.org/10.6084/m9.figshare.20346723.v1.32

Figshare: 20140814 Dicty Stream #2 ROI E6 Bi3 Primary + mode 019 0.11pA PI_0.ita, https://doi.org/10.6084/m9.figshare.20346717.v1.33

Figshare: 20151023 ND7 5 3D FI-DE+ 0.04pA Ar1500 0.48nA FG ON 015.itm, https://doi.org/10.6084/m9.figshare.20346741.v1.34

Extended data

Figshare: Figure 2.tif, https://doi.org/10.6084/m9.figshare.20347146.v1.35

Figshare: Figure 3.tif, https://doi.org/10.6084/m9.figshare.20347152.v1.36

Figshare: Figure 1.tif, https://doi.org/10.6084/m9.figshare.20347149.v1.37

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Version 1

Reviewer Report 22 February 2023

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Jonathan Chubb
Laboratory for Molecular Cell Biology and Department of Cell and Developmental Biology, University College London, London, UK

This study uses spatial mass spectrometry approaches to explore molecular heterogeneity across cells during the aggregation phase of Dictyostelium. The study identified specific signals which showed striking variability along the cell axis, with orientation towards a likely source of chemoattractant. This is really impressive and the approach might be very useful, as it is unbiased, rather than based on prior knowledge of the signalling pathways identified using more traditional molecular approaches. The study is mostly very clearly described, even for a non-expert in mass spectrometry, and well presented.

I have a few comments that the authors may wish to consider:

It took a while to work out what the high magnification images in Figure 1 represent. It would be good to expand the narrative in the legend and/or label the figure to orient the reader. It also took a while to realise I was looking at streaming-again-some expanded legend material or some marking on the figure to show the aggregation centre/direction of migration would help.

In Figure 3, perhaps it would help the reader to know how you assigned the polarity of the cells from the optical image (they are somewhat round) although the ion images themselves are compelling. In addition-this is a 3D approach-would it be possible to have a 3D representation of the ion images in the main text eg. Fig 3G-I? This might be informative, even in the absence of molecular identity. I do not know the method, so maybe a "z-stack" cannot be done.

In the discussion, I would have liked to see a little thinking about what types of molecules might generate these distributions. This would align the study more to the literature, and so bring it more attention. For example are the sharp gradients-eg. Fig3 G-I, compatible with what is known about the distribution of actin, cAMP, or something else?

Is the work clearly and accurately presented and does it cite the current literature?
Is the study design appropriate and is the work technically sound? Yes

Are sufficient details of methods and analysis provided to allow replication by others? Yes

If applicable, is the statistical analysis and its interpretation appropriate? Yes

Are all the source data underlying the results available to ensure full reproducibility? Yes

Are the conclusions drawn adequately supported by the results? Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Dictyostelium, signalling, gene expression.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 04 October 2022

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Serge Della Negra
CNRS-IN2P3, Institut de Physique Nucléaire, Orsay, France

This manuscript presents a study by mass spectrometry imaging of Dictyostelium discoideum aggregation. Dictyostelium discoideum is a model organism used to investigate many cellular processes including chemotaxis, cell motility, cell differentiation, and human disease pathogenesis. Key questions can be addressed using Dictyostelium as a model organism. The goal of the authors is the study of D. discoideum cells during chemotaxis using 3D - mass spectrometry imaging (3D-MSI). The instrument used for this research is IONTOFV LMIS Bismuth Source and Argon Cluster Gun.

The presentation of the goal is clear and also the different experimental steps with the preparation of the samples already studied by this team, as well as the imaging techniques that are allowed by the IONTOFV instrument. It is clear that the authors know their instrumentation well with the associated bibliography. Their results and the ionic images thus obtained benefit
from the best techniques with the BA-DE and the improvement of the signal-to-noise ratio permitted by the use of 3D MSI approach. The highlighting of two different regions with a peripheral concentration probably membranous is clearly imaged.

Personally, I would like the authors to present the mass area of interest (MW: 220-241 daltons) in high resolution and also the precise masses and uncertainties.

The authors are not optimistic in their conclusions for future determinations of these molecules, despite the new M6 and hybrid developments of IONTOF. I would have liked to have some values knowing that an accumulation of data on several ROI is always possible.

Regarding the chemotaxis objective, the authors could specify some assumptions, these molecules are in relation with:
- the recognition process;
- the polarisation process;
- or the displacement process.

Dictyostelium has cAMP receptors uniformly distributed on the surface of its plasma membrane. The receptors are coupled to heterotrimeric G proteins, the activation of different signalling pathways are described\(^1\). Is there a link with these processes? It should be noted that Dictyostelium cells use cAMP as described in this paper which allows subsequent cells to reach the centre of the aggregate. There is an asymmetry between where the concentration of chemotactant is greatest, i.e. at the front of the cell, and where the concentration of chemotactant is lowest. There are illustrations of this polarisation of chemotactic cells\(^2\). This requires knowledge of the direction of progression. Numerous studies on proteins and the different signalling steps have been described and indications can be found.

For mobility there are also studies that have demonstrated a three-phase cycle:
1) The emission of protrusions at the front of the cell resulting in membrane deformation. These forces are obtained thanks to the polymerisation of actin filaments.
2) The adhesion of these protrusions to the substrate. The adhesion of the protrusions to the substrate to pull the cell forward. Cellular adhesion involves many molecular assemblies, including integrin-like proteins.
3) Detachment and retraction of the cell's rear end. Once the cell is well stretched on the surface, it must contract before it can extend again. In particular, it involves assemblies of myosin II and actin filaments. Same question as above?

I don't know if these relationships are possible but if the observation described in this article is related to aggregation it would be necessary to show the image of isolated cells (this point will demonstrate the link with the aggregation of these molecules in the membrane) and cells in the process of aggregation or to include in the conclusion to continue these studies with these points.

Regardless of my questions and suggestions this article can be published with a minimum of modification. Especially if possible a conclusion or discussion in relation to the knowledge obtained elsewhere about this chemotaxis would be interesting.

**References**
1. Kimmel AR, Parent CA: The signal to move: D. discoideum go orienteering. *Science*. 2003; **300**
2. Comer F, Parent C: PI 3-Kinases and PTEN. *Cell*. 2002; **109** (5): 541-544 Publisher Full Text

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** My main activity for many years concerns the fundamental research on particle-solid interaction. These fundamental studies, which covered a large energy range from keV to GeV for atomic ions, led me to develop new molecular and metallic cluster ion beams. I obtained the first high energy fullerenes beams which I accelerated to 40 MeV and metal aggregates using LMIS sources in the keV to 10 MeV range in the early 1990's. Again, fundamental studies on secondary emission and material modification induced by these complex beams led me to a new source development for accelerating metallic nanoparticles in the 2000s. At Orsay I led a new project Andromede: High velocity molecule and Nano-particle projectiles: Probe of nano domains and environmental nano objects. This project is focused on the following scientific area: Nano-particles, Surface analysis, Mass spectrometry, Biology, Astrochemistry, catalysis, solid-particle Interaction.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**
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