We would like to thank both reviewers for their thoughtful comments and suggestions regarding our manuscript. We have addressed all the reviewers’ concerns below following their quoted comments. Comments about minor grammatical, spelling or style concerns were not elaborated below, but simply addressed in the revised manuscript.

**Reviewers' comments:**

**Reviewer #1:**

1. “The use of the term raster scanning may be misleading. Raster scanning can imply a serpentine pattern of the sample below the sprayer. What is actually done is unidirectional scanning and lane stepping.”
   1. We agree that “unidirectional scanning and lane stepping” is a more accurate term. We had originally used raster scanning for simplicity, but have since corrected the usage of the term to unidirectional scanning.
2. “The authors talk only about untargeted imaging but targeted imaging is also important.”
   1. The discussion of untargeted MSI was intended to highlight the usefulness of MSI in the discovery-mode of analysis, which is particularly relevant to biological samples. Even so, targeted analysis also plays a key role in bioanalytical chemistry, therefore we have since added in a statement about the use of MSI for targeted imaging and the benefits of having a known target.
3. “Page 2: Like SIMS or MALDI, but maybe not as stringent, surfaces in DESI also need to be relatively flat and mounted exactly horizontal or false images can result (see reference 21).”
   1. We have added a statement in the introduction regarding the need for a planar sample for the most commonly used MSI methods. We have addressed the issue of the horizontal mounting of the sample in the Discussion section of the paper.
4. “Page 2: WRT Wiseman 2006 as first demonstration of DESI imaging there is actually a Kertesz and van Berkel paper that was in print slightly earlier. That paper was not referenced here.”
   1. We would like to apologize for the oversight in referencing the work of Van Berkel and Kertesz (now included as Ref. 13), and have given that group proper acknowledgement. We had highlighted the work of Wiseman (2006) because the focus of the procedure is the analysis of biological samples, which Wiseman et al pioneered using DESI, but the work of Van Berkel is the first demonstration of imaging by DESI. Therefore we have modified that section of the introduction appropriately.
5. “Page 3: Thaw mounting is often done with just heat of finger under slide. This might be a better alternative than pressing down on the tissue with another slide, which risks tissue damage.”
   1. When the sectioned tissue sample slides over the blade, onto the pressure plate, we directly press the warm slide onto the section, which is sufficient to mount the tissue section onto the slide without further handling. Alternatively, the sectioned tissue can be lifted and laid onto a cold slide, then using the heat of a finger, the slide is heated from underneath securely mounting the section to the slide. In our protocol, we were referring to the first method of thaw mounting in our protocol. By either method, we agree that no slide or finger should come into contact with the section on the top side of the slide as it would significantly damage the tissue or change chemical distributions. We believe that we have clarified this by removing part of our description and adding a clarifying statement about handling of the slides.

**Reviewer #2:**  
*Major Concerns:*

1. “The claim "Ultimately, gas phase ions are produced through ESI-like processes following the ion evaporation or charge residue models" appears to be unjustified. That ion generation from DESI-yielded analyte-containing droplets follows the mechanisms of ESI is likely (and believed by many), yet this is yet to be experimentally proven. Furthermore, recent studies indicate that ion generation in ESI can occur through other processes than the ion evaporation and charge residue models, leaving the claim incomplete for ESI and thus for DESI as well. Please remove or rephrase the claim.”
   1. We agree that the exact mechanism of ion formation has yet to be proven experimentally. We have since included a statement to clarify the remaining uncertainty on the ionization mechanism.
2. “I think Steps 1.7.1, 1.7.2, and 1.7.3 should be moved to Section 2 as they contain variables that likely require optimization (e.g., spray solvent composition, flow rate, nebulizer gas pressure, and spray voltage).”
   1. We had included the initialization of the equipment in Section 1.7 for the interest of time to start while waiting for the tissue section to thaw as a practical measure. These variables do require optimization for different experimental set-ups as well as sample type. We had included specific values for these variables because they are regularly used in our lab for this particular sample. We have since added a statement within section 2.1.1 to clarify that these variables should be optimized for different set-ups or samples.
3. “Please consider including steps to optimize the variables identified in 3), if applicable.”
   1. We have added a statement about the impact of spot size on image resolution to the Procedure section (3.1.1) in addition to the existing point made in section 3.2.1 and within the Discussion. We feel that this guidance is sufficient for a reader to make an informed decision regarding the desired motion parameters and resulting perceptions of image resolution without belaboring the optimization.
4. “In Step 2.5.1, the protocol recommends moving the sample slide to avoid depleting the material (brain tissue) during optimization. The protocol however fails to account for chemical changes that are intrinsic to the sample and can present upon moving the stage. For example, the corpus callosum to the cerebral cortex have different chemical composition, thus moving the point of analysis between these anatomical regions (without recognizing this) could mislead the user in finding optimal conditions for ionization. Please address this concern and, alternatively, recommend a negative control.”
   1. We agree that the different chemical compositions in the tissue make it difficult to make a precise interpretation of the effects of each step within the optimization process. We have elaborated on this point within the protocol (2.5.1), recommending a focus on the thalamus of the section, which would have a slightly more homogeneous composition. We have also added the suggestion of using red Sharpie® for preliminary optimization; however changing the sample surface and chemical class of the analyte will still not necessarily provide the final optimized set of parameters, but may require less tuning.
5. “Executing Step 2.6 will void the conditions optimized per Step 2.5. Thus, please move Step 2.6 before 2.5 or refer back to Step 2.5 to call for the need for re-optimization via Steps 2.1 and on.”
   1. We agree that the process of optimization of the DESI ion source is cyclical and that all variables are interrelated. Therefore in section 2.7.1, we have added a note to highlight the need for re-adjustment of other variables following the tuning of one.
6. “Please comment in the discussion section on the protocol's adaptability to performing MSI in three dimensions at ambient conditions as already demonstrated by DESI.”
   1. While 3D imaging was not our original focus, we agree that 3D imaging is at the forefront of the field and of great importance. We have since added a brief discussion on 3D imaging in the discussion per your recommendation.

*Minor Concerns:*

1. “Please evaluate whether ion suppression effects due to the OCT compound would be of concern in DESI MSI, that users should be aware of.”
   1. The primary reason that minimal OCT is used, rather than embedding the sample is due to ion suppression from the OCT. We have added a statement in section 1.3.2 regarding this issue to make the reader aware of the reasoning.
2. “Please correct "translational stage" to "translation stage"”
   1. We have corrected this term throughout the document.