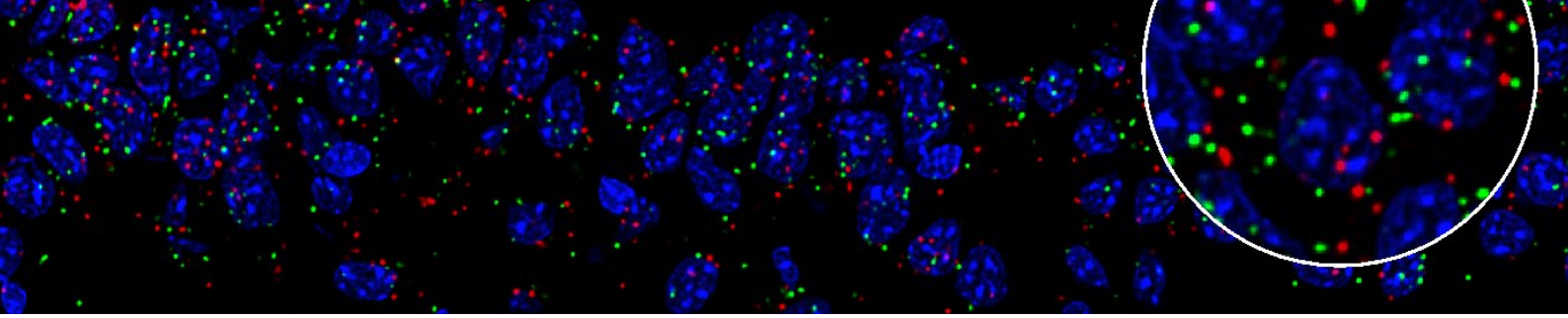

SUPER-RESOLUTION MICROSCOPY INCREASES AMPLICON SPOTS DETECTION EFFICIENCY IN SPATIAL TRANSCRIPTOMICS

Spatial transcriptomics is a rapidly growing research field that aims to provide molecular maps of the RNA transcriptome of single cells within tissues with preserved spatial information.

Imaging-based spatial biology uses epifluorescence microscopy to directly identify transcripts and visualize multiple targets in situ. However, a common challenge arises when the observed field of view (FOV) becomes saturated with fluorescence targets (optical crowding) and the number of genes that can be visualized is limited by the diffraction of light. Consequently, it is not possible to reliably and uniquely identify each individual transcript using this imaging approach.

In a recent study published in **Cells MDPI journal**, Dr. A. Linares and his collaborators investigated the effect of **structured illumination microscopy (SIM)** on the performance of single-gene transcript detection in spatial transcriptomics experiments. In particular, in this work, the authors explore the effect of the **DeepSIM super-resolution (SR) system** in combination with both low- and high-magnification objectives on the localization of individual amplicon spots.

In this application note, we report some examples of how the CrestOptics DeepSIM SR system (https://crestoptics.com/deepsim/) was crucial to improving RNA spot detection performance compared to widefield (WF) and confocal (CF) modes. Detailed descriptions of all the outstanding results in this work can be found in the **complete paper**.

SIM enhances contrast, resolution, and spot detection performance

The authors carried out **hybridization-based in situ sequencing (HybISS)** on a mouse brain coronal section, targeting four highly expressed genes (Actb, Gapdh, Atp1a3 and Slc17a7). Moreover, in order to increase the spot density per channel, only two channels were used for the visualization of all four genes.

As a standard method of reference, the sample was visualized using WF (**Figure 1**, left panels) and with a 25x silicon oil immersion plan apochromatic objective (1.05 NA). Then, the same FOVs were analyzed using SIM (**Figure 1**, right panels). The comparison of the obtained images revealed that the diffraction-limited spots imaged in WF mode contain significant blur and poor contrast and are not clearly visible as individual objects (Figure 1, left). **DeepSIM, on the other hand, produced individual spots with enhanced contrast and resolution** (**Figure 1**, right).

Since deconvolution has been shown to improve spot detection during the image processing of in situ sequencing experiments, the improvement in image quality provided by SIM was compared to the one obtained

when applying deconvolution to the WF images. **As shown in Figure 1, although deconvolution on WF images enhances image contrast with a deblurring effect (Figure 1, middle panels), the improvement in resolution is still limited compared to that achievable with SIM.**

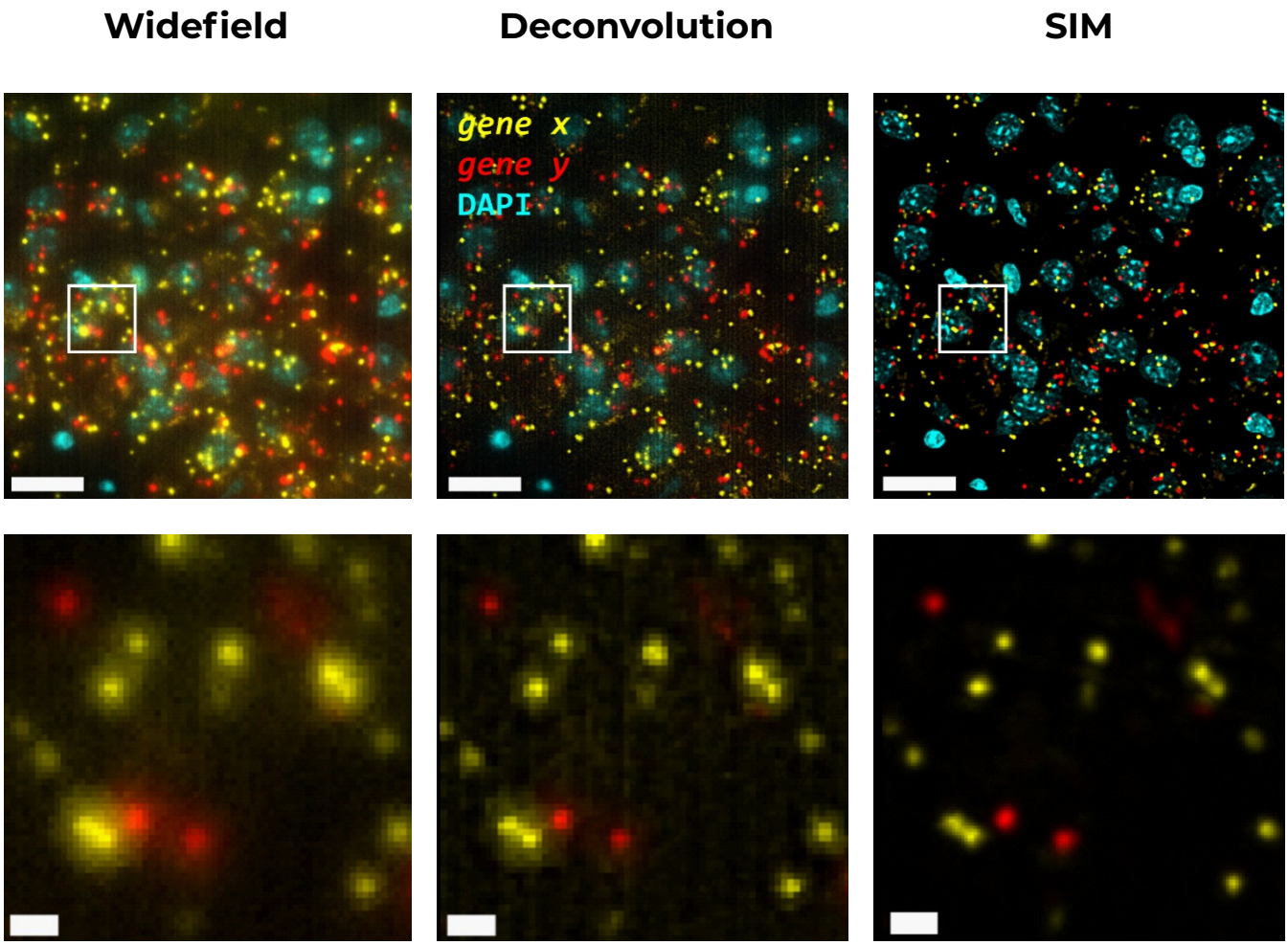

Figure 1: Comparison of spot resolution between WF (left), deconvolution (middle) and SIM (right). The sample was stained with DAPI for nuclei visualization, Cy3 (Actb and Gapdh) and Cy5 (Atp1a3 and Slc17a7) for gene transcript detection. Images in the lower row correspond to the boxes of the images in the upper row- Scale bars: 20 um (upper) and 2 um (lower).

In Figure 2 the quantification of the spots of the same FOVs acquired with WF or SIM is shown. DeepSIM (https://crestoptics.com/deepsim/) enhances spot detection performance in different ways:
-SIM detects a larger number of bright spots compared to WF.
-Most of the bright spots in SIM acquisitions had a lower intensity in WF images.
-SIM was able to rescue low-intensity spot signals that were poorly visible in WF mode or even confused with the background (Figure 2A, white arrows).

The spot quantification (Figure 2B) revealed that a higher fraction of bright and dim RNA

spots were identified with SIM as a result of contrast and resolution improvements achievable with DeepSIM (https://crestoptics.com/deepsim/). In this regard, a new population of dim spots appeared in the SIM acquisitions (left-most pink bars in Figure 2B) and corresponds to faint signals from undetected spots in WF images (white arrows in Figure 2A). Moreover, thanks to the resolving ability of DeepSIM (https://crestoptics.com/deepsim/) over signals in close proximity, SIM was able to perform a decrowding of the fluorophores and detect multiple spots. Overall, the use of SIM increases the number of detected spots by about 40%.

In addition, the authors also quantified the number of detected spots with SIM or deconvolution against those detected in WF mode for specific FOVs with increasing transcript density (Figure 2C). Using deconvolution as a pre-processing step in the image analysis pipeline improves spot detection; however, only SIM showed twice as many spots for some FOVs compared to those detected using WF.

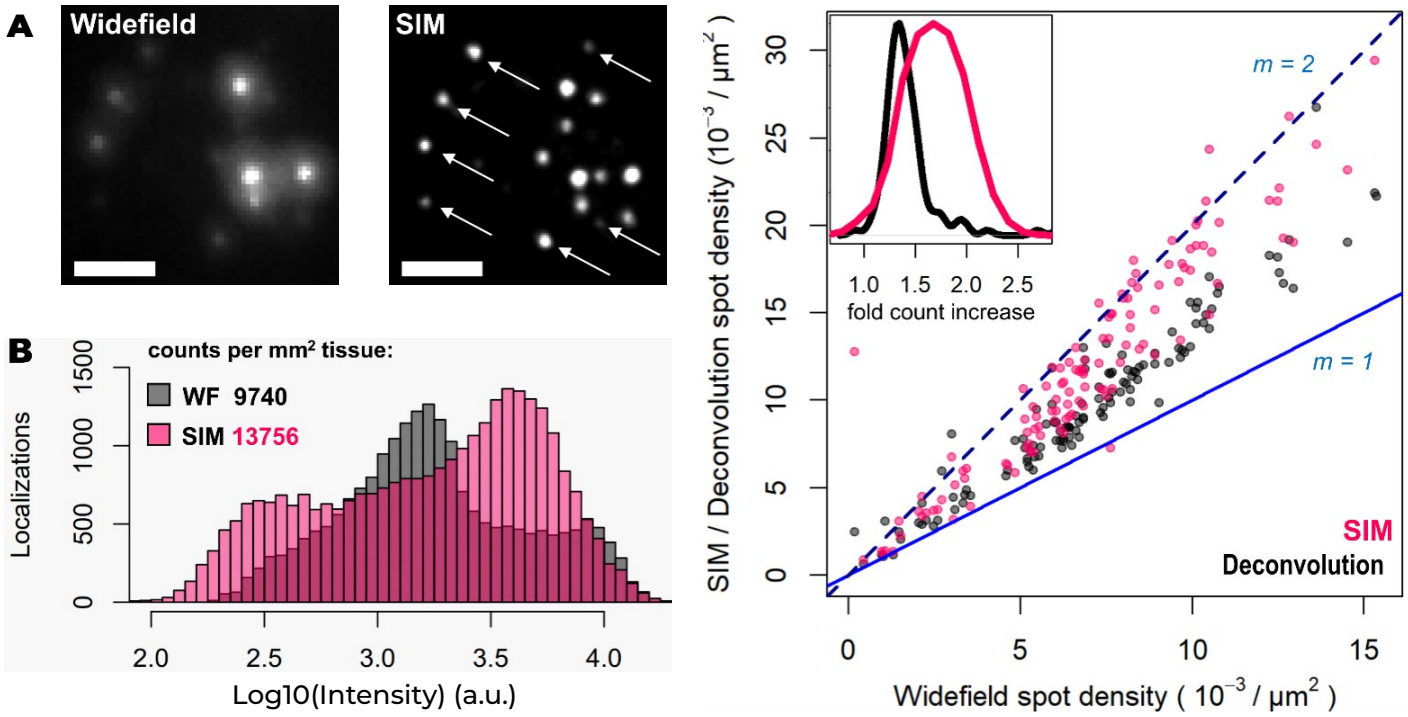

Figure 2: SIM enhances spot detection performance improving spot quantification. (A) WF and SIM comparison of individual transcripts; white arrows indicate super-resolved spots previously undetected. (B) Spot localization count distribution in mouse brain tissue imaged in WF (grey) and SIM (pink) modes. (C) The plot shows the ratio of localization counts for each FOV analyzed when comparing WF against deconvolution (grey dots) and SIM (pink dots). Blue and dotted blue lines correspond to a localization ratio of 1 and 2, respectively. The inner graph shows the distribution of the number of fold count increases for deconvolution (black curve) and SIM (pink curve). Scale bars: 5 um.

Altogether these results indicate that the use of DeepSIM in combination with a 25x silicon oil immersion objective enhances contrast and improves resolution providing up to a two-fold increase in spot detections of amplified single mRNA transcripts.

SIM enhances spot detection performance in highly clustered regions

As the effect of SIM is more pronounced for higher densities, Linares et al. tested even denser areas. In order to increase the spot density, they used a 275 genes panel encoded in six imaging cycles, so that for

any given cycle, there were about 50 genes per channel visualized. Each channel had a particular optical density of spots (**Figure 3C**) and they refer to the grouped genes as group x, group y and group z for the Cy3, Cy5 and Cy7 respectively. For this experiment, the authors used a 20x air objective (0.8 NA) since it is most commonly used for this kind of experiment.

As displayed in Figure 3, the use of **DeepSIM improved contrast, reduced background and increased resolution, similar to what was observed with the 25x objective**. In particular, images in WF mode revealed blurry and unresolved spots. The use of deconvolution improved resolution and spot detection; however, **DeepSIM provided the highest improvement, with a more noticeable effect as spot density increased (Figure 3C). These data demonstrated that the use of SIM improves both the resolution and the overall quality of imaged fluorescent objects, which renders more accurate and reliable spot detection.**

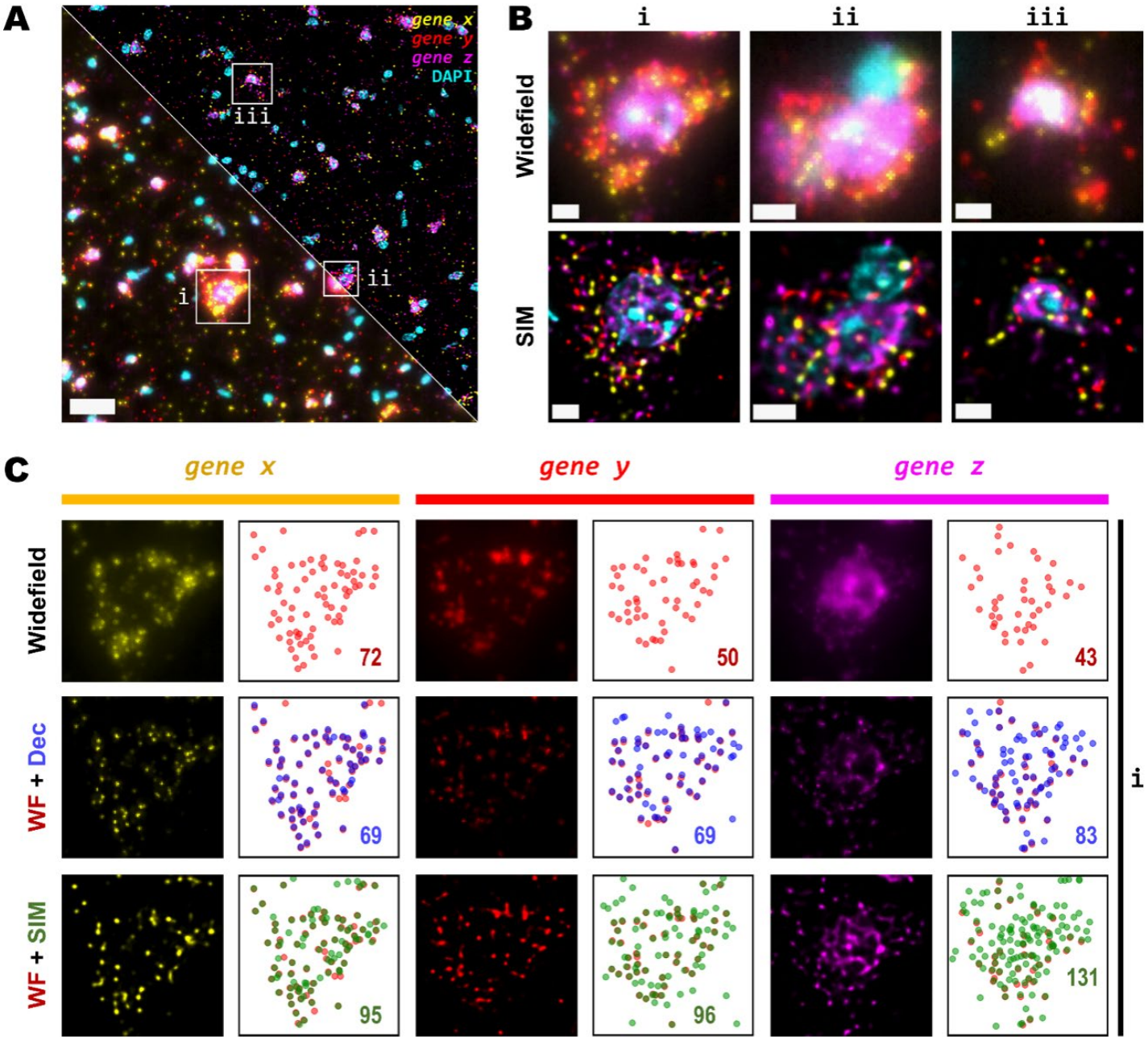

Figure 3: RNA spot detection sensitivity is increased in super-resolved images at moderate magnification and NA. (A) Single FOV of a mouse brain coronal section. Three sets of 50 genes are visualized in each channel (Cy3, Cy5 and Cy7). Bottom-left and upper-right corners correspond to WF and SIM images respectively. (B) Zoomed views of the boxed regions in (A) for WF (upper row) and SIM (lower raw). (C) Comparison of resolution and contrast increase for each gene channel between WF, deconvolved and SIM images. Next to each fluorescence image, a plot shows the position of all detected spots for WF (upper row, red), deconvolution (middle row, blue) and SIM (lower row, green), Numbers indicate total spot detection for each channel (group x, group y and group z). Scale bars: (A) = 30 um; (B) = 5 um.

Moreover, the authors investigated the spot detection performance in mid-to-low density areas as well as within those clusters **(Figure 4A, B). As shown in Figures 4A and B, as the spot density increases, the effect of the SIM is even more evident.** A detailed examination of spot detection as a function of density showed that using **SIM allows for the recovery of larger numbers compared to WF (Figure 4C).** However, at low-to-medium densities, the use of SIM showed no significant difference compared to the use of deconvolution at low magnification (20x). **Spot detection from SIM images recovered almost four times as many spots from the high-density and clustered areas compared to that from WF (Figure4 B, C) and about twice as many when compared to WF plus deconvolution (Figure 4C).**

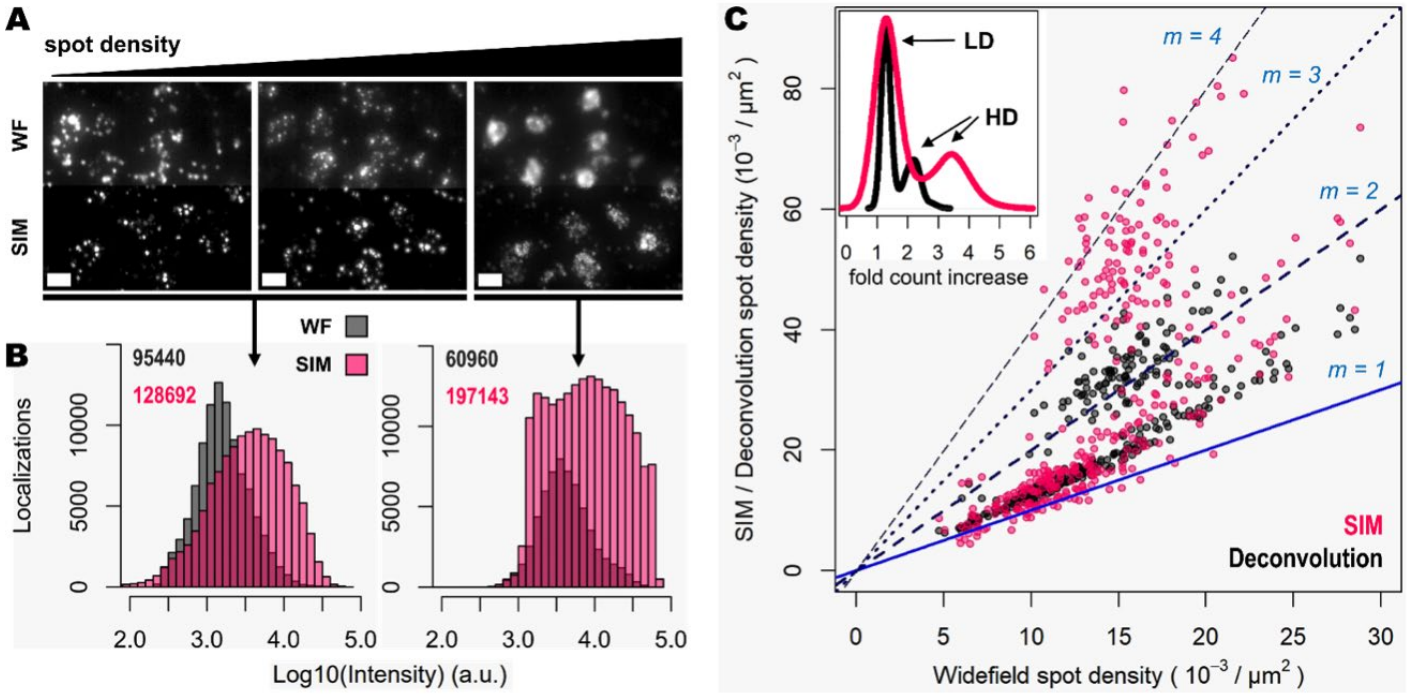

Figure 3: DeepSIM improves the detection of highly clustered transcripts. (A) Three different groups of 50 genes with increasing density are shown (WF, upper row; SIM lower row). (B) Spot localization count distribution of images acquired in WF (grey) and SIM (pink). The left histogram corresponds to the localization count of relatively low-expression genes; the right histogram corresponds to the localization count of a higher-expressed gene with crowded zones. The total number of localized spots for each condition is shown. (C) Plot showing the ratio of spot detection density when comparing WF against deconvolution (grey dots) and SIM (pink dots) imaging modes. Blue and dotted blue lines correspond to an increasing spot density ratio from 1 to 4. The inner graph shows the distribution of the number of fold count increases for deconvolution (black curve) and SIM (pink curve). The count increase distributions that correspond to low-density (LD) and High-density (HD) gene transcripts are indicated by the black arrows. Scale bars: 10 um.

These data demonstrated that the use of DeepSIM when imaging with a conventional 20x air objective significantly benefits spot detection at high densities and clusters of amplified single RNA transcripts.

Conclusions

In this study, A. Linares and colleagues investigated for the first time the effect of SIM on the performance of single-gene transcript detection in spatial transcriptomics experiments. The authors performed direct mRNA-targeted HybISS for multiple genes in mouse coronal brain tissue sections and evaluated spot detection performance in WF and confocal (CF) images (data not shown, please refer to the complete paper) versus those with SIM in combination with 20x, 25x and 60x (data not shown, please refer to the complete paper) objectives.

In order to have the possibility to easily switch

between different imaging modalities (WF, CF and SIM), the authors used a microscope equipped with **X-Light V3 CF spinning disk** and a **DeepSIM X-light SR system** thus performing three imaging modalities in one setup.

In general, SR provided by the SIM module delivers a good enough performance to accurately and reliably detect targeted transcripts in spatial transcriptomics experiments. The SIM spot detection performance is better than that of WF or CF imaging methods, with or without deconvolution imaging post-processing, and this improvement is even more evident in regions of the sample with a high density of fluorescent spots.

In ISS multiplex experiments, transcripts identification depends on the correct localization of single fluorescent spots; in this context, **for signal decoding and transcript identification, SIM provides increased localization precision.**

Regions with the highest transcripts density can arise by looking at a large number of genes and, in this sense, **DeepSIM has the potential capacity to improve the dynamic range of gene expression measurements in ISS experiments.**

In conclusion, SIM increases the detection efficiency of gene transcript spots compared to WF and CF modes, especially in highly crowded areas. DeepSIM's ability to double the performance of even low-magnification objectives, such as 20x or 25x, makes this system a great candidate for spatial transcriptomics studies.

Methods

All the acquisitions presented in this application note were performed with the **CrestOptics X-Light V3 spinning disk** and **DeepSIM X-Light SR**.For further information about **CrestOptics products**, please visit our **product page**.

Images were acquired in various channels according to the experiments, using laser excitations at 405, 477, 546, 638 and 749 nm. Appropriate filters for DAPI, GFP, Cy3, Cy5 and Cy7 were used.

Stack images were acquired with 0.8 NA CFI Plan Apochromat Lambda D 20x air objective (Nikon) and 1.05 NA CFI Plan Apo Lambda S 25x silicone oil objective (Nikon) with z-steps of 1 um and 0,5 um respectively.

Acknowledgments

Data presented in this application note are courtesy of **Dr. A.H. Crevenna** and **Dr. A. Linares** as published in **Linares et al., 2023** and have been reused following approval from the authors. Re-use of images from this article is done under the terms and conditions of the **Creative Commons Attribution (CC BY 4.0) license**.

For a more complete view of all the brilliant results obtained in this work, please refer to the **complete paper** .

We want to acknowledge **Dr. A.H. Crevenna** and **Dr. A. Linares** for their support in drafting this application note.