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Super resolution microscopy and membrane contact site detection: Challenges and opportunities

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*equal contribution

Membrane contact sites (MCS) are sites where two organelles come in close proximity, from 10-80 nm, without fusing. Their morphological detection is best accomplished by electron microscopy, with recent developments of FIB-SEM tomography providing exquisite 3D views of interaction between organelles. However, EM is limited to fixed cell analysis, is relatively slow and is challenged when it comes to localizing proteins associated with the MCS. Fluorescence microscopy brings the potential for multi-channel live cell analysis of MCS. Colocalization analysis of diffraction limited confocal microscopy can detect relative changes in contact site formation but is limited to 200-250 nm lateral (XY) and 400-500 nm axial (Z) resolution, far beyond that of the actual contact site.

Super-resolution microscopy (SRM) enhances resolution of fluorescent microscopy beyond the diffraction limit. Single molecule super resolution microscopy provides nanometer resolution and with live cell single particle tracking able to track MCS tethers, but labeling efficiency limits detection of all MCS in the cell. Stimulated emission depletion (STED) microscopy provides 40 XY nm resolution in 2D mode, with confocal Z resolution, and 100-120 nm XY and 200-250 Z resolution in 3D mode. Structured illumination microscopy (SIM) achieves a similar two-fold resolution improvement with increased speed of acquisition and reduced photobleaching compared to 3D STED. Deconvolution can improve “resolution” and spectral deconvolution provide capability of imaging up to 8 different organelles. Current 3D SRM does not achieve resolution able to detect MCS and quantification is based on user-determined organelle segmentation.

To enhance detection of MCS from 3D STED image volumes we developed MCS-DETECT, a multichannel differential correlation analysis algorithm that reconstructs the interface between organelles at sub-pixel precision without segmentation, resulting in highly sensitive MCS detection robust to variations in local signal or background intensity differentials. MCS-DETECT was validated at the cell level based on its ability to reconstruct and differentiate elongated, tubular ribosome-studded mitochondria-ER contact sites (riboMERCs) from smaller smooth contacts and larger, flat contacts induced by mitochondria-ER linker expression. MCS-DETECT was further shown to have markedly increased specificity compared to both colocalization analysis and the split-GFP-based contact site sensor (SPLICS). MCS-DETECT outputs closely align with contact site metrics obtained by 3D electron microscopy. Ongoing work addresses future challenges of applying super-resolution microscopy and MCS-DETECT to other super-resolution modalities, live cell acquisition and multi-organelle MCS.

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Super resolution microscopy and membrane contact site detection: Challenges and opportunities

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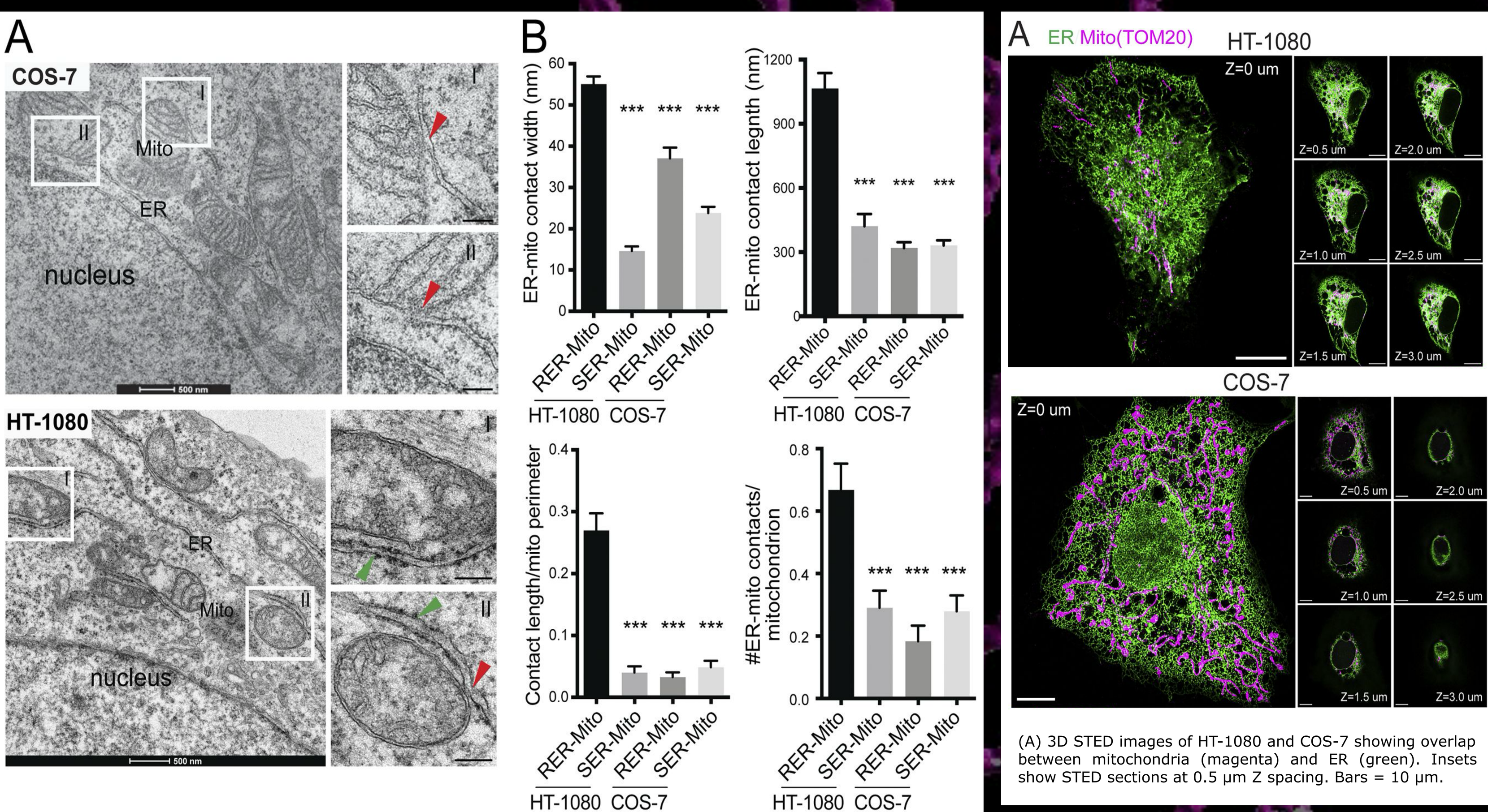
Membrane contact sites (MCS) are sites where two organelles come in close proximity, from 10-80 nm, without fusing. MCS morphological detection is best accomplished by electron microscopy (EM). Fluorescence microscopy brings the potential for multi-channel live cell analysis of MCS. Diffraction limited confocal microscopy is limited to 200-250 nm lateral (XY) and 400-500 nm axial (Z) resolution while 3D stimulated emission depletion (STED) and structured illumination microscopy (SIM) provide a two-fold resolution improvement. Current 3D SRM does not achieve resolution able to detect MCS. To enhance detection of MCS from 3D STED image volumes we developed MCS-DETECT, a multichannel differential correlation analysis algorithm that reconstructs the interface between organelles at sub-pixel precision without segmentation, resulting in highly sensitive MCS detection robust to variations in local signal or background intensity differentials. MCS-DETECT was validated at the cell level based on its ability to reconstruct and differentiate elongated, tubular ribosome-studded mitochondria-ER contact sites (riboMERCs) from smaller smooth contacts and larger, flat contacts induced by mitochondria-ER linker expression. MCS-DETECT was further shown to have markedly increased specificity compared to both colocalization analysis and the split-GFP-based contact site sensor (SPLICS). MCS-DETECT outputs closely align with contact site metrics obtained by 3D electron microscopy. Ongoing work addresses future challenges of applying super-resolution microscopy and MCS-DETECT to other super-resolution modalities, live cell acquisition and multi-organelle MCS.

Distinct MERCs in COS-7 and HT-1080 cells



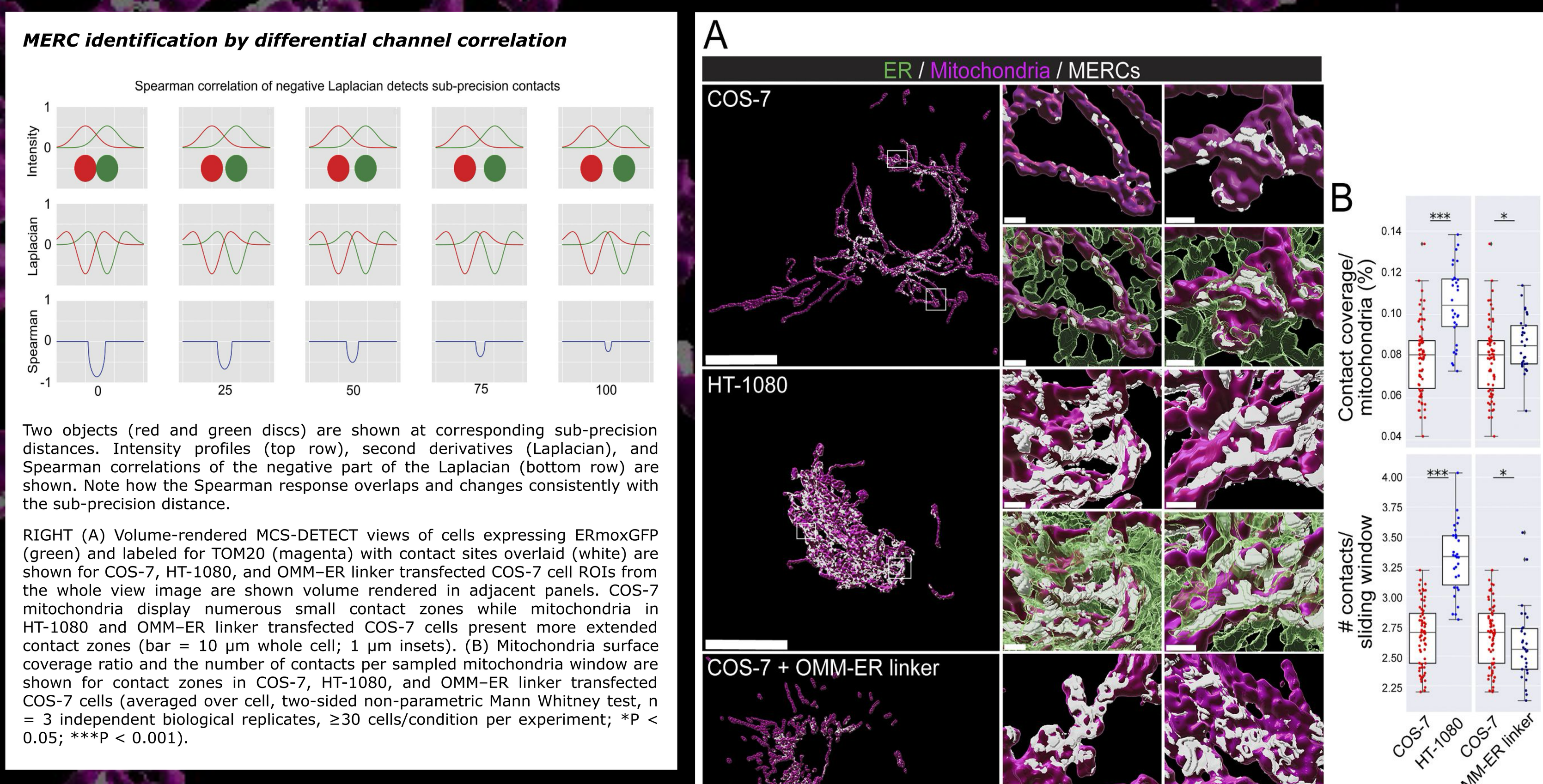
(A) Representative EM images of HT-1080 and COS-7 cells. Insets show rough ER–mitochondria contacts (RER-mito) in HT-1080 cells (green arrowheads) and smooth ER–mitochondria contacts (SER-mito) in HT-1080 and COS-7 cells (red arrowheads). (B) Quantification of contact width, contact length, contact length relative to mitochondria perimeter, and number of contacts per mitochondria profile are shown for SER-mito and RER-mito contacts in HT1080 and COS-7 cells.

(A) 3D STED images of HT-1080 and COS-7 showing overlap between mitochondria (magenta) and ER (green). Insets show STED sections at 0.5 µm Z spacing. Bars = 10 µm.

- ❖ By EM, HT-1080 cells contain elongated rough ER–mitochondria contacts far longer than the shorter, smooth ER–mitochondria contacts in COS-7 cells
- ❖ While HT-1080 cells present increased overlap between ER and mitochondria than COS-7 cells in whole-cell 3D STED image stacks, determining what exactly is a contact site is challenging

TOOLS
Membrane contact site detection (MCS-DETECT) reveals dual control of rough mitochondria–ER contacts

MCS-DETECT reports on sub-precision contacts



MERC identification by differential channel correlation

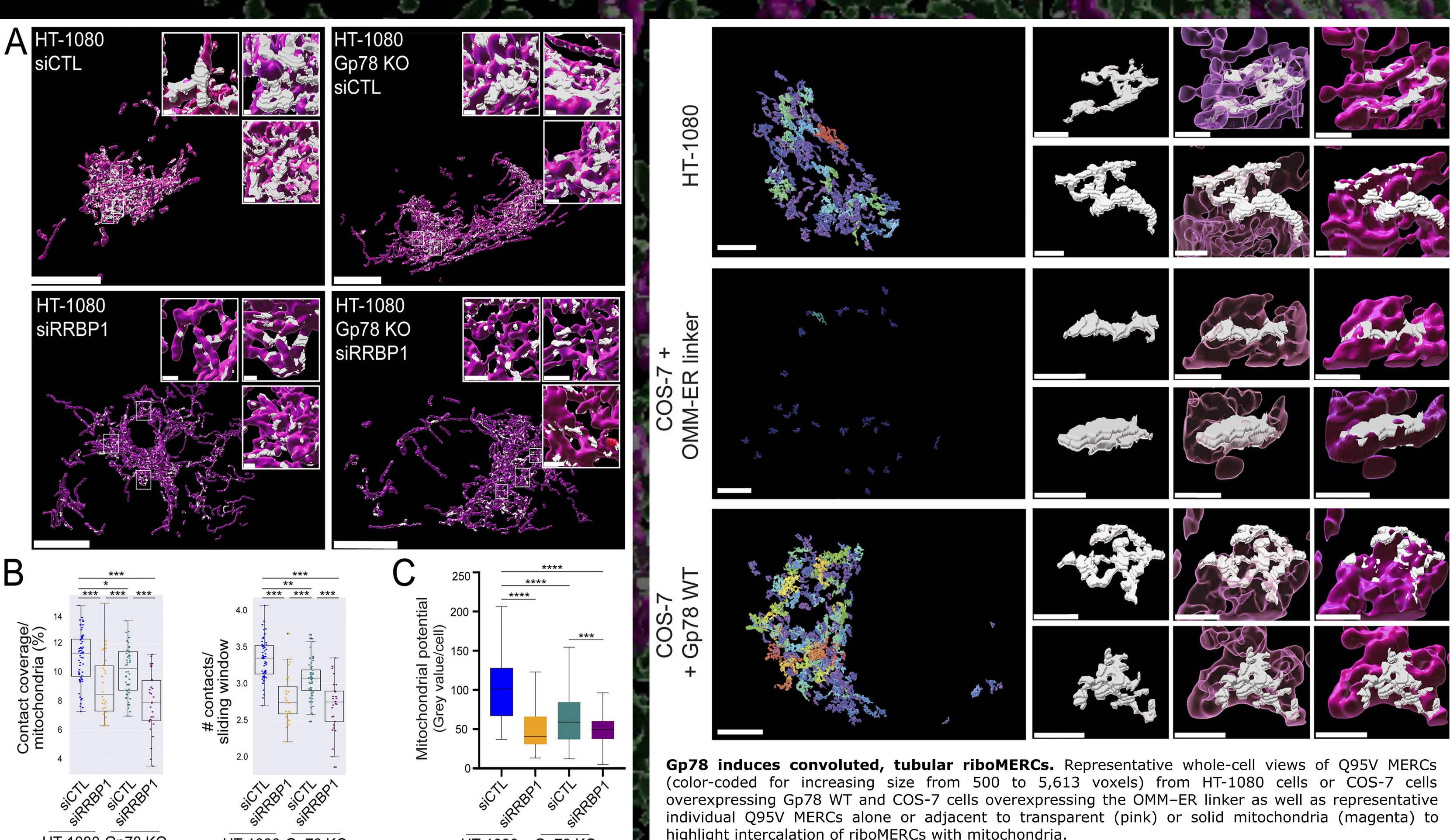
Two objects (red and green discs) are shown at corresponding sub-precision distances. Intensity profiles (top row), second derivatives (Laplacian), and Spearman correlations of the negative part of the Laplacian (bottom row) are shown. Note how the Spearman response overlaps and changes consistently with the sub-precision distance.

RIGHT (A) Volume-rendered MCS-DETECT views of cells expressing ERmoxGFP (green) and labeled for TOM20 (magenta) with contact sites overlaid (white) are shown for COS-7, HT-1080, and OMM–ER linker transfected COS-7 cell ROIs from the whole view image are shown volume rendered in adjacent panels. COS-7 mitochondria display numerous small contact zones while mitochondria in HT-1080 and OMM–ER linker transfected COS-7 cells present more extended contact zones (bar = 10 µm whole cell; 1 µm insets). (B) Mitochondria surface coverage ratio and the number of contacts per sampled mitochondria window are shown for contact zones in COS-7, HT-1080, and OMM–ER linker transfected COS-7 cells (averaged over cell, two-sided non-parametric Mann Whitney test, n = 3 independent biological replicates, ≥30 cells/condition per experiment; *P < 0.05; ***P < 0.001).

- ❖ MCS-DETECT identifies contact sites based on the negative Spearman correlation of the second differential, approximated by the Laplacian operator
- ❖ To validate contact site detection in the absence of ground truth, we compared HT-1080 and COS-7 cells, based on known differences by EM, and expressed an ER-OMM linker in COS7 cells to induce MERCs

PERSPECTIVE
AI analysis of super-resolution microscopy: Biological discovery in the absence of ground truth

Dual control of tubular riboMERCs

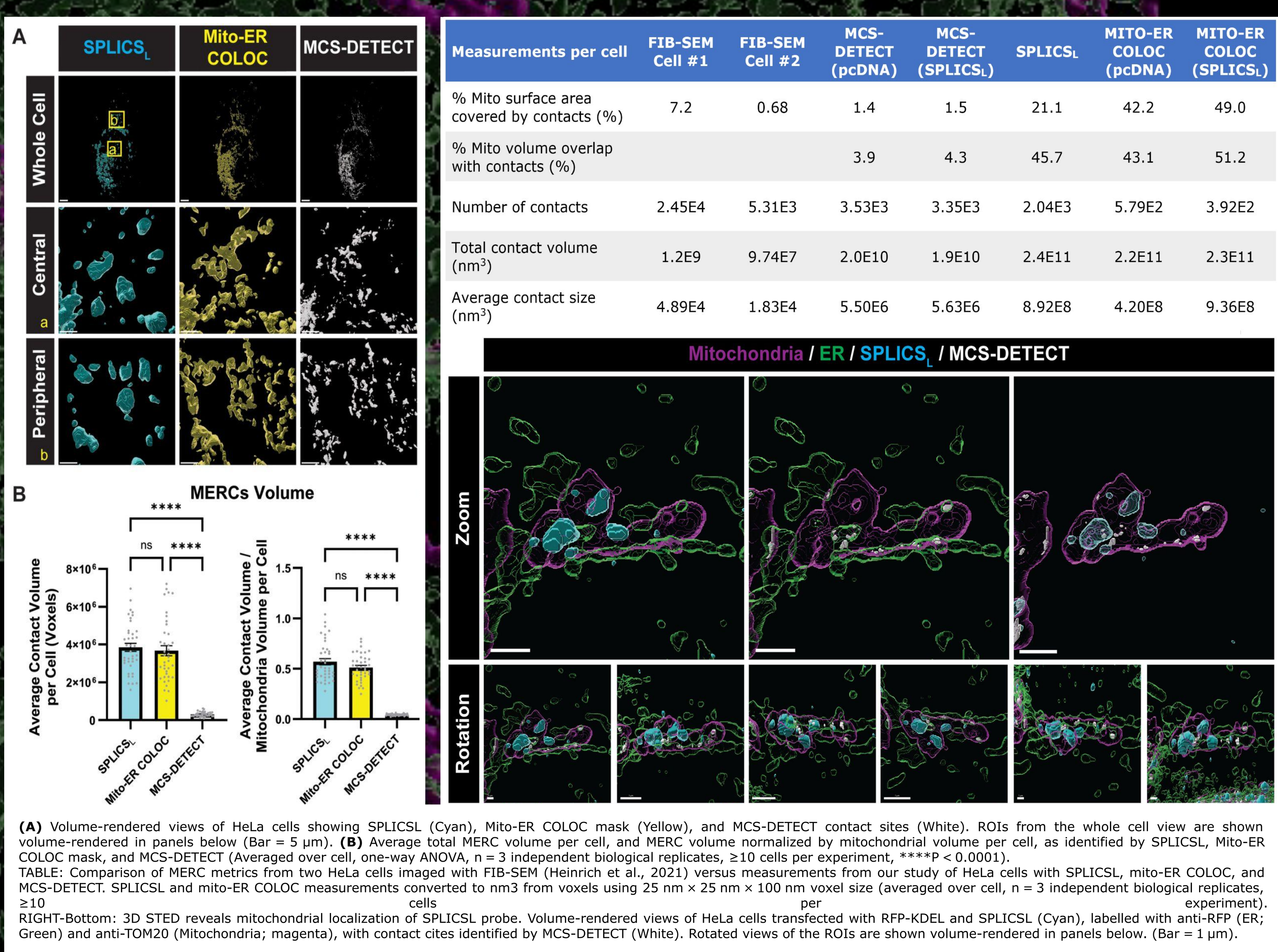


Gp78 induces convoluted, tubular riboMERCs. Representative whole-cell views of Q95V MERCs (color-coded for increasing size from 500 to 5,613 voxels) from HT-1080 cells or COS-7 cells overexpressing Gp78 WT and COS-7 cells overexpressing the OMM–ER linker as well as representative individual Q95V MERCs alone or adjacent to transparent (pink) or solid mitochondria (magenta) to highlight intercalation of riboMERCs with mitochondria.

MCS-DETECT captures MERC changes induced by RRBP1 knockdown. (A) Volume-rendered MCS-DETECT views of HT-1080 WT and Gp78 KO cells treated with either siControl or siRRBP1. Mitochondria are labeled with TOMM20 (red) and MERCS are visualized in white. Bar = 10 µm whole cell; 1 µm insets. **(B)** Mitochondria surface coverage ratio and the number of contacts per sampled mitochondria window are shown for contact zones in HT-1080 WT and Gp78 KO cells treated with either siControl or siRRBP1. Averaged over cell, two-sided non-parametric Mann–Whitney test, n = 3 independent biological replicates, ≥30 cells/condition per experiment; *P < 0.05; **P < 0.01; ***P < 0.001. **(C)** HT-1080 cells transfected with either siControl or siRRBP1 were labeled with MitoView633. Integrated density of MitoView633 per cell was quantified. n = 3 independent biological replicates; >50 cells/condition per experiment; ***P < 0.0001; ****P < 0.0001; Tukey post hoc test.

- ❖ Knockout and overexpression of Gp78 ubiquitin ligase controls riboMERC size
- ❖ Expression of the RRPB1-SYNJ2BP tether is required for riboMERC expression

More selective MERC detection by MCS-DETECT



Measurements per cell	FIB-SEM Cell #1	FIB-SEM Cell #2	MCS-DETECT (pcDNA)	MCS-DETECT ($SPLICS_L$)	$SPLICS_L$	MITO-ER COLOC (pcDNA)	MITO-ER COLOC ($SPLICS_L$)
% Mito surface area covered by contacts (%)	7.2	0.68	1.4	1.5	21.1	42.2	49.0
% Mito volume overlap with contacts (%)			3.9	4.3	45.7	43.1	51.2
Number of contacts	2.45E4	5.31E3	3.53E3	3.35E3	2.04E3	5.79E2	3.92E2
Total contact volume (nm^3)	1.2E9	9.74E7	2.0E10	1.9E10	2.4E11	2.2E11	2.3E11
Average contact size (nm^3)	4.89E4	1.83E4	5.50E6	5.63E6	8.92E8	4.20E8	9.36E8

(A) Volume-rendered views of HeLa cells showing SPLICSL (Cyan), Mito-ER COLOC mask (Yellow), and MCS-DETECT contact sites (White). ROIs from the whole cell view are shown volume-rendered in panels below (Bar = 5 µm). **(B)** Average total MERC volume per cell, and MERC volume normalized by mitochondrial volume per cell, as identified by SPLICSL, Mito-ER COLOC mask, and MCS-DETECT (Averaged over cell, one-way ANOVA, n = 3 independent biological replicates, ≥10 cells per experiment, ****P < 0.0001).
TABLE: Comparison of MERC metrics from two HeLa cells imaged with FIB-SEM (Heinrich et al., 2021) versus measurements from our study of HeLa cells with SPLICSL, mito-ER COLOC, and MCS-DETECT. SPLICSL and mito-ER COLOC measurements converted to nm3 from voxels using 25 nm × 25 nm × 100 nm voxel size (averaged over cell, n = 3 independent biological replicates, ≥10 cells per experiment).
RIGHT-Bottom: 3D STED reveals mitochondrial localization of SPLICSL probe. Volume-rendered views of HeLa cells transfected with RFP-KDEL and SPLICSL (Cyan), labelled with anti-RFP (ER; Green) and anti-TOM20 (Mitochondria; magenta), with contact cites identified by MCS-DETECT (White). Rotated views of the ROIs are shown volume-rendered in panels below. (Bar = 1 µm).

- ❖ MCS-DETECT mitochondria coverage was significantly smaller than colocalization analysis or SPLICSL, and closely matched contact site metrics obtained by 3D EM
- ❖ STED analysis localized a subset of the SPLICSL label to mitochondria

Comparative Analysis of SPLICS and MCS-DETECT for Detecting Mitochondria-ER Contact Sites (MERCs)

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MCS fluorescent detection challenges:

- ❖ **3D resolution**
  - ➢ Voxel-based super-resolution still limited to 100 nm lateral resolution, larger than the distance between organelles
  - ➢ Single particle tracking offers some possibilities
  - ➢ Analysis of super resolution should be based on interaction and not colocalization
- ❖ **Live cell**
  - ➢ Speed of acquisition ok for 2D but whole cell 3D live cell imaging remains slow (1-2 seconds/stack)
  - ➢ Light sheet can address the speed issue but not the resolution issue
  - ➢ Need to validate that fluorescent reporters (organelle targeted and bicomplementation) do not impact organelles and faithfully reproduce contacts
- ❖ **Multi-organelle contacts**
  - ➢ Ideally we should image multiple organelles (à la Sarah Cohen) at a resolution sufficient to detect all contacts between all organelles but see above …
  - ➢ DNA-PAINT offers some interesting possibilities but limited to fixed cells
- ❖ **Validation: how do we know that detected contacts are real?**
  - ➢ Gold standard is correlative light electron microscopy (CLEM) but it is limited to fixed cells
  - ➢ Our validation approach is based on weak supervision, identifying differences between groups, and was successful for riboMERCs that present very distinct shapes and features, but how useful will it be for smaller MERCs?

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Review
Closing the multichannel gap through computational reconstruction of interaction in super-resolution microscopy
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