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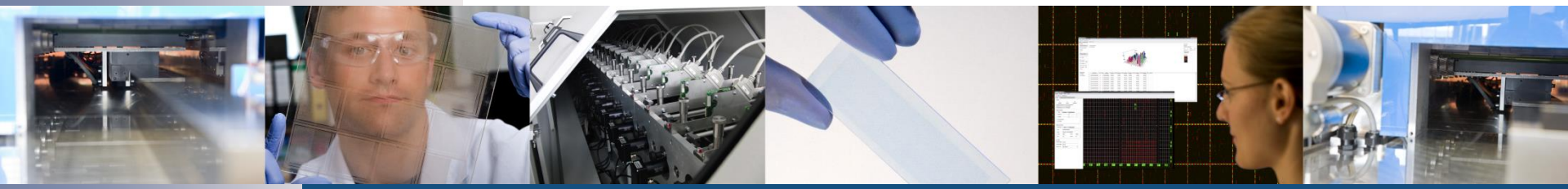

Comparison of High Resolution PEPperMAP® Epitope Mappings with Low Resolution Epitope Mappings

PEPperPRINT GmbH

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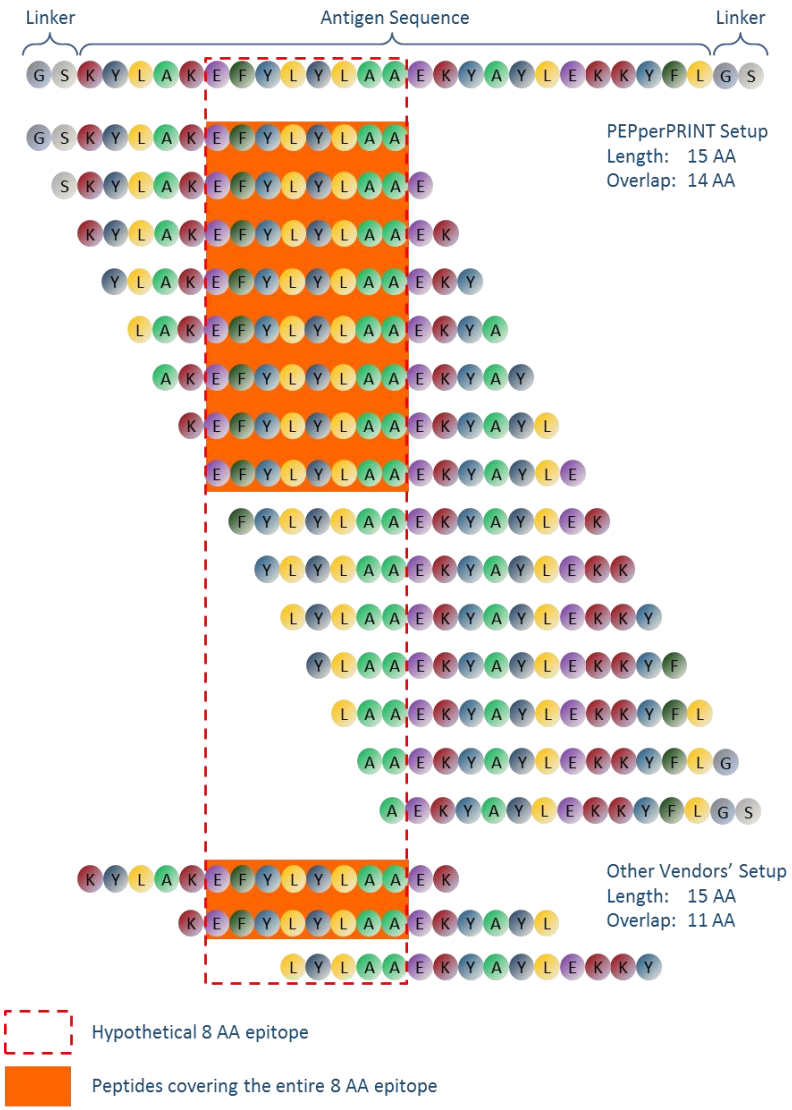
Introduction

For peptide microarray-based epitope mappings, an antigen sequence is usually translated into overlapping peptides. To save costs, the peptide-peptide overlap is frequently reduced to e.g. 10 or 11 amino acids with 15mer peptides, as provided by most commercial suppliers. The peptide-peptide overlap, however, plays an important role for epitope prediction. A reduced peptide-peptide overlap hampers the exact identification of conserved core motifs, the unambiguous itemization of adjacent epitopes in polyclonal samples, or the discrimination of non-specific binders from real epitopes.

Due to our proprietary laser printer-based on-chip synthesis, PEPperPRINT is able to routinely translate antigens into peptide microarrays with a maximum peptide-peptide overlap, e.g. 14 amino acids with 15mer peptides, to provide the maximum amount of epitope coverage and prediction at reasonable costs. Therefore, PEPperMAP® Epitope Mappings are typically done with PEPperCHIP® Peptide Microarrays based on a given antigen translated into overlapping peptides with a maximum peptide-peptide overlap.

To demonstrate the influence of the peptide-peptide overlap on epitope mapping and prediction, we generated peptide microarrays with antigen CENPA translated into overlapping peptides. The systemic sclerosis antigen consists of 140 amino acids that were further elongated by neutral GSGSGSG linkers at the N- and C-terminus to avoid truncated peptides. Based on 15mer peptides, we selected a maximum peptide-peptide overlap of 14 amino acids resulting in 140 different peptides per array, and compared it with a reduced overlap of 11 amino acids (36 different peptides per array). The resulting peptide microarrays were assayed with two different systemic sclerosis patient sera to investigate the prediction of CENPA autoantibody epitopes in relation of the peptide-peptide overlap.

Overlapping Scheme



The scheme on left highlights how the peptide-peptide overlap affects the number of peptides and data points based on a hypothetical 25 amino acid antigen elongated by C- and N-terminal GS linkers. Based on 15mer peptides and a peptide-peptide overlap of 14 amino acids, the hypothetical 8mer epitope EFYLYLAA (red dotted box) is covered by 8 adjacent peptides (orange box) with detailed and clear information on the epitope length and the conserved core motif.

Based on 15mer peptides and a peptide-peptide overlap of only 11 amino acids, however, the hypothetical 8mer epitope EFYLYLAA (red dotted box) is only covered by 2 adjacent peptides (orange box) with unclear epitope boundaries, since both adjacent peptides contain the hypothetical epitope sequence in the middle.

Materials and Methods

Microarray Content:	The sequence of the antigen CENPA was translated into 15mer peptides with peptide-peptide overlaps of 14 and 11 amino acids resulting in arrays with 140 and 36 different peptides printed in duplicate (280 or 72 peptide spots each array). The corresponding peptide arrays were further framed by Flag and HA control peptides (74 spots each control peptide for 14 aa overlap arrays, and 70 spots each control peptide for 11 aa overlap array).
Samples:	Human systemic sclerosis patient sera SSc1 and SSc2
Incubation Buffer:	PBS, pH 7.4 with 0.05% Tween 20 and 10% Rockland blocking buffer
Washing Buffer:	PBS, pH 7.4 with 0.05% Tween 20 (3x1 min after each assay)
Blocking Buffer:	Rockland blocking buffer MB-070 (30 min before the first assay)
Assay Conditions:	Serum dilution of 1:5000 in incubation buffer; incubation for 16 h at 4°C and shaking at 140 rpm
Secondary Antibody:	Goat anti-human IgG(H+L) conj. DyLight680; 30 min staining at RT and a dilution of 1:5000
Control Antibodies:	Monoclonal anti-HA (12CA5)-DyLight680, monoclonal anti-FLAG(M2)-DyLight800; staining in incubation buffer at a dilution of 1:1000 for 1 h at RT
Scanner:	LI-COR Odyssey Imaging System; scanning offset 1 mm, resolution 21 µm, scanning intensities red/green of 7/7
Microarray Data:	Microarray Data CENPA High Resolution Mapping.xlsx, Microarray Data CENPA Low Resolution Mapping.xlsx
Microarray Identifier:	000725_04 and 000725_05 (five array copies each microarray)

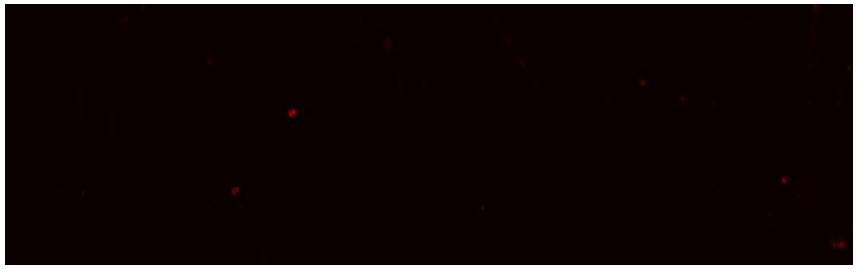
Experimental and Data Analysis

Pre-staining of both peptide array variants was done with the goat anti-human IgG(H+L) conj. DyLight680 antibody at a dilution of 1:5000 to investigate background interactions with the CENPA-derived peptides that could interfere with the main assays. Subsequent incubation of the peptide microarrays with human sera SSc1 and SSc2 at dilutions of 1:5000 in incubation buffer was followed by staining with the secondary antibody and read-out at a scanning intensity of 7 (red). HA and Flag control peptides framing the peptide arrays were finally stained as internal quality control to confirm the assay quality and the peptide microarray integrity (scanning intensities red/green: 7/7).

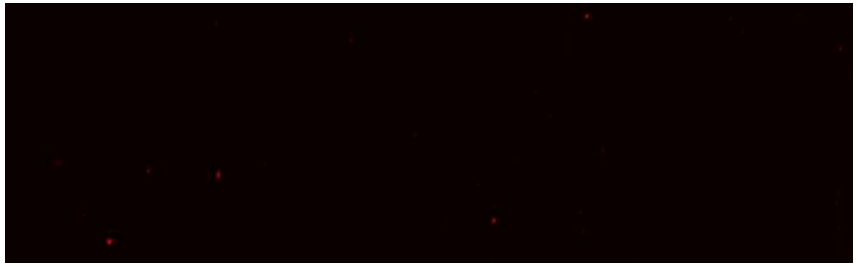
Quantification of spot intensities and peptide annotation were done with PepSlide® Analyzer and summarized in the Excel files Microarray Data CENPA High Resolution Mapping.xlsx and Microarray Data CENPA Low Resolution Mapping.xlsx. A software algorithm breaks down fluorescence intensities of each spot into raw, foreground and background signal (see “Raw Data” tabs), and calculates the standard deviation of foreground median intensities (see “Mapping Summary” tabs). Based on averaged foreground median intensities, intensity maps were generated and binders in the peptide maps highlighted by an intensity color code with red for high and white for low spot intensities.

We further plotted averaged spot intensities of all assays against the antigen sequence from the N- to the C-terminus of CENPA to visualize overall spot intensities and signal to noise ratios (see “Intensity Plot” tabs). The intensity plots were correlated with peptide and intensity maps as well as with visual inspection of the microarray scans to identify peptides and consensus motifs that interacted with the plasma samples. In case it was not clear if a certain amino acid contributed to antibody binding, the corresponding letters were written in grey.

Pre-Staining with the Secondary Ab



14 aa overlap CENPA arrays:
goat anti-human IgG(H+L) conj. DyLight680 antibody, 1:5000

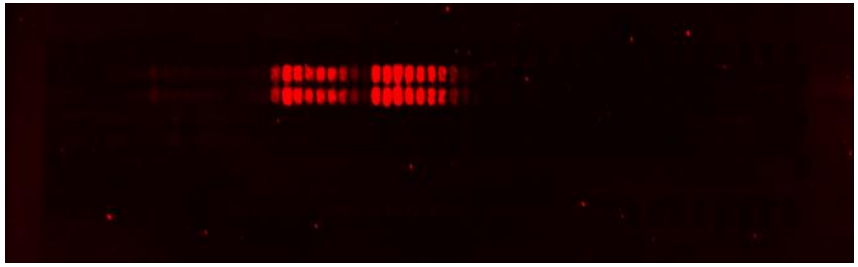


11 aa overlap CENPA arrays:
goat anti-human IgG(H+L) conj. DyLight680 antibody, 1:5000

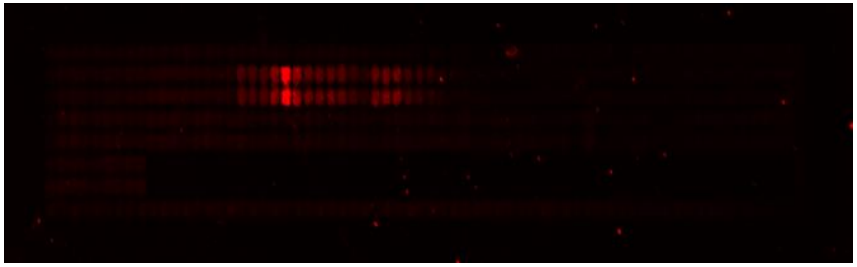
After 10 min pre-swelling in standard buffer and 30 min in blocking buffer, both peptide array variants were initially incubated with the secondary goat anti-human IgG(H+L) conj. DyLight680 antibody at a dilution of 1:5000 for 30 min at room temperature to analyze background interactions with the antigen-derived peptides. At scanning intensity of 7, we did not observe any background due to non-specific binding of the secondary antibody on both peptide arrays. Data quantification with PepSlide® Analyzer was neither possible nor required, since the absence of any spot pattern hampered alignment of the microarray grid.

Microarray Scans, 14 aa Overlap

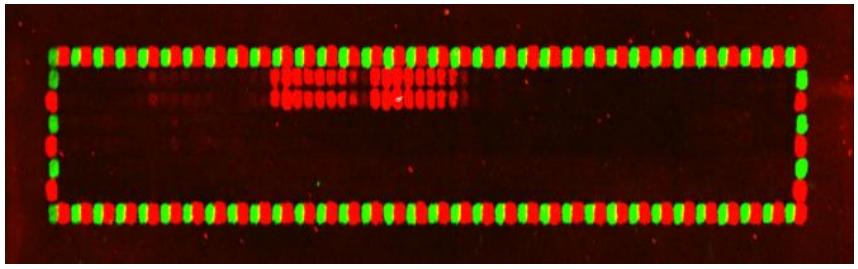
Sample SSc1



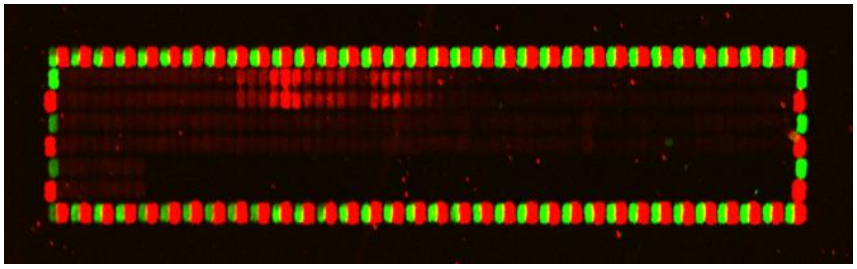
Sample SSc2



Control Staining



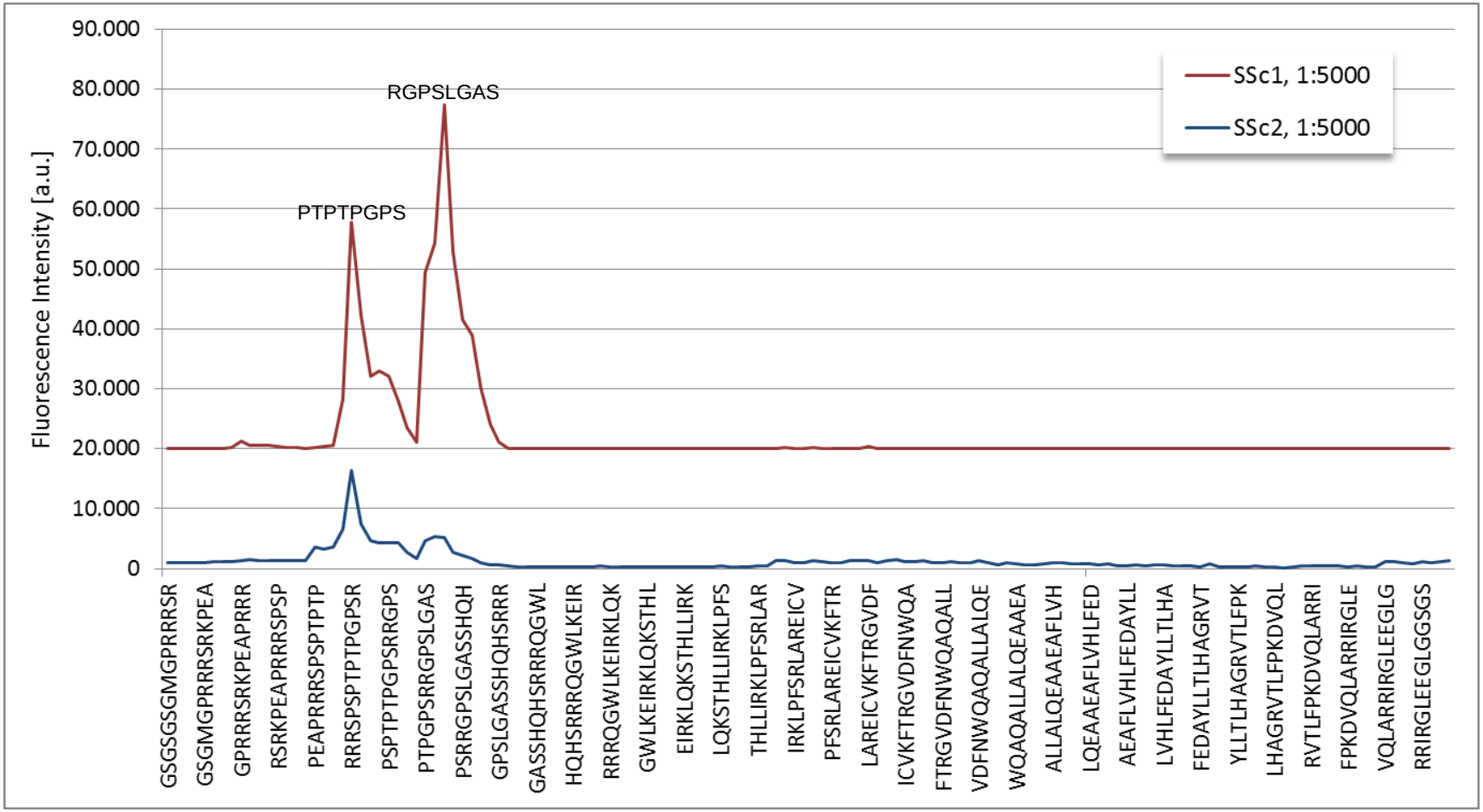
Control Staining



Two different peptide microarrays with maximum peptide-peptide overlap of 14 aa were incubated with human serum SSc1 and SSc2 at a dilution of 1:5000 in incubation buffer. After each incubation, staining with the secondary goat anti-human IgG(H+L) conj. DyLight680 antibody was followed by read-out at a scanning intensity of 7.

On both arrays, we observed strong and well-defined interactions with two clear epitope-like spot patterns formed by rows of adjacent peptides with a consensus motif. The final staining of the HA and Flag control peptides framing the peptide arrays gave rise to the expected and well-defined spot pattern and validated the overall peptide microarray integrity.

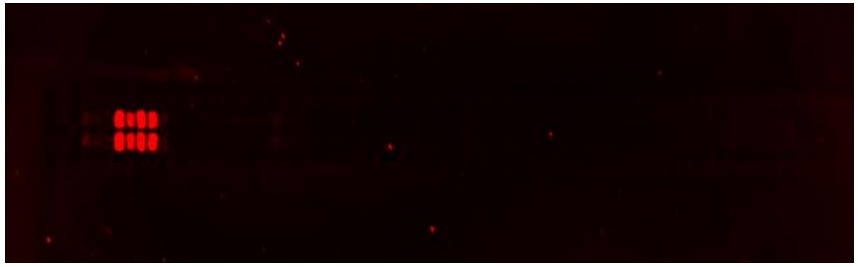
Intensity Plots, 14 aa Overlap



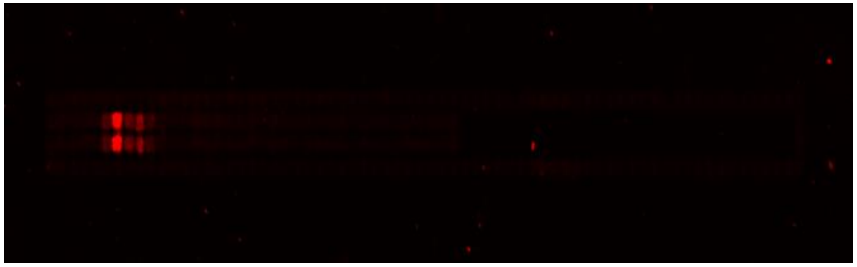
The intensity plots of the CENPA peptide arrays highlighted the very strong response of serum SSc1 with spot intensities close to the limit of the dynamic range of the microarray scanner. Please note that the SSc1 plot was leveled to 20.000 a.u. to provide a clearer data overview. Compared to sample SSc1, sample SSc2 showed a slightly weaker response. We clearly observed two different epitopes close to the N-terminus of CENPA, which can be easily identified due to the maximum peptide-peptide overlap of 14 aa; the consensus motifs of both epitopes were PTPTPGPS and RGPSLGAS.

Microarray Scans, 11 aa Overlap

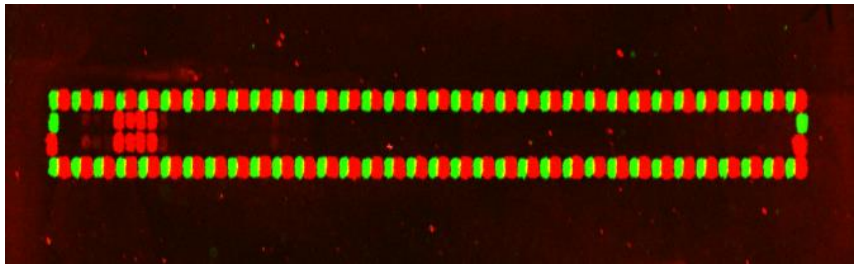
Sample SSc1



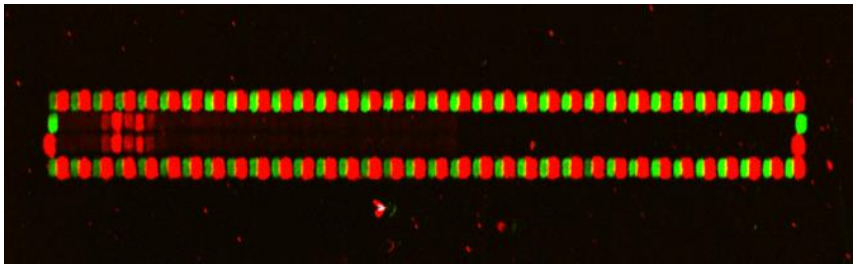
Sample SSc2



Control Staining



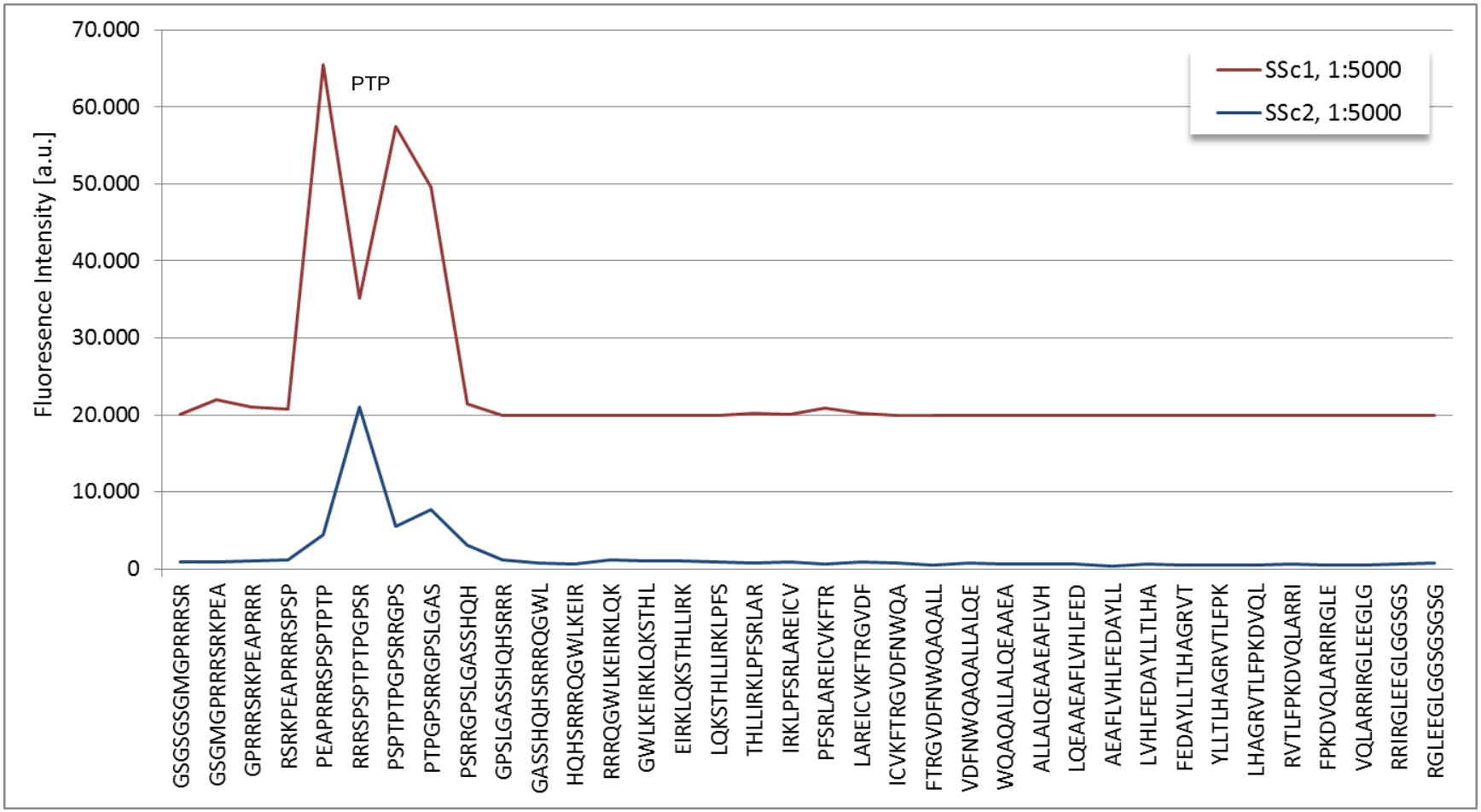
Control Staining



Two different peptide microarrays with a low peptide-peptide overlap of 11 aa were incubated with human serum SSc1 and SSc2 at a dilution of 1:5000 in incubation buffer. After each incubation, staining with the secondary goat anti-human IgG(H+L) conj. DyLight680 antibody was followed by read-out at a scanning intensity of 7.

On both arrays, we observed a single strong and well-defined interaction with a clear epitope-like spot pattern formed by a row of adjacent peptides with a consensus motif. The final staining of the HA and Flag control peptides framing the peptide arrays gave rise to the expected and well-defined spot pattern and validated the overall peptide microarray integrity.

Intensity Plots, 11 aa Overlap



The intensity plots of the CENPA peptide arrays highlighted the very strong response of serum SSc1 with spot intensities close to the limit of the dynamic range of the microarray scanner. Please note that the SSc1 plot was leveled to 20.000 a.u. to provide a clearer data overview. Compared to sample SSc1, sample SSc2 showed slightly weaker response. In contrast to the high resolution epitope mapping, however, we only observed a single main signal close to the N-terminus of CENPA corresponding to a single apparent consensus motif PTP; the two different N-terminal epitopes cannot be resolved with a reduced overlap of 11 amino acids.

Conclusion

The autoimmune epitope mappings of human systemic sclerosis patient sera SSc1 and SSc2 were done against antigen CENPA translated into 15mer peptides with a maximum peptide-peptide overlap of 14 amino acids as well as with a reduced peptide-peptide overlap of 11 amino acids. The high resolution and low resolution peptide microarrays with the antigen-derived peptides were incubated with the human sera at a dilution of 1:5000 followed by staining with the secondary goat anti-human IgG(H+L) conj. DyLight680 antibody and read-out with a LI-COR Odyssey Imaging System. Quantification of spot intensities and peptide annotation were done with PepSlide® Analyzer.

Sera SSc1 and SSc2 generally showed strong responses with excellent signal-to-noise ratios against the N-terminal region of antigen CENPA. While serum SSc1 exhibited spot intensities close to the detection limit of the microarray scanner, serum SSc2 was slightly less reactive. We clearly identified two different N-terminal epitopes in each serum with the maximum peptide-peptide overlap of 14 amino acids: PTPTPGPS and RGPSLGAS in serum SSc1, and very similar SPTPTP as well as PSRRGPSLGAS in serum SSc2. With a reduced overlap of 11 amino acids, we observed only one signal with an apparent single epitope PTP in each serum. Only the curve shape in the intensity plot hints at the presence of two different epitopes, which cannot be separated due to reduced peptide-peptide overlap.

The reduced peptide-peptide overlap of 11 amino acids, as provided by most commercial suppliers, clearly had a negative and inaccurate impact on the outcome of such low resolution epitope mappings. A high resolution epitope mapping with a maximum peptide-peptide overlap and the full peptide coverage, however, enabled the detailed and comprehensive identification of the epitopes of patient sera SSc1 and SSc2.

Our data shows that a maximum peptide-peptide overlap is required for the itemization of adjacent epitopes, the exact identification of conserved core motifs and the determination of the actual epitope length, and demonstrates the advantages of PEPperPRINT's cost-effective PEPperMAP® high resolution epitope mapping services.

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