APPLICATION NOTE

Fluorescent Multiplex Immunohistochemistry with Tyramide Signal Amplification

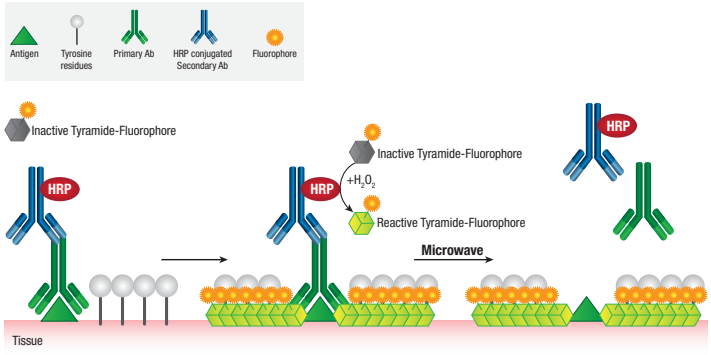
Introduction

Fluorescent multiplex immunohistochemistry (mIHC) is a method that enables simultaneous detection of multiple proteins of interest in formalin-fixed paraffin-embedded (FFPE) tissue sections. There are various approaches to fluorescent multiplexing:

1. **Direct immunofluorescence:** involves the use of multiple antigen-specific primary antibodies conjugated to distinct fluorophores. The disadvantage of this approach is limited sensitivity for targets of low abundance due to lack of signal amplification.
2. **Indirect immunofluorescence:** antigen detection is mediated via conjugated secondary antibodies specific to the species of the host in which each primary antibody was raised. This approach provides modest signal amplification but is limited by the number of available host species, e.g. rabbit, mouse, rat, and others.
3. **Deposition assays:** involve the use of enzyme-labeled antibodies and tyramide-fluorophore conjugates. This approach is unhindered by host species and isotype concerns, while providing ample signal amplification. It is the focus of this Application Note.

Tyramide-based fluorescent mIHC involves HRP-catalyzed deposition of fluorophore-conjugated tyramide molecules at the site of the antigen or in its immediate vicinity (**Figure 1**). This can markedly enhance the level of signal amplification. Moreover, tyramide deposition is mediated through its covalent binding to tyrosine residues on the antigen and its neighboring proteins. The permanent nature of this binding allows for heat-mediated removal of primary/secondary antibody pairs, while preserving the fluorescence signal associated with the antigen. This facilitates the sequential use of multiple primary antibodies of the same host species or isotype without the concern for crosstalk, thereby greatly enabling the potential to multiplex.

Figure 1: Basic principles of tyramide-based fluorescent mIHC



While chromogenic IHC is compatible with a limited degree of multiplexing, there are numerous benefits to adopting a multiplexing approach to IHC that relies on tyramide-based fluorescent detection (see **Table 1**). Firstly, patient tissue samples are rare, thus collecting maximal information from a single tissue section is of great value. Secondly, concurrent examination of 5 or more proteins/biomarkers, their spatial relationship and frequency of co-expression, all in the context of preserved tissue architecture, can offer insight into disease progression. In addition, multiple primary antibodies of the same species/isotype can be used when multiplexing in a serial fashion, something that is difficult to achieve when doing mIHC relying on signal amplification approaches that do not involve tyramide. This greatly simplifies panel design. Notably, multiplex detection of 5 or more proteins often relies on utilizing fluorophores with overlapping emission spectra. This mandates spectral unmixing, which not only ensures that the signal from each protein of interest is differentiated from the rest, but also provides the capability to take into account and subtract the signal arising from tissue autofluorescence across the entire visible spectrum.

The benefits of this methodology combined with the development of automated slide scanning platforms as well as software for quantitation are making fluorescent mIHC an increasingly powerful tool in the analysis and characterization of disease progression.

Table 1: Comparison of key features provided by fluorescent versus chromogenic multiplex immunohistochemistry

Fluorescent mIHC	Chromogenic mIHC
Fluorescent readout using widefield or confocal fluorescence microscopy	Chromogenic readout using brightfield microscopy
Detection of > 2-6 targets (5 + DNA)	Detection of up to 4 targets
Autofluorescence prevalent in violet, blue, and green channels	Autofluorescence is not an issue
Tissue architecture preserved but not visible	Tissue architecture preserved and visible
Robust resolution of co-expressed proteins as well as proteins that do not co-localize	Robust resolution of proteins that do not localize to the same sub-cellular compartment
Software available that can streamline quantitation, providing an objective analysis of the level of target expression and co-localization	Semi-quantitative readout, subjective interpretation of key parameters, such as the level of target expression, spatial proximity and co-localization

This Application Note aims to demonstrate the optimization steps that need to be carried out to successfully perform a fluorescent multiplex IHC experiment using an antibody panel to detect the following targets: PD-L1, B7-H4, FoxP3, CD8α, and Cytokeratin (CK) in FFPE sections of a human ovarian serous carcinoma. Note that a nuclear counterstain was performed with the inclusion of DAPI. While this panel is designed for researchers interested in the field of tumor immunology, multiplexing with specifically tailored panels can be applied to any field or disease of interest.

Method

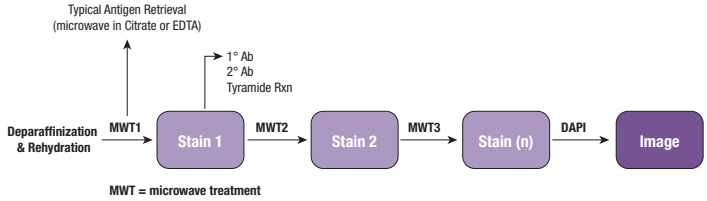
Tissue sections from a human ovarian serous carcinoma were processed and analyzed in the following manner (see **Figure 2** for an overview):

1. **Deparaffinization/Rehydration:** to prepare for antigen retrieval, tissue sections on slides were deparaffinized and rehydrated with the use of xylene and successive treatments with ethanol and dH_2O, respectively.
2. **Antigen Retrieval:** extensive optimization was performed to ensure maximal unmasking of each epitope to allow for efficient binding of the primary antibody.

3. **Antibody Titration:** prior to staining, the optimal dilution for each primary antibody was determined empirically to ensure maximal fluorescence intensity and minimal background signal for each target of interest.
4. **Staining:** incubation with primary antibodies was performed under humidified conditions at room temperature using SignalStain® Antibody Diluent #8112. Subsequent incubation with SignalStain® Boost IHC Detection Reagent (HRP, Mouse) #8125 or (HRP, Rabbit) #8114 was performed.
5. **Image Acquisition/Analysis:** the Nuance® multispectral slide analysis system (PerkinElmer) was used. Note that other multispectral imaging platforms such as Vectra®, Mantra®, and others can also be used.

   Multispectral images were processed using the inForm® Tissue Finder™ pattern recognition software (PerkinElmer).

Figure 2: Schematic representation of the fluorescent mIHC workflow



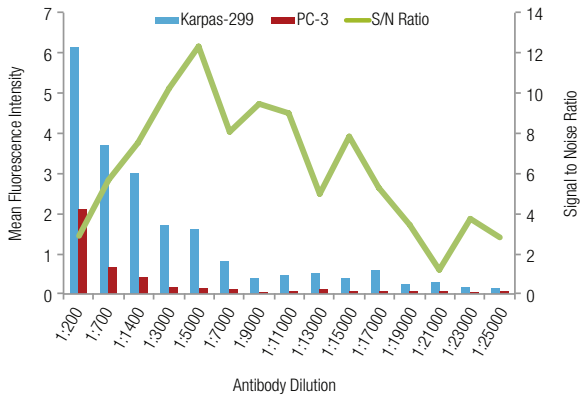
Results

1. **Titration:** Prior to performing a multiplex experiment, the optimal concentration of each primary antibody needs to be determined in a singleplex setting, wherein a single target is to be detected. To this end, an extensive dilution series of the primary antibody targeting human PD-L1 was conducted to determine the dilution point that gives rise to maximal fluorescence signal intensity combined with the highest signal to noise (S/N) ratio (**Figure 3**). This was done on sections of paraffin-embedded cell pellets known to express PD-L1 at high levels (Karpas-299) as well those with negligible levels of PD-L1 expression (PC-3) as positive and negative controls, respectively.

   At 1:1400 the signal strength (represented as mean fluorescence intensity per cell) and S/N ratio are sufficiently high, making this an optimal dilution for this particular antibody. While S/N continues to rise at higher dilutions, signal intensity declines significantly, making dilutions beyond this point sub-optimal.

   We highly recommend applying this approach to establish the optimal dilutions for all antibodies to be used in a multiplex experiment.

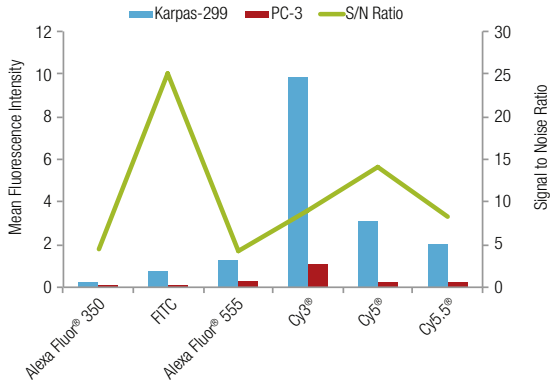
Figure 3. PD-L1 E1L3N® Titration in Cy5®



2. **Antibody Fluorophore Pairing:** The objective of this optimization step is to achieve a balance of signal intensities within the panel such that the fluorescence signal originating from targets of high abundance does not overshadow that of targets with lower abundance. To this end, it is good practice to pair antibodies detecting targets with low expression with the brightest fluorophores and vice versa. We recommend analyzing a matrix composed of optimized primary antibodies and each available fluorophore.

   Figure 4 shows that when paired with PD-L1, Cy3® clearly gives rise to the highest signal intensity, but exhibits a low S/N ratio. FITC, on the other hand, yields a higher S/N ratio but produces a weak fluorescence signal. Pairing with Cy5® yields a more desirable balance of signal versus noise exhibiting both moderate fluorescence signal intensity and S/N ratio (**Figure 4**). This makes Cy5® the most suitable fluorophore to pair with PD-L1.

Figure 4. PD-L1 E1L3N® Fluorophore Comparison



3. **Order Optimization:** The order in which antibodies in a multiplex panel are applied to a tissue section must be optimized to ensure that multiple rounds of heating do not compromise the integrity of the epitope of interest. The tolerance of the deposited fluorophore to successive rounds of heat treatment must also be considered. For this reason the signal intensity and S/N ratio generated by each primary antibody within each slot of the multiplex panel must be assessed using the pre-optimized antibody-fluorophore pairs to ensure that the fluorescence signal is not affected by the relative position within the panel. Note that for the purposes of order optimization each tissue section was stained only once and subjected to microwave treatment the same number of times irrespective of staining order (see **Table 2**). For instance, in the first position, the tissue was subjected to microwave treatment for 5 consecutive times after it was stained to detect PD-L1 with Cy5®, and in the last position (position 5) the unstained tissue was microwaved 5 times prior to staining and fluorophore deposition. In the first instance we are measuring the heat tolerance of the tyramide-Cy5® deposits, while in the second scenario we are assessing the resilience of the PD-L1 epitope. Both factors have the potential to influence signal intensity.

   As indicated in **Figure 5**, the signal intensity of deposited Cy5® at the site of antibody-bound PD-L1 decreases with each successive round of microwave treatment with the S/N ratio at lowest levels in the first and last slots in the panel. The highest S/N ratios are observed at positions 2 and 4, suggesting that the epitope for PD-L1 is minimally affected by heat treatment (**Figure 5**). When taking into consideration the optimal order and strength of signal intensity of the other antibodies within the panel PD-L1, when paired with Cy5®, is best positioned as second, even though this position yields moderate signal intensity.

Figure 5. PD-L1 E1L3N® Order Optimization

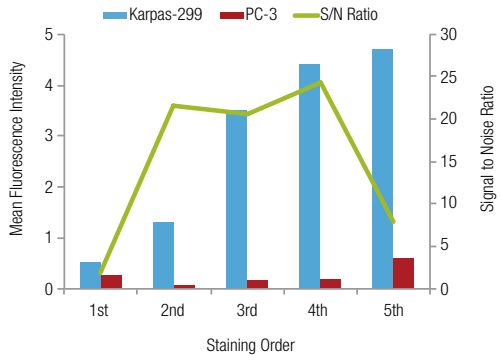


Table 2. The relationship between staining order and microwave treatment

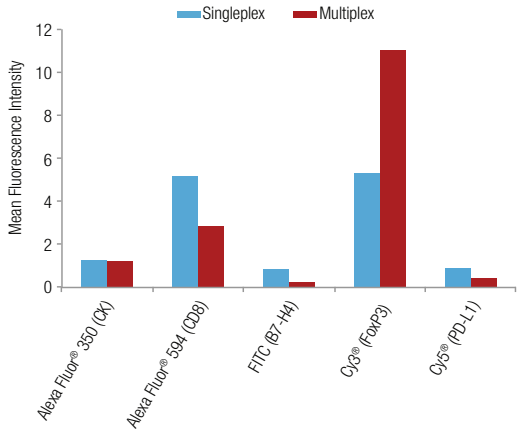
Staining Order	1st	2nd	3rd	4th	5th
Number of microwave treatments prior to incubation with primary antibody and fluorophore deposition	1	2	3	4	5
Number of microwave treatments after fluorophore deposition	5	4	3	2	1

4. **Construction of a Multispectral Library:** Fluorescence signal originating from each target and associated fluorophore within the panel was used to build a spectral library that would allow for linear unmixing when multiple antibodies/fluorophores were used in a multiplex fashion. In essence, this defines the emission spectra for each fluorophore and helps the imaging software recognize each fluorescence signal as distinct from the rest. A pseudocolor was arbitrarily assigned to each fluorophore. Notably, black pseudocolor was assigned to the full spectrum of autofluorescence originating from unstained tissue. This allowed for the artifactual signal associated with tissue autofluorescence to be subtracted during image acquisition and processing, one of the key benefits of multispectral imaging.

   Note that the assigned pseudocolors for the individual target/fluorophore pairs are listed in **Table 3**.

5. **Singleplex versus Multiplex Staining:** In an effort to understand how multiplex staining can affect the mean fluorescence intensity (MFI) obtained per target, a comparison was made whereby the signal intensity of each target of interest was examined in a singleplex (a single stain) or multiplex (multiple rounds of staining) context. Note that regardless of the multiplicity of the stain all stained tissue sections were heated and cooled the same number of times to eliminate variability due to microwave treatment. On average, in 4 out of 5 targets tested, multiplexing caused some level of decline in MFI (**Figure 6**). This could be attributed to a phenomenon known as 'antigen sheltering' whereby initial tyramide-fluorophore deposits may preclude subsequent deposition events at the site of a target or in its vicinity by masking target-specific epitopes.

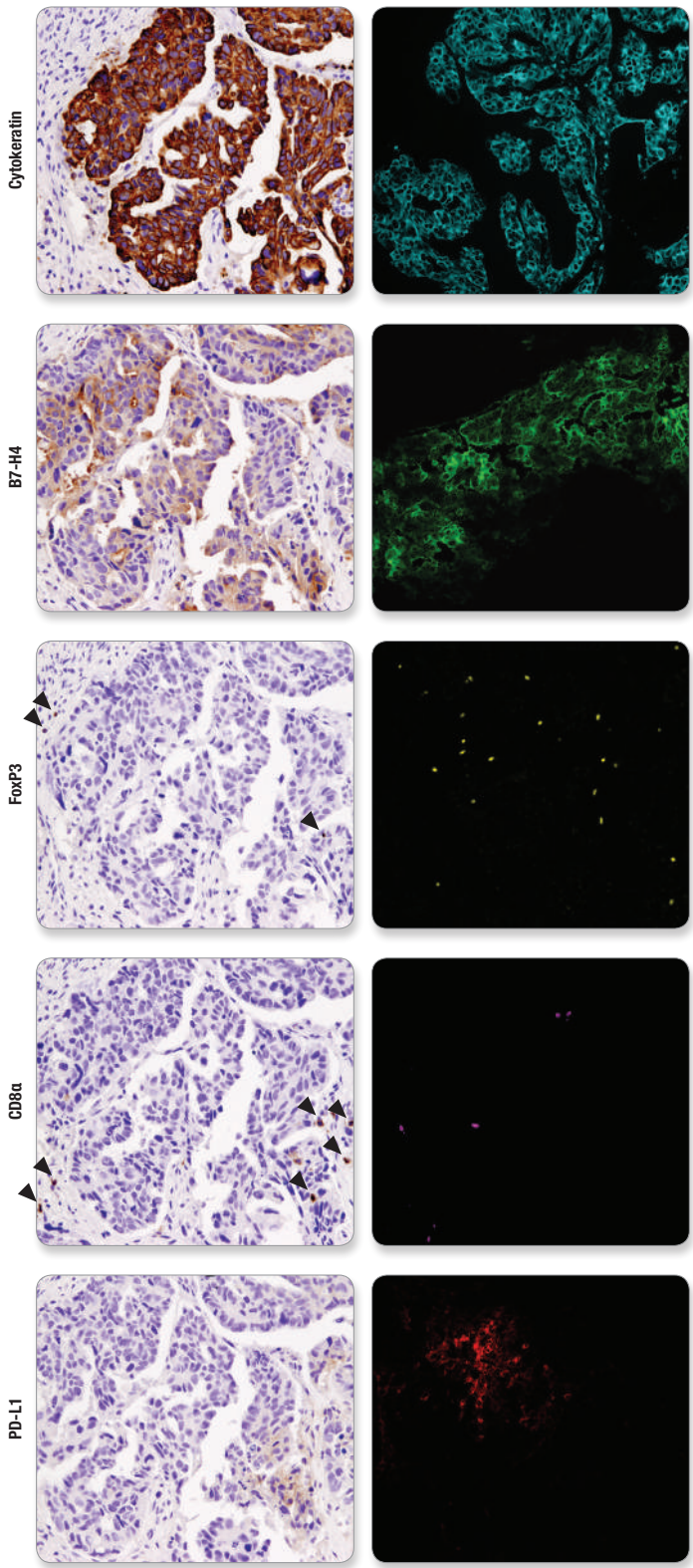
Figure 6. Singleplex vs. Multiplex Controls



6. **Chromogenic versus Fluorescent Detection:** Singleplex staining was performed on serial sections to evaluate the pattern and level of expression of individual proteins of interest using a chromogenic versus fluorescent detection system (**Figure 7**). Both detection systems reveal equivalent detail of protein level and distribution.

   Note that the images taken were of different fields of view. Arrows pinpoint cells expressing the indicated target.

Figure 7. Chromogenic vs. Fluorescent Detection of Individual Targets



7. Multiplex Staining (5 targets + nuclear counterstain): Tissue sections of a human ovarian serous carcinoma were stained with the multiplex panel described in detail in **Table 3**. An image composite is shown along with each individual unmixed stain comprising the composite image (**Figure 8**).

A panel of this composition can reveal the spatial relationship of tumor epithelial cells (often expressing cytokeratin), the components of the tumor microenvironment, and tumor infiltrating lymphocytes (TILs). Studies have shown that expression of PD-L1 in the tumor microenvironment is correlated with positive clinical outcome in response to immune checkpoint therapy (1). This information combined with the knowledge of the extent of tumor infiltration by TILs (the so called 'immunoscore') (2) and, importantly, the ratio of CD8 positive effector T cells versus FoxP3 positive regulatory T cells (3), can help better inform therapeutic strategies.

Figure 8. Fluorescent Multiplex IHC Analysis of a 5-Plex Panel (5 targets + a nuclear counterstain)

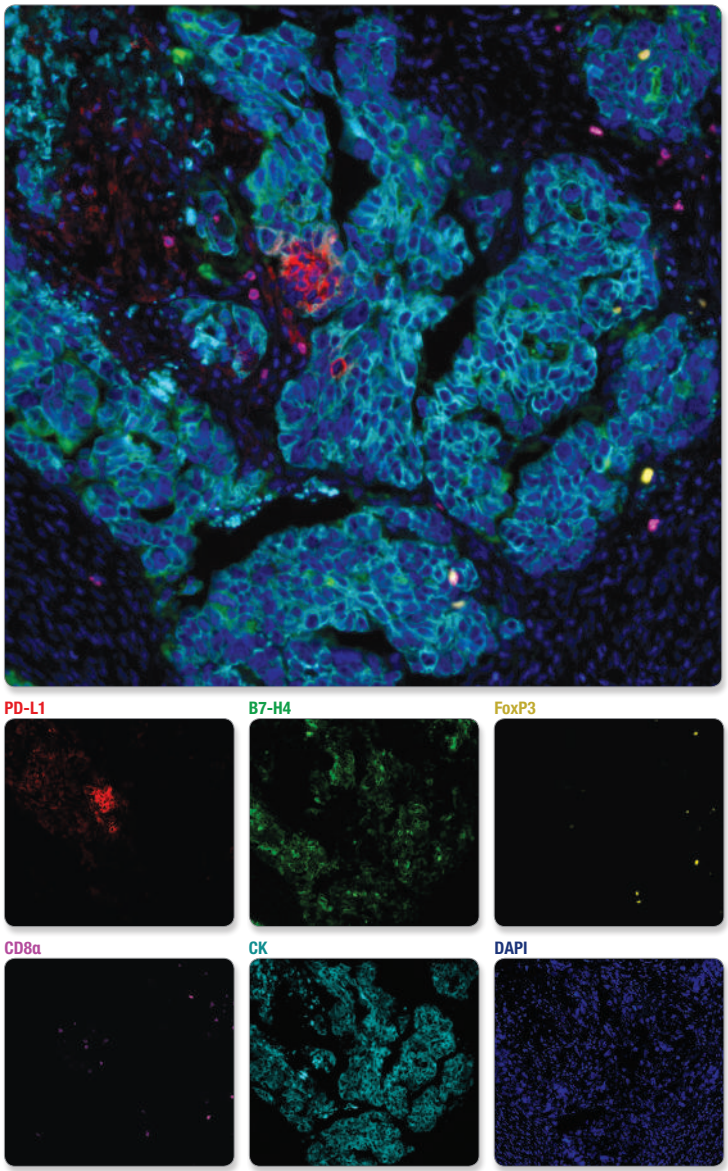


Table 3: Optimized conditions for staining with a 5-plex antibody panel: PD-L1, B7-H4, CD8, FoxP3, and Cytokeratin

CST Antibodies/ Reagents	Target	Cell Type	Dilution	Order	Fluorophore	Pseudocolor
CD8α (C8/144B) Mouse mAb (IHC Specific) #70306	CD8α	Cytotoxic T cells	1:200	1st	**Alexa Fluor® 594** TSA® Kit #25; T20935 (ThermoFisher Scientific)	Magenta
PD-L1 (E1L3N®) XP® Rabbit mAb #13684	PD-L1	Tumor cells and cells within the tumor microenvironment	1:1400	2nd	**Cy5®** TSA® Plus Cyanine 5 System #NEL745001KT (PerkinElmer)	Red
B7-H4 (D1M8I) XP® Rabbit mAb #14572	B7-H4	Tumor cells	1:1000	3rd	**FITC** TSA® Plus Fluorescein System #NEL741001KT (PerkinElmer)	Green
FoxP3 (D2W8E™) Rabbit mAb (IHC specific) #98377	FoxP3	Regulatory CD4 T cells	1:100	4th	**Cy3®** TSA® Plus Cyanine 3 System #NEL744001KT (PerkinElmer)	Yellow
Pan-Keratin (C11) Mouse mAb #4545	Cyto-keratin	Epithelial cells	1:500	5th	**Alexa Fluor® 350** TSA® Kit #17; T20927 (ThermoFisher Scientific)	Cyan
ProLong® Gold Antifade Reagent with DAPI #8961	DNA	Cell nuclei	Undiluted	Last	DAPI	Blue

Conclusion

In summary, fluorescent multiplex IHC involving HRP-catalyzed tyramide deposition offers several key advantages: heightened signal amplification for targets with low to moderate expression; simplified panel design wherein any primary antibody of choice, irrespective of host species or isotype, can be used; concurrent detection of multiple targets in a preserved tissue context, eliminating the need for large amounts of valuable tissue material and deepening our understanding of spatial organization and proximity of biomarkers critical to our understanding of disease mechanism and progression.

Importantly, it would be remiss not to stress that the use of primary antibodies rigorously validated for IHC on FFPE tissue and exhibiting exceptional sensitivity and specificity for the protein of interest is a prerequisite for successful and reliable fluorescent multiplex IHC staining.

References

1. Mahoney, K.M. and Atkins, M.B. (2014) *Oncology* 28, 39-48.
2. Galon, J. et al. (2014) *J.Pathol.* 232, 199-209.
3. Jacobs, J.F. et al. (2012) *Lancet Oncol.* 13, 32-42.

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