TECHNICAL NOTE

Top and bottom measurements of intracellular NanoBRET with the VICTOR Nivo.

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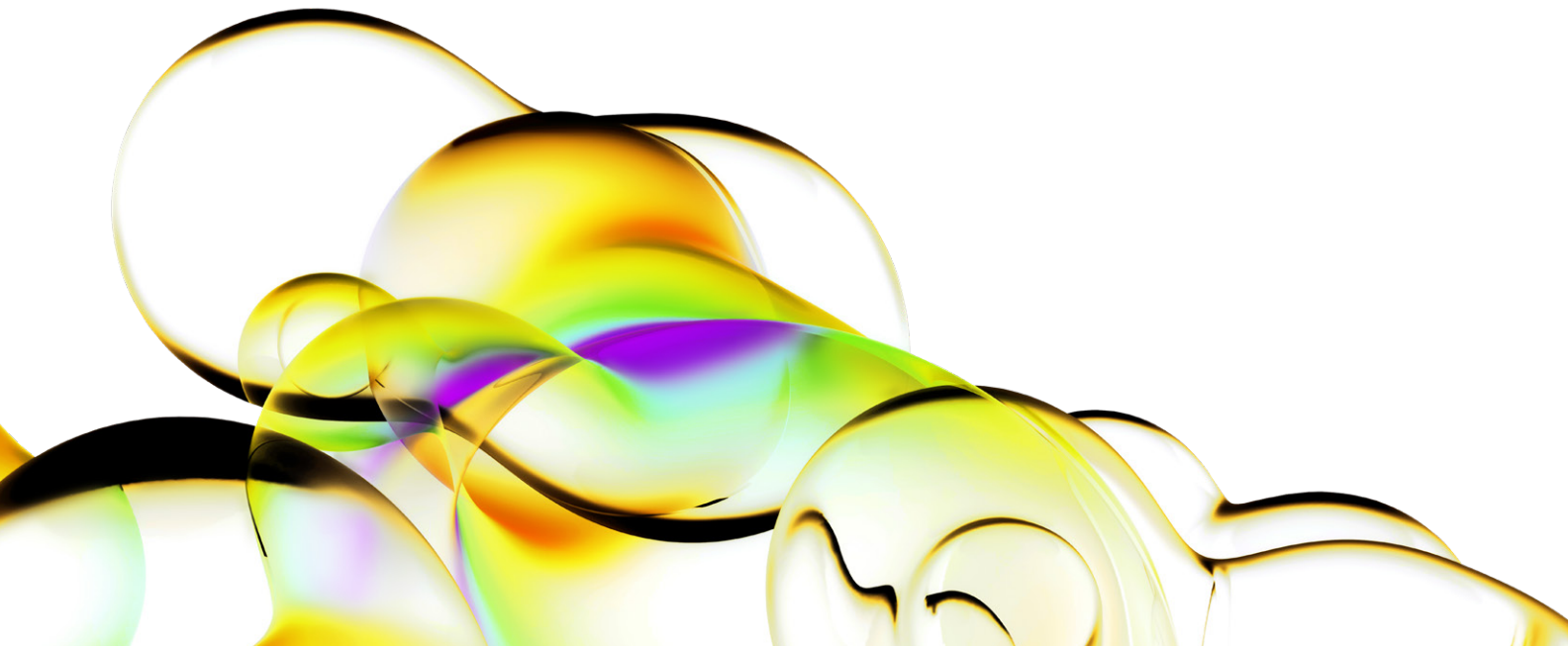
Introduction

Bioluminescence Resonance Energy Transfer (BRET) assays are gaining increased popularity amongst scientists. The small and very bright luciferase NanoLuc® used in BRET assays vastly increases the signal of these assays compared to standard assays using other bioluminescent proteins. Utilizing NanoLuc® leads to improved signal to background ratios and a broad dynamic range at low and more native protein expression levels. This even enables BRET imaging to study protein-protein interactions within individual cells.[1]

In this technical note, we demonstrate the top and bottom reading capabilities of the VICTOR Nivo™ Multimode Microplate Reader (Figure 1) to detect BRET within living cells using a fluorescent protein or fluorescent dye as acceptor molecules. The energy transfer from the donor NanoLuc® to either YFP or a commercially available HaloTag® Ligand as acceptors were detected and measured from the top in opaque white microplates usually used for Luminescence assays. Additionally, the BRET signal in the cells was examined in black plates with clear bottom that allow for bottom measurements on a plate reader and the determination of transfection efficiencies or orthogonal assay readouts in imaging devices.



VICTOR Nivo's browser based control software simplified the process of testing many different parameters. It enables combining kinetic reads for donor plus acceptor signal in top and bottom orientation in one measurement protocol. Furthermore, up to 32 filters are stored directly inside the flexible filter wheel and are automatically retrieved by the system when needed. And the direct export of results in Microsoft® Excel® format for further analysis provides an easy workflow.

Figure 1: The VICTOR Nivo Multimode Microplate Reader enables intracellular BRET and NanoBRET™ assays from the top and below the plate.

Materials and methods

Fusion proteins

Two independent experiments were performed. Experiment 1 used a custom YFP-NanoLuc® fusion protein to measure BRET from NanoLuc® to YFP and investigate its signal stability over time. The plasmids encoding the YFP-NanoLuc® fusion protein and the NanoLuc® control vector were published by Jiho Kim and Regis Grailhe.[1] Experiment 2 used the commercially available NanoBRET™ Positive Control Vector that encodes a NanoLuc®-HaloTag® fusion protein, tethering together the NanoLuc® donor and HaloTag® acceptor proteins to ensure efficient energy transfer (Promega, order number #N1581). The latter fusion protein was used to compare performance of two different plate types.

Cell preparation

Experiment 1

HeLa cells were transiently transfected to express either NanoLuc® or YFP-NanoLuc® fusion protein following the jetPEI® reverse transfection protocol for HTS (Polyplus Transfection, order number #101). For this 10 K cells per well were seeded in a black 384-well microplate with a clear plate bottom (Revvity CellCarrier Ultra, order number #6057300). Crosstalk between neighboring wells was excluded by seeding the cells at intervals of at least three wells. The final volume resulted in 50 µL per well and the cells were incubated overnight. Directly before measurements, 50 µL of Nano-Glo® Substrate (Promega, order number #N1571) was added from a 2x dilution in 1% FCS containing phenol red free medium to start the BRET experiment.

Experiment 2

HeLa cells were transiently transfected to express either NanoLuc® or NanoLuc®-HaloTag® fusion protein following the jetPEI® reverse transfection protocol for HTS (Polyplus Transfection, order number #101). 10 K cells per well were seeded in a black 384-well microplate with a clear plate bottom (Revvity CellCarrier Ultra, order number #6057300) and in a white opaque 384-well microplate (Revvity CulturPlate, order number #6007680). Crosstalk between neighboring wells was excluded and the cells were incubated overnight in 50 µL per well. An additional wash step prior to HaloTag® staining was done. HaloTag® NanoBRET™ 618 Ligand (Promega, order number #G980A) was titrated in medium with 1% FCS and without phenol red to 50 µL final solution per well. Six dilutions were applied (1:500, 1:1000, 1:2000, 1:4000, 1:8000 and 1:16000) and incubated for one hour, after which the cells were washed once with 1% FCS containing phenol red free medium. Directly before measurements, 50 µL Nano-Glo® Substrate (Promega, order number #N1571) was added from a 2x dilution in 1% FCS containing phenol red free medium to start the NanoBRET™ experiment.

VICTOR Nivo measurement

Optimal Z-focus height for both top and bottom measurements of each plate type were determined on a NanoLuc® positive sample using a Z-Focus scan on the VICTOR Nivo Multimode Microplate Reader. To test the BRET signal stability for Experiment 1, kinetic measurements were performed every two minutes for at least an hour.

Table 1: Measurement settings and filters on VICTOR Nivo that were used for experiment 1 and 2 (CWL = central wavelength, BW = bandwidth).

	Experiment 1: NanoLuc®/YFP	Experiment 2: NanoLuc®/ HaloTag® 618 Ligand
Measurement time	1000 ms	1000 ms
Emission spot size	2 mm	2 mm
Measurement type	Dual Emission	Dual Emission
Filter donor	NanoLuc®: 460/80 nm (CWL/BW) (Revvity, Order Number #HH35000920)	NanoLuc®: 460/80 nm (CWL/BW) (Revvity, Order Number #HH35000920)
Filter acceptor	YFP: 540/30 nm (CWL/BW) (Revvity, Order Number #HH35000926)	HaloTag® 618 Ligand: 645/75 nm (CWL/BW) (Revvity, Order Number #HH35000940)
Z-focus height top	8 mm	8 mm
Z-focus height bottom (if applicable)	0.6 mm	0.6 mm

Top and bottom measurements were combined in a single measurement protocol and due to the VICTOR Nivo's large filter storage wheel and automatic filter selection all filters were loaded into the instrument upfront. All measurement settings for both experiments are shown in Table 1. The data was exported in Excel format (.xlsx) directly from the VICTOR Nivo browser-based control software and analyzed using GraphPad Prism. Emission intensities of donor and acceptor and their acceptor/donor ratio per well were used for evaluation. Crosstalk into neighboring wells was calculated using the following equation: *Crosstalk [%] = (average (N) – average (E))/(average (S) – average (E))*100,* with N: empty neighbor wells above, below, left and right of sample well; E: empty wells far away from sample well and not influenced by crosstalk; S: sample well.

Results

Investigation of BRET signal stability (Experiment 1)

The emission signals of NanoLuc® and the BRET induced emission of YFP were measured one hour after NanoLuc® substrate was added. For this experiment a CellCarrier-384 Ultra Microplate (which has also been used successfully for BRET applications on the Revvity Operetta CLS™ High-Content Analysis System[2]) was used to allow top and bottom measurements (Figure 2). Both emission signals decline very similarly over time, but positive signals can be measured up to one hour after they begin. The raw results for measurements from the top of the plate are approximately 1.5 times higher than for bottom measurement, but the kinetic profiles of the samples are very similar for both measurement modalities. As BRET occurs only in cells expressing the YFP-NanoLuc® fusion protein, cells expressing NanoLuc® alone show a clear signal in the NanoLuc®, but only a weak crosstalk signal from NanoLuc® in the YFP channel (red curves). For both top and bottom measurements, the difference in signal levels of the two replicates for YFP-NanoLuc® expressing cells (green curves) are based on varying transfection efficiencies and hence unequal YFP-NanoLuc® expression levels in the two wells (confirmed on Operetta CLS™, data not shown). In contrast, the NanoLuc® expressing cells (red curves) show such variation only for the top measurement. This can be explained by air bubbles which can cause this deviation.

A common approach to evaluate BRET measurements is using the acceptor/donor ratio. Although the raw NanoLuc® and YFP signals decrease (Figure 2), the YFP/NanoLuc® ratio stays very stable over time at 0.5 for YFP-NanoLuc® cells and 0.05 for the control cells expressing NanoLuc® only (Figure 3). This indicates that manual dispensing of the substrate is sufficient for this experiment and dispensers are not needed. Furthermore, the ratio of both replicates results in almost identical curves compared to the high variation in intensity between the replicates visible in Figure 2. This demonstrates the advantage of referencing the acceptor signal to the amount of donor signal, because the data quality becomes independent on the measurement time after substrate addition, the expression levels or the measurement orientation.

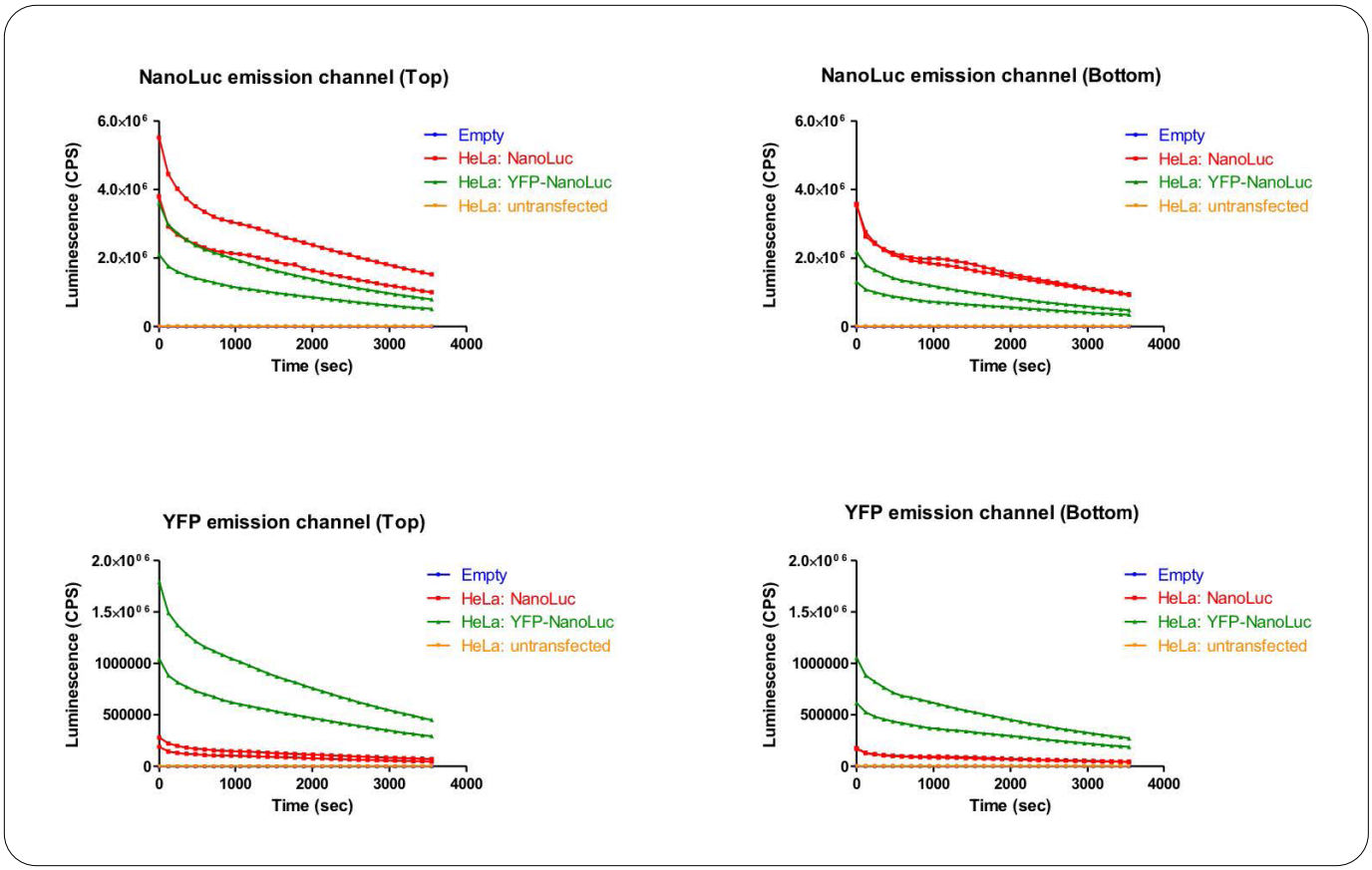


Figure 2: Emission of NanoLuc® (upper row) and YFP (lower row) detected separately over 1 hour measured either from the top (left) or bottom (right) of the plate. If BRET occurs, the YFP emission is only detected in cells expressing the YFP-NanoLuc® fusion protein. Empty wells and wells containing non-transfected cells were measured as control. Curves of the same color represent one replicate each.

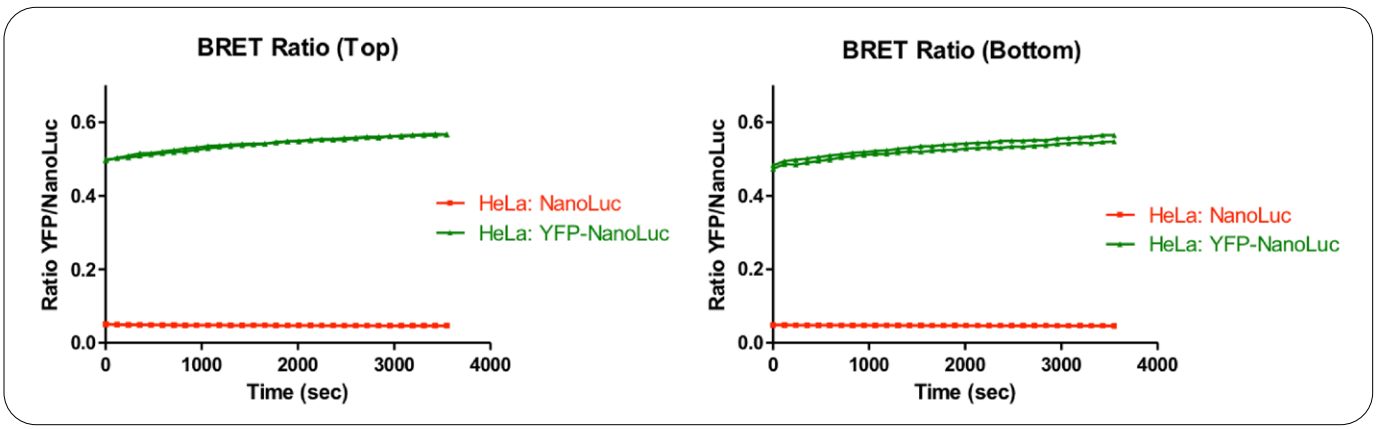


Figure 3: Kinetics of the BRET ratio for top (left) and bottom (right) measurements resulting from the NanoLuc® and YFP signals shown in Figure 2. Ratios of both replicates are shown as individual curves.

Comparison of plate type and measurement orientation (Experiment 2)

Luminescence measurements in a clear-bottom plate from below are advantageous, because:

- Signal to background and assay robustness can be enhanced, if working with adherent cells (as shown below in Table 2).
- Plate seals or lids can be applied to reduce contamination or evaporation without interfering with the measurement.
- Cell-based luminescence assays can be measured, but also imaged using the same plate. This is often used for quality control of the cell layer or for orthogonal imaging assays.
- Interference of liquid "artifacts" such as meniscus effects or air bubbles is prevented.
- Top measurements of adherent cells in high density formats such as 1536-well plates always suffer from signal reduction due to the narrow wells, whereas measurements from the bottom do not.

To better understand how an imaging capable plate performs in comparison to a regular white luminescence assay plate, NanoBRET™ measurements were performed in two plate types using cells either expressing NanoLuc® alone (control) or the NanoLuc®-HaloTag® fusion protein (Figure 4).

Using a black and clear-bottom plate for NanoBRET™ assays on the VICTOR Nivo is as reliable as using a white luminescence assay plate. In our test system and under the conditions mentioned the imaging compatible plate resulted in even better assay windows than the regular plate (Table 2). Additionally, the crosstalk into empty neighboring wells of the sample wells is as low as for the regular luminescence assay plate (Figure 5). Moreover, whereas the crosstalk in white plates is wavelength dependent (crosstalk of HaloTag® Ligand emission at 645 nm > crosstalk of NanoLuc® emission at 460 nm), black plates uniformly produce crosstalk at less than 0.5%.

Table 2: Plate dependent assay windows calculated based on the NanoBRET™ ratios from Figure 4. NanoBRET™ ratios of the HaloTag® 1:500 dilution (High Sample) and the sample without any HaloTag® added (Low Sample) are compared.

Plate	Orientation	High sample	Low sample	High/ Low
CellCarrier	Top	0.1349	0.0031	43.52
CellCarrier	Bottom	0.1242	0.0029	42.83
CulturPlate	Top	0.1846	0.0054	34.19

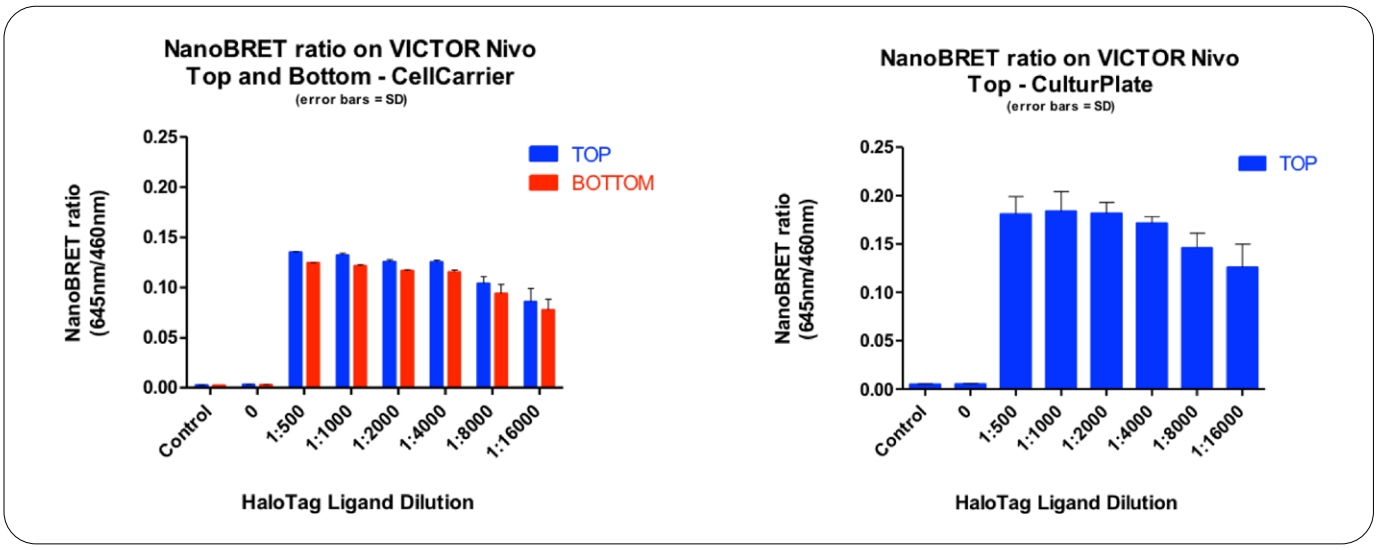


Figure 4: Comparison of NanoBRET™ measurements using either a black imaging capable (CellCarrier-384 Ultra) or regular white luminescence microplate (CulturPlate-384). Cells either express NanoLuc® alone (Control) or the NanoLuc®-HaloTag® fusion protein, which was stained with varying concentrations of HaloTag® 618 Ligand (0 – 1:16000). Error bars represent one standard deviation (SD) of three replicates each.

Both plate types as well as top and bottom measurements perform very similarly and a decrease in NanoBRET™ ratio due to the dilution of HaloTag® 618 ligand is clearly visible (Figure 4). The distinct error bars in the CulturPlate are based on plate handling variability resulting from manual pipetting. Control cells expressing the NanoLuc® only show almost no NanoBRET™ similar to the cells that were not treated with HaloTag® 618 ligand (0). The latter and the cells that received the highest concentration of HaloTag® 618 ligand (1:500) were used as low and high sample to estimate the assay window for both plate types (Table 2).

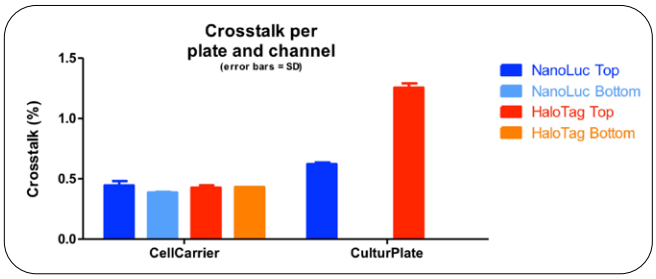


Figure 5: Crosstalk of light emission into empty neighboring wells for each plate, channel and measurement orientation.

Conclusion

- BRET signals can be reliably measured on the VICTOR Nivo using both, top or bottom measurement orientation.
- Bottom BRET measurements are ideal for adherent cells and circumvent artifacts produced at the top of the well by lids, seals or the liquid meniscus.
- In the test system shown here, imaging compatible plates result in better data quality than regular luminescence assay plates.
- Data evaluation using the BRET ratio is strongly recommended, as the data quality becomes independent on the measurement time after substrate addition, the expression levels or the measurement orientation.
- Even though the VICTOR Nivo can be equipped with dispensers, in the BRET experiments shown here the signal is stable enough to allow for manual substrate addition.
- Flexible VICTOR Nivo software simplified the assay workflow.

References

1. Kim, J., & Grailhe, R. (2016). Nanoluciferase signal brightness using furimazine substrates opens bioluminescence resonance energy transfer to widefield microscopy. Cytometry Part A, 89(8), 742-746.
2. Foitzik, A., Grailhe, R., Haenel, F., Letzsch, S., Trask, J., & Schreiner, A. (2018). Bioluminescence Resonance Energy Transfer for High Content Imaging. SLAS Poster 2018.

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