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High-throughput analysis of endogenous fruit glycosyl hydrolases using a novel chromogenic hydrogel substrate assay†

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A broad range of enzyme activities can be found in a wide range of different fruits and fruiting bodies but there is a lack of methods where many samples can be handled in a high-throughput and efficient manner. In particular, plant polysaccharide degrading enzymes – glycosyl hydrolases (GHs) play an important role in fruit development and ripening processes by modulating the plant cell wall. Knowledge about these enzymes is important for research in fruit development and also important for industry regarding postharvest properties. Although advances in genetic control and cell wall biochemistry have led to a more profound understanding of the importance of GH activity and regulation, current methods for determining glycosyl hydrolase activity are lacking in throughput and fail to keep up with data output from transcriptome research. Here we present the use of a versatile, easy-to-handle, multiplexed and highly reproducible method using CPH assays where different fruits have been screened for enzyme activity. Additionally, the importance and impact of the extraction method and buffer conditions on the assay are investigated. We will show that one experimental setup can be used for testing all enzymes.

Introduction

Plant polysaccharide degrading enzymes – glycosyl hydrolases (GHs) play an important role in fruit development and ripening processes by modulating the plant cell wall as described by literature.[1–5]

The disassembly of the cell wall especially modification of the neutral sidechains of pectin are one of the initial events leading to fruit ripening although differences are observed between fruits.[5] One important enzyme that is upregulated in pepper and apple is galactosidase, removing the galactan side chains from pectin.[5–7] In other fruits like pear and apricots it is the loss of arabinose and xylose that is the first sign accompanying ripening[5] also observed in strawberries.[8]

Management of GH activity in fruits has industrial importance with regard to fruit storage, softening and many other aspects.[9,10] However, the postharvest activity of GHs is observed to be different in analyzed apple cultivars. Some apple cultivars as "Golden Delicious" will become mealy quickly after harvest while other cultivars like "Fuji" keep crisp.[11] This difference in texture was shown to be correlated with different expression profiles of β-galactosidase and α-L-arabinofuranosidase activity with "Golden Delicious" fruits having the highest activity. Change in storage conditions can affect enzyme activity highlighting that measurement of the induced enzyme activity is an important factor to assess.

Activity of glycosyl hydrolase enzymes also plays important roles in fruit defense against bacteria and fungi.[12,13] Some of the bacteria and fungi associated with degradation of fruits produce polysaccharides that can be degraded by GHs. These polysaccharides are pullulan and curdlan respectively produced by specific species of fungi and bacteria. One curdlan-producing bacterium example is *Alcaligenes faecalis* that was observed on bananas.[14] Plant defense against fungi is mediated through fungus-related polysaccharides and plant cell wall damage-related structures[15] which will not be part of this study.

However, many of the functions of these enzymes remain insufficiently investigated or unclear.[16] Although advances in genetic control and cell wall biochemistry have led to a more profound understanding of the importance of GH activity and regulation[17] the current methods for determining glycosyl hydrolase activity are lacking in throughput and fail to keep up with data output from transcriptome research.[18] Furthermore, with respect to the particular case of fruits, sample handling and control is crucial for reproducible research results.[19,20] In all literature mentioned above it is apparent that each enzyme type requires another experimental setup with new buffers and incubation methods.[6–8,11]

Chromogenic Polymer Hydrogel (CPH) substrates are synthesized from purified polysaccharides and proteins and then

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chemically dyed and crosslinked rendering them chromogenic and insoluble. As such, their flexibility in terms of different assay layouts such as 96-well plates and agar plates has already been demonstrated in previous work.[18,21] Additionally, the possibility of dying the substrates with dyes of different colors has also been demonstrated in abovementioned work giving the assay an additional level of flexibility and high-throughput nature.

Here we present the use of a versatile, easy-to-handle, multiplexed and highly reproducible method using CPH assays[18] where different fruits have been screened for enzyme activity. Additionally, the importance and impact of the extraction method and buffer condition on the assay are investigated. In comparison with existing methods, here we show that one experimental setup can be used for testing all enzymes.

Results and discussion

To demonstrate that the CPH substrate assay can be used in a high-throughput fashion for screening endogenous enzymes from fruits, we picked 21 different fruits and screened their enzymatic degradation specificity and the correlated activity against 20 different CPH substrates that were synthesized as described previously.[18]

Our aim is to demonstrate the broad range of enzyme activities that can be found in a wide range of different fruits and how so many samples can be handled in a high-throughput and efficient manner. In this study, we picked fruit samples without detailed control of their ripening stage or growth conditions. However, we are aware that studies focused on particular fruits with monitored origin, growth conditions and other variables should be more aware of the details and conditions pertaining to that fruit alone. This complexity is something that this assay can easily accomplish as it is flexible in layout and allows for many different experimental setups.

To push the limit of this already multiplexed assay and increase the throughput even further, we mixed 2 different substrates in different colors in each well effectively enabling analysis of 192 different samples in one 96-well plate.

As depicted in Fig. 1 (for a full set of substrates used see Fig. S-1†), the overall enzymatic activity was evaluated side-by-side at 3 different pH values of 4.0, 7.0 and 10.0 to broaden the spectrum of the analysis combined with a commercial enzyme known to degrade the particular substrate as a positive control and a point of reference.

We found that there is a wide diversity in enzymatic activity across the range of different fruits tested as well as due to the different pH values and different substrates used. Most of the enzymes had the highest activity at pH 7.0 with some being more active at pH 4.0 except for protease activity on yellow CPH-casein which was higher at pH 10.0 for the dry fig sample. The values presented in Fig. 1 represent the overall enzymatic activity, since the enzymes were not purified or enriched in any way prior to analysis.

The analytical approach presented is intended for quick and high-throughput evaluation of the overall enzymatic activity on specific substrates. For more detailed information about specific enzymes – more in-depth studies on purified enzymes would be required. Nonetheless, the analytical approach

	y-CPH-amylose			y-CPH-amylopectin			r-CPH-pachyman			r-CPH-curdlan			y-CPH-β-glucan (barley)			y-CPH-β-glucan (yeast)			y-CPH-xylan			y-CPH-galactomannan			y-CPH-casein		
pH	**4.0**	**7.0**	**10.0**	**4.0**	**7.0**	**10.0**	**4.0**	**7.0**	**10.0**	**4.0**	**7.0**	**10.0**	**4.0**	**7.0**	**10.0**	**4.0**	**7.0**	**10.0**	**4.0**	**7.0**	**10.0**	**4.0**	**7.0**	**10.0**	**4.0**	**7.0**	**10.0**
positive control	0	48	29	0	100	100	54	54	0	90	87	1	100	52	0	64	97	0	26	6	0	0	94	9	1	58	69
mango	1	37	1	0	61	4	4	1	0	80	11	0	0	2	1	1	1	1	0	1	1	0	1	1	0	1	1
papaya	1	24	0	0	29	1	2	6	0	9	87	2	0	3	1	1	1	0	0	1	0	56	32	0	1	68	6
litchi	0	1	6	0	2	10	0	1	2	0	1	2	0	3	7	0	1	5	1	2	6	0	1	0	0	1	4
avocado	0	10	4	0	5	8	3	24	2	20	29	3	0	1	6	1	5	4	0	1	4	0	1	0	0	1	4
tomato	0	2	1	1	1	1	20	54	12	94	94	93	0	1	1	6	22	1	1	1	1	0	1	1	0	1	1
banana	0	2	1	0	1	2	53	3	0	94	16	1	0	1	2	5	2	1	0	1	1	0	1	0	0	1	1
pear	0	1	0	0	2	1	5	3	0	15	6	1	0	1	1	1	1	1	0	1	0	0	1	0	0	0	0
passion fruit	0	0	0	0	0	0	1	2	3	69	74	2	1	1	1	15	17	27	0	0	0	0	0	0	7	10	25
nashi pear	0	0	1	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1
clementine	0	13	5	0	10	11	4	5	0	36	38	1	0	4	11	1	2	7	0	2	6	1	3	0	1	1	8
orange	0	17	6	0	15	14	6	7	1	52	54	1	1	4	15	3	5	8	0	2	9	0	1	0	1	66	5
red pepper	0	14	1	0	6	1	12	55	3	91	81	47	0	2	1	5	16	1	0	1	1	0	1	2	0	2	1
green pepper	0	6	1	0	3	2	0	31	1	1	85	5	0	2	2	1	5	1	0	1	1	0	1	2	0	1	1
yellow pepper	0	16	1	0	7	1	16	57	4	82	85	61	1	2	1	6	17	1	0	1	1	0	1	1	0	2	1
onion	0	1	2	0	1	5	4	8	1	63	61	3	0	1	5	1	3	4	3	1	3	1	1	0	0	1	4
white onion	0	0	0	0	0	0	10	9	0	66	61	2	0	0	0	2	3	0	0	0	0	0	0	0	0	1	1
red onion	0	1	3	0	2	6	14	18	1	80	81	3	0	2	5	3	6	4	1	1	4	0	2	0	1	2	4
apricot	0	0	0	0	1	1	0	0	0	3	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	1
dry apricot	0	0	1	0	1	2	0	0	0	1	2	0	0	1	2	0	0	1	0	1	1	0	0	2	0	1	2
fig	0	14	1	0	9	1	5	18	1	19	91	4	0	1	1	2	5	1	0	1	1	0	1	1	1	73	2
dry fig	2	3	2	1	2	3	5	13	1	28	95	2	2	3	3	2	5	2	2	2	2	1	2	0	1	37	67

0 50 100

Fig. 1 Selection of 21 different fruits and vegetables were tested towards 9 different CPH substrates at three different conditions (pH 4.0, 7.0 and 10.0). Note the overwhelmingly higher enzymatic activity towards curdlan in comparison with other substrates. The highest calculated value was set to 100 and all other data normalized.

presented demonstrates the overall enzymatic response towards the specified substrates and based on prior knowledge[18] the enzymes acting on polysaccharides are most likely glycosyl hydrolases and lyases or other enzymes such as lytic polysaccharides monooxygenases (LMPOs). The full table with the results shown as numerical values for all the CPH substrates tested can be found in the Fig. S-1.† The absorbance values have been normalized to values between 0 and 100 for convenience of presentation.

The activity of enzymes geared towards pH 7.0 and lower was expected according to the pH values of the apoplastic fluid recorded previously.[22] We have also demonstrated that some of the activities had other functions such as defense against pathogens and were not necessarily pertaining to the intrinsic processes in the fruits themselves. As observed across the range of fruits tested shown in Fig. 1, there is an overwhelmingly higher activity towards CPH-curdlan, a substrate derived from curdlan from *Alcaligenes faecalis* – a bacterial extracellular polysaccharide.[23] To verify that claim, we repeated the enzyme screening with a selection of fruits (Fig. S-2†) and additionally performed Comprehensive Microarray Polymer Profiling (CoMPP, Fig. S-3†) on these fruits. CoMPP analysis provides information about the relative polysaccharide content and composition of the material tested[24,25] and in this case, it is clear that the enzymatic activities recorded did not correlate with the actual polysaccharide composition of that particular fruit sample. This was expected, as not all polysaccharides of the fruit are intended to be digested by the fruit itself as part of fruit ripening.[26] Again, curdlan is a very good example, as the drastically high enzymatic activity against this β-1,3-glucan was not reciprocated by the β-1,3-glucan structures detected with the BS-400-2 antibody[27] (Fig. S-3†).

After homogenization, there is a high chance that, as cellular structures of the tissues are destroyed, the enzymes, amongst other cellular components, are released into the same medium where they can inevitably interact with each other. Modes of such interactions, especially with regard to enzymatic activity, can be degradation, inhibition and synergistic or antagonistic effects which can be unnatural in a sense that they do not reflect that homeostatic conditions and processes of an intact fruit.

Extraction of enzymes from fruit samples takes a long enough time to allow for these effects to be amplified, because of extended exposure to such unnatural conditions and could significantly affect the results of the analysis.

With respect to these specific problems regarding endogenous enzymes from fruits and possibly other plant organs and tissues as well as the need for tight control during their growth and sample preparation, we investigated whether we could bypass extracting the enzymes from fruit by applying the fruit homogenates suspended in a buffer directly to the assay plate. The amount of homogenized material required for the experiments was as little as 15 mg per well demonstrating the effectiveness and responsiveness of the assay. As a control and proof-of-concept we ran the same experiments with enzyme extractions under different conditions. The results of this analysis showed, as shown in Fig. 2, that in most cases where raw material was applied *versus* the extracts – the activity was comparably similar except for the orange sample where the activity recorded for the raw material was considerably higher than that of the extract. A positive control was included as a point of reference.

Even though the demonstrated differences in Fig. 2 are not striking, it is safe to assume that minimal sample processing between the harvest/homogenization time and the actual analysis is ultimately beneficial for the accuracy of the analysis. Additionally, having the ability to work with raw material with virtually no processing prior to analysis adds yet another level of advantage to this assay setup. Additional data on how different methods of homogenization reflect upon the results obtained for enzymatic activity can be found in Fig. S-4.†

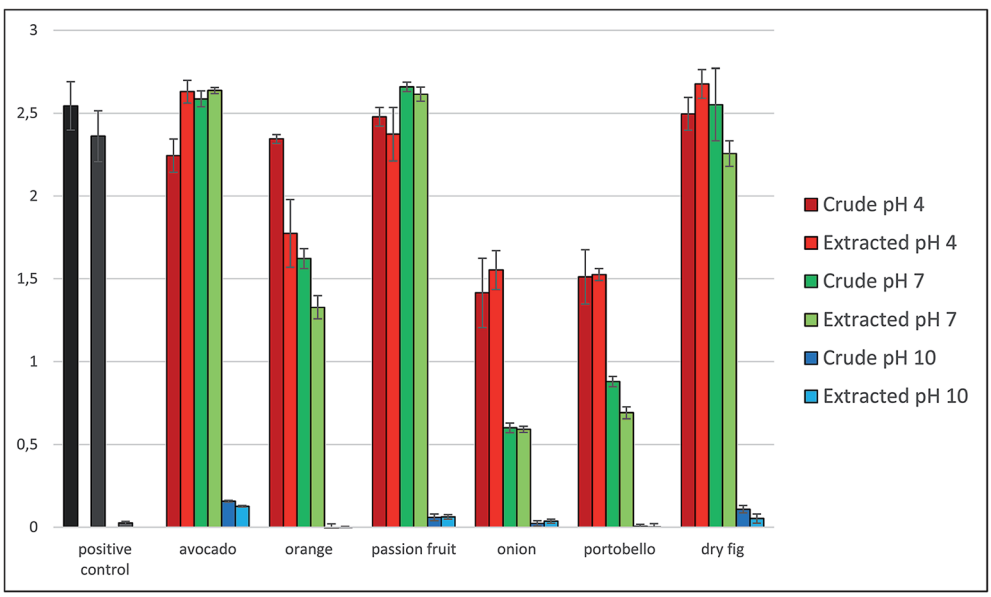

Fig. 2 Activity of crude and extracted material towards CPH-curdlan at three different pH conditions (pH 4.0, 7.0 and 10.0).

With our assay setup relying on absorbance in the near-UV and VIS range, we expected that some of the natural pigments from fruits could interfere with our analysis by raising the absorbance value and therefore the result would not reflect the actual absorbance that was a result of CPH substrate degradation. To address that, we recorded the absorbance spectra of the 21 fruit extracts at pH 4, 7 and 10 between 350 nm and 750 nm as shown in Fig. 3 and discovered that many of the fruits do have absorbance spectra in the range where we record the absorbance for enzyme degradation assays. Since we can easily synthesize all of the used substrates in 4 different colors as described previously,[18] it is easy to circumvent this problem by using the CPH substrate dyed with dye with an absorbance maximum outside of the interference range of the natural pigments. Alternatively, the absorbance of the extract can be easily recorded before the experiment and then subtracted from the absorbance obtain after enzymatic digestion of the CPH substrate.

With this study, we have demonstrated a significant increase in throughput and flexibility in analysis of endogenous enzymes from fruits. A 96-well format of the assay enables quick analysis and miniscule sample volumes suitable, the flexibility in the choice of the CPH substrate dye eliminates interference from inherent plant pigments and finally the potential of using raw material minimizes sample processing and the time between sample harvesting and biochemical analysis. In addition, by combining 2 substrates of different colors in one well we have effectively doubled the throughput of the assay.

We believe that minimal sample-processing combined with a quick, easy-to-use and high-throughput assay is an optimal

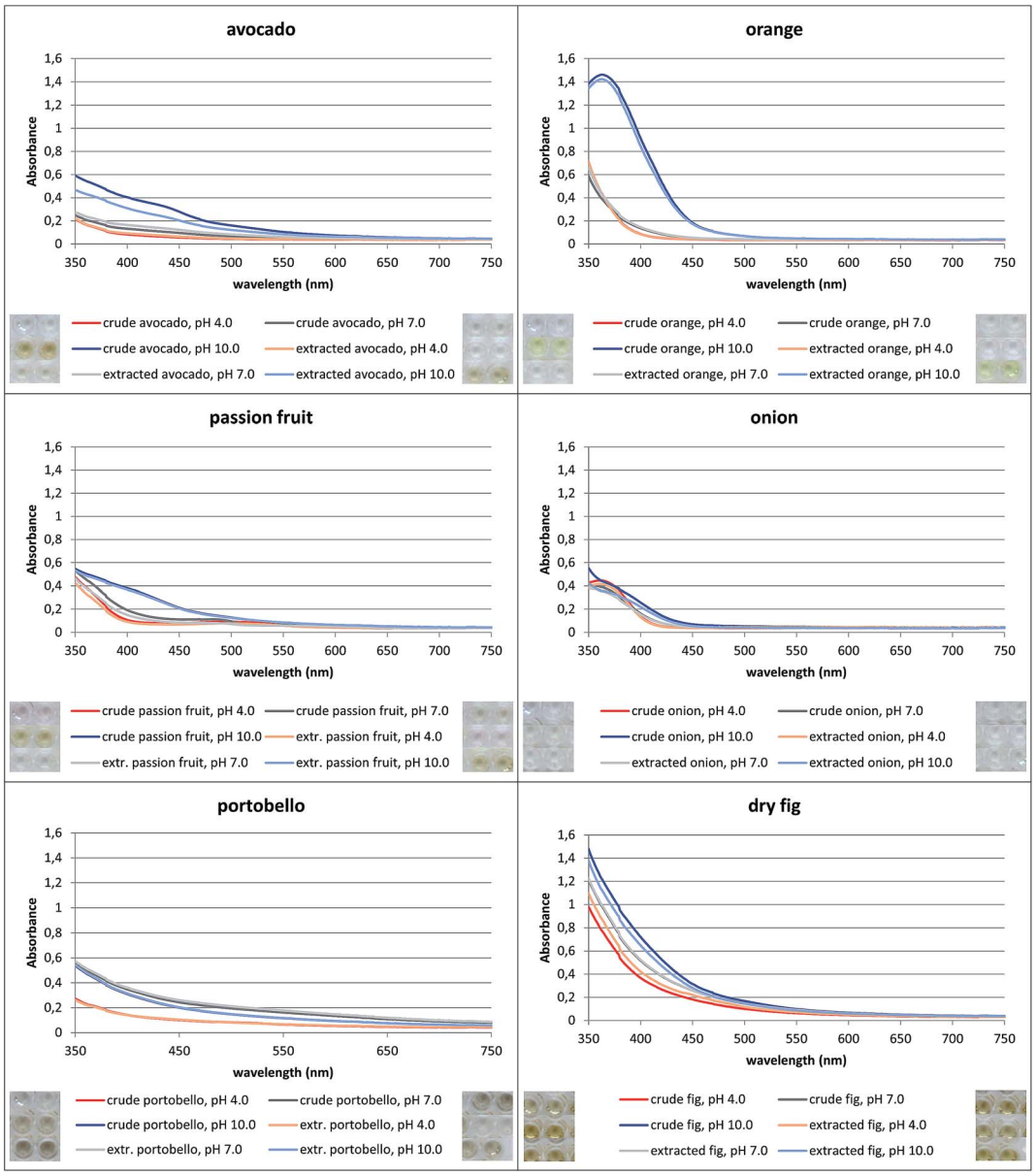

Fig. 3 Spectra showing the inherent absorbance of 6 different samples: avocado, orange, passion fruit, onion, portobello and dry fig.

solution for analysis of the endogenous enzymatic activity in fruits because of the fragile balance between the actual enzymatic activity that can be detected and the perishable nature of enzymes, proteins and other components of fruit and other plant tissues after they have been homogenized. For more detailed studies on the specificity, activity and degradation products of any particular enzyme from the mixture present in the homogenate, additional methods such as LC-MS would need to be employed.

Existing activity assays for each enzyme have to be done separately for every enzyme and with different test setups significantly increasing the time, cost and amount of materials needed for the experiment. As such, we envisage that this assay will be an important initial screening tool in the field of studies investigating fruit development, ripening, storage and shelf-life where stringent control of growth conditions and time are crucial for valid and reproducible results. In conclusion, we have described a quick, easy, and high-throughput technique enabling the use of a single method for measurement of the activity of both many enzymes and against many substrates simultaneously. In addition, we have shown that crude extracts can be used directly with our assay. This will speed up the analysis process even further.

Experimental section

Reagents, enzymes and microorganisms

Fruits were purchased at a local grocery store and used immediately. CPH substrate kits containing CPH substrates were obtained from GlycoSpot (Copenhagen, Denmark) and used as per manufacturer's instructions. The mixtures of substrates in the assay plates are described in the Table S-1.† Blue, red and green substrates were always mixed with a yellow substrate to achieve the best spectral separation of absorbance maxima. Rhamnogalacturonan I from potato was isolated and purified as published previously.[28]

The assay plates for assaying enzyme activities from raw and extracted material contained only blue CPH substrates.

Fruit enzyme extraction

The plant tissue was frozen in liquid nitrogen and ground grinded using a blender or mortar and pestle and 2 g of each fruit sample were transferred into a 15 ml Falcon tubes, 4 ml of 40 mM sodium acetate buffer pH 5.0 containing 1 M NaCl were added for extraction. The samples were either used directly for enzyme screening or were incubated on ice for 3 hours with regular shaking. After extraction, the tubes were spun down at 2700 × *g* for 10 min at 4 °C, and the supernatant was collected and used for the assay.

CPH substrate assays

The CPH substrates were activated following the provided protocol (GlycoSpot, Copenhagen, Denmark) and then 125 μl of 200 mM buffer (sodium acetate buffer, pH 4.0, sodium phosphate buffer, pH 7.0 or sodium carbonate, pH 10.0) were added. Fruit extract (30 μl, homogenized or the supernatant after 3 h of extraction) was added to each well and each sample was added to 3 wells. The plates were sealed and incubated at 25 °C with shaking at 130 rpm for 20 h. Then the solutions were spun down at 2700 g for 10 min and 120 μl supernatant of each well transferred to a fresh plate. The absorbance at 404 nm (yellow), 517 nm (red), 595 nm (blue) and 630 nm (green) was detected using a plate reader. The average values and standard errors presented in the figures were calculated based on signals from the 3 wells used for each sample.

Comprehensive microarray polymer profiling (CoMPP) analysis

The plant material was freeze-dried, homogenized to yield a powder-like material. After producing the alcohol-insoluble residue, the material was analyzed as described previously.[24,25] A sequential extraction is used starting with 1,2-diaminocyclohexanetetraacetic acid (CDTA) to extract pectic polysaccharides followed by NaOH extracting primarily hemicelluloses.

Statistical analysis

Standard error values were calculated for all measurements and included in the figures. The data point for the CoMPP is a result of four five-fold dilutions. Standard errors for all data points are shown in the ESI with Fig. S-6† corresponding to Fig. 1, S-7 to S-1 and S-8 to S-2.†

Conflict of interest

Authors declare no competing interests.

Author contributions

JS performed most of the experiments, helped design the study and wrote the manuscript; TFL performed some of the experiments; SKK performed some of the experiments, helped design the study and wrote the manuscript, WGTW helped design the study, BJ prepared the rhamnogalacturonan I from potato, helped design the study and wrote the manuscript.

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