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Phosphothioated Oligodeoxynucleotides Induce Nonspecific Effects on Neuronal Cell Adhesion in a Growth Substrate-Dependent Manner

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Synthetic phosphothioated (PTO) oligodeoxynucleotide (ODN) sequences are commonly used for a variety of applications that benefit from nuclease protection. The PTO modification is implemented mainly in antisense ODN, but also in ODN that were shown to activate members of the toll-like receptor (TLR) family such as TLR3 (poly-I:C), TLR8 (ssRNA), and TLR9 (CpG). Neurons are routinely plated on surfaces coated with either cationic substances such as poly-L-ornithine (PLO), polyethylenimine (PEI), poly-L-lysine or ECM components such as laminin, collagen, or fibronectin. We found that PTO-ODN aimed at activating TLR9 induces a non-TLR9-specific detachment phenotype in cortical neurons plated on either laminin or PEI, but not on PLO. This phenotype was correlated with decreased viability and was partially inhibited when caspase-3 was inhibited with Ac-DEVD-CMK. This finding suggests that the use of PTO-ODN can cause nonspecific effects on cell adhesion that could compromise interpretation of data from experiments using PTO-ODN.

Key words: phosphothioate; ODN; neurons; adhesion; toll-like receptors

The phosphothioate (PTO) modification of oligonucleotides contains one sulfur atom in place of an oxygen atom in the internucleotide linkage of DNA or RNA. This modification is extremely useful for antisense oligodeoxynucleotide (ODN)-mediated gene expression inhibition (Williams et al., 1997), because of more resistance to nuclease degradation than natural DNA or RNA with maintainance of the ability to bind to complementary nucleic acid sequences. Another application that utilizes PTO oligonucleotides is the activation of certain members of the toll-like receptor (TLR) family of receptors. TLRs are a family of pattern-recognition receptors (PRR) recognizing molecules that are broadly shared by pathogens and are referred to as *pathogen-associated molecular patterns* (PAMPs). Each TLR is activated by ligands that originate in different classes of pathogenic microorganisms. Certain TLRs recognize foreign nucleic acids. TLR3 recognizes dsRNA and polyinosine-polycytidylic acid (poly-I:C). TLR7 and TLR8 both recognize ssRNA, and TLR9 recognizes unmethylated bacterial or viral DNA or synthetic CpG oligonucleotides (Kawai and Akira, 2007). The above-mentioned ligands are commonly used to study different aspects of TLR3, TLR8, and TLR9 activation. These ligands are generally synthesized with a PTO modification in their backbone. This modification renders the ODN better protected against cellular nucleases, allowing prolonged activation of the desired TLR. Full PTOs are more stable than partially modified oligonucleotides, but, because of their extreme stability and their hydrophobic character, they can also have toxic effects on living cells. The study of TLRs has extended beyond the limits of immune-related cells, and TLRs are expressed on a variety of nonimmune cells; for example, neurons (Tang et al., 2007), astrocytes, and oligodendrocytes (Bsibsi et al., 2000). The role of TLRs in general and of TLR9 in particular has not been deciphered yet in postmitotic neurons. CpG ODNs are synthetic DNA ODNs that contain CG motifs and are known to activate TLR9 in immune cells. They are considered to mimic unmethylated CG islands that exist in bacterial and viral DNA. Three types of CpG ODN (types A, B, and C) were found accord-

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ing to their ability to activate dendritic or B cells. Inhibitory sequences were also found according to their ability to counteract the activation of immune cells by CpG ODNs (Krieg et al., 1998). In this report, we provide evidence for a non-TLR9-specific activity conferred by PTO-CpG ODNs in cortical neurons grown on different adhesion-promoting substances.

MATERIALS AND METHODS

Materials

Resazurin (Alamar blue, 500 μM stock concentration, dissolved in PBS), polyethylenimine (PEI), and poly-L-ornithine (PLO) were purchased from Sigma (St. Louis, MO). *Escherichia coli* DNA was purchased from Invivogen (San Diego, CA). The following CpG ODNs were purchased from Invivogen: 1826 ODN: 5′-tccatgacgttcctgacgtt-3′, 1826 control ODN: 5′-tccatgagcttcctgagctt-3′, 1688 ODN: 5′-tccatgacgttcctgatgct-3′, 1688 control ODN: 5′-tccatgagcttcctgatgct-3′, 2088 inhibitory ODN: 5′-tcctggcggggaagt-3′. All ODNs were synthesized with a full phosphothioate backbone. Caspase-3 inhibitor III (Ac-DEVD-CMK) and wortmannin were purchased from Calbiochem (La Jolla, CA). Neurobasal medium and the B27 medium supplement were purchased from Invitrogen (Carlsbad, CA).

Neuronal Cultures

Dissociated cell cultures of cortical fragments were established from 18-day Sprague-Dawley rat embryos as previously described (Mattson and Kater, 1988). Neurons were plated on PEI (0.005%), laminin (10 μg/ml), or PLO (15 μg/ml)-coated 48-well plates and were maintained with Neurobasal medium supplemented with B27 (1:50, v:v). All treatments were incubated for 72 hr unless otherwise indicated.

Cell Viability

Cell viability was measured using the resazurin dye (10 μM final concentration). Resazuring was added directly to the cultures for 30 min of incubation at 37°C. Fluorescence was then measured with a fluorescence microplate reader. Excitation wavelength was 540 nm and emission wavelength 595 nm.

Statistical Analysis

Student's *t*-test was used to determine significance in all viability measurements.

RESULTS

Phosphothioated CpG ODN Cause Detachment of Neurons From PEI-Coated Surfaces

Cortical neurons incubated with 1826 CpG ODN (5 and 10 μM), a type B CpG ODN, showed a detachment phenotype (Fig. 1A). This was characterized by clustering of neuronal cell bodies and axonal processes into thick bundles. This effect was dose dependent and could be visible as early as 48 hr (data not shown). A mild effect on viability was conferred by P-CpG ODN in doses of 5 μM and higher (Fig. 1B). Incubation with the 1826 ODN control sequence (with a GC motif instead of CG) gave identical results. Moreover, incubation of the cells with 1688 CpG ODN (also a type B CpG ODN) and its control ODN resulted in the same detachment phenotype (Fig. 2A). ODN 2088 was reported to inhibit the activity of both ODN 1826 CpG and ODN 1688 CpG. When ODN 2088 was added to neurons either with or without the 1826 or 1688 CpG ODN, the detachment phenotype was evident (Fig. 2A). This implies an effect for P-CPG ODN on the cells that is not specific for TLR9 activation. Similarly to PTO-ODN 1826, all the different PTO-CpGs induced mild but significant decreases in cell viability (Fig. 2B).

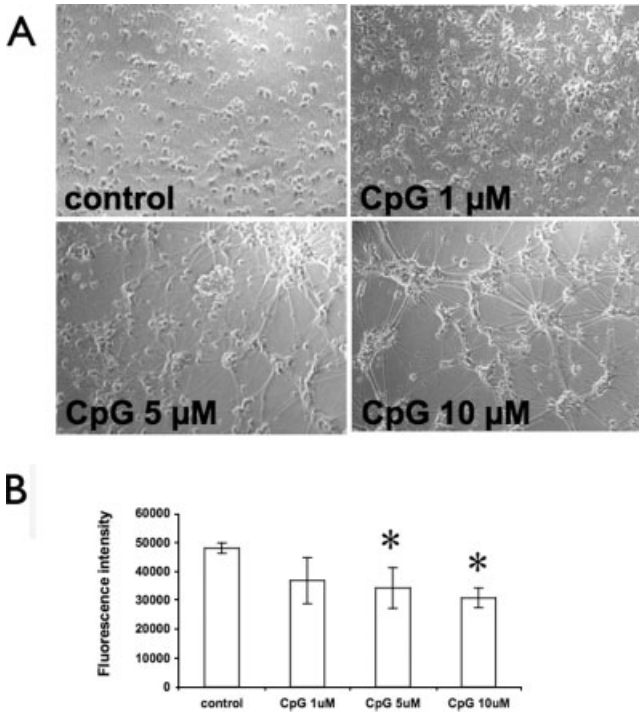

Fig. 1. P-ODN-induced detachment of cortical neurons plated on PEI. **A:** Cortical neurons were plated on PEI-coated dishes. At DIV7, cells were treated with increasing doses of P-ODN 1826 (1, 5, and 10 μM), and, after 72 hr, cells were imaged. **B:** Viability measurements of P-ODN 1826 were conducted using the viability dye resazurin. $^{\star}P < 0.05$ vs. control.

E. coli DNA Does Not Induce Detachment of Neurons

To verify that the detachment phenotype was not mediated through TLR9, we used *E. coli* DNA, the natural ligand for TLR9. Increasing doses of *E. coli* DNA of up to 30 μg/ml did not induce any morphological change in the cells (Fig. 3A). Moreover, no effect on the viability of the cells was noticed (Fig. 3B).

Caspase-3 Inhibitor Counteracts the Effect Conferred by 1826 CpG ODN

The decrease in viability following treatment of the cells with PTO-CpG suggested that an apoptotic process

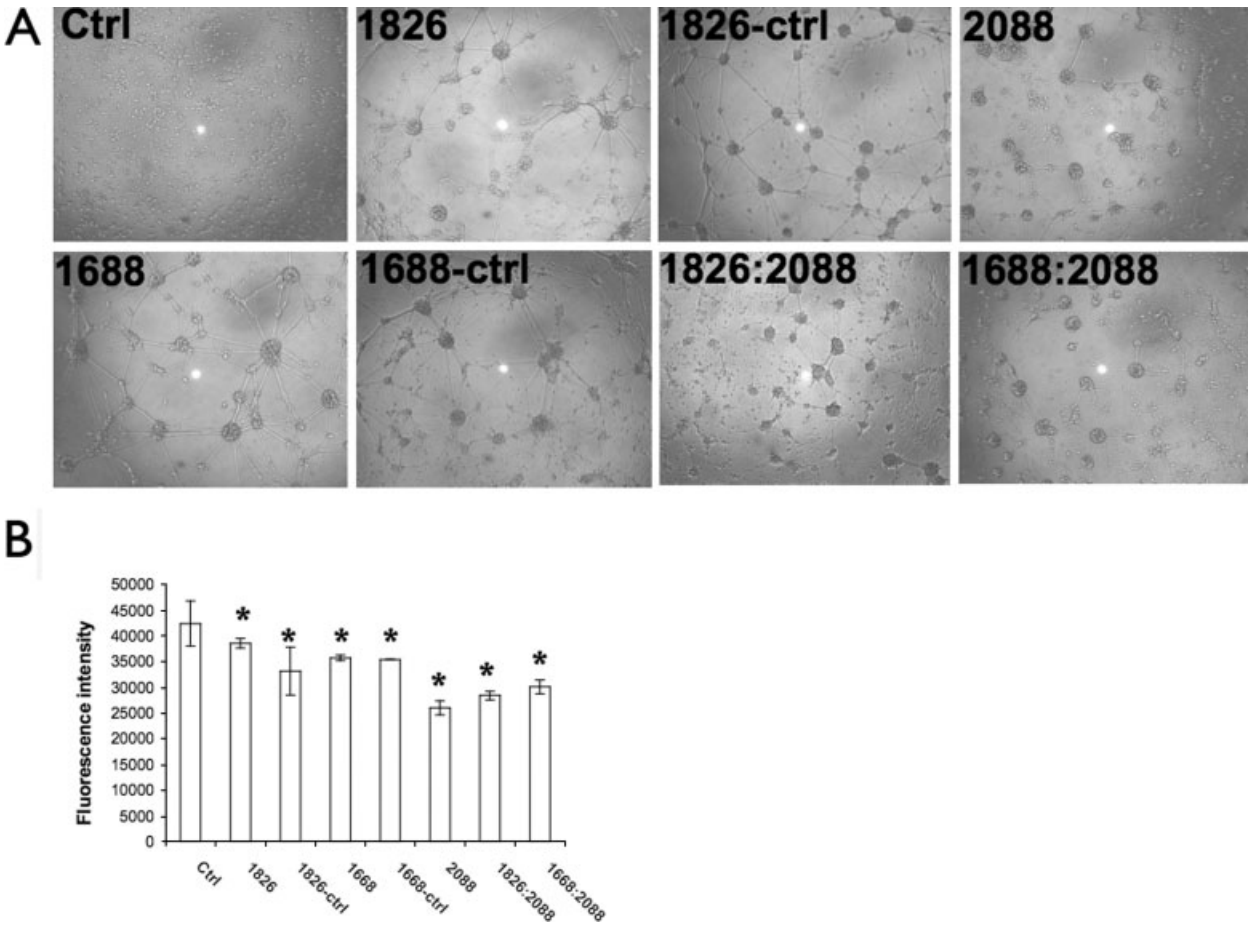

Fig. 2. Detachment phenotype of PTO-ODN is not sequence specific. **A:** Cortical neurons were plated on PEI-coated dishes. At DIV7, cells were treated with different p-CpG ODNs and their control sequences (10 μM for all the ODNs): 1826, 1688, 1826-ctrl, 1688-ctrl, the inhibitory ODN 2088, and combinations of 2088 with either 1826 or 1688. After 72 hr, cells were imaged. **B:** Viability measurements of PTO-ODN were conducted using the viability dye resazurin. $^{\star}P < 0.05$ vs. control.

was involved. When a cell-permeable caspase-3 inhibitor (Ac-DEVD-CMK) was added 2 hr prior to PTO-ODN addition, the detachment phenotype was partially inhibited (Fig. 4). This effect was not reversed, however, when wortmannin, a PI3K inhibitor, was used (data not shown).

The Detachment Effect Is Dependent on the Coating Substrate

Our results were in contrast to those of Iliev et al. (2004), who recently described the effects of CpG ODN on neurons. In their study, the authors used PLO to facilitate neuronal attachment and did not encounter the detachment phenotype that we observed. We therefore investigated whether different coating substrates confer different effects. Indeed, when cortical neurons were grown on PEI or laminin substrates and treated with PTO-ODN CpG, the same detachment effects were observed (Fig. 5). However, when neurons were cultured on PLO-coated plates, no detachment effect was seen (Fig. 5).

DISCUSSION

TLR were initially shown to be functional on immune-related cells. However, many cell types and tissue types have since emerged as sources for TLR expression and, therefore, as potential targets for TLR activation by different ligands. The detachment phenotype, induced by PTO-ODN at concentrations of 5 μM and higher, was characterized by clustering of the cortical neuron cell bodies and bundling of their axons. This phenotype is typical for detachment of neurons because of their inability to attach to the surface. This effect was

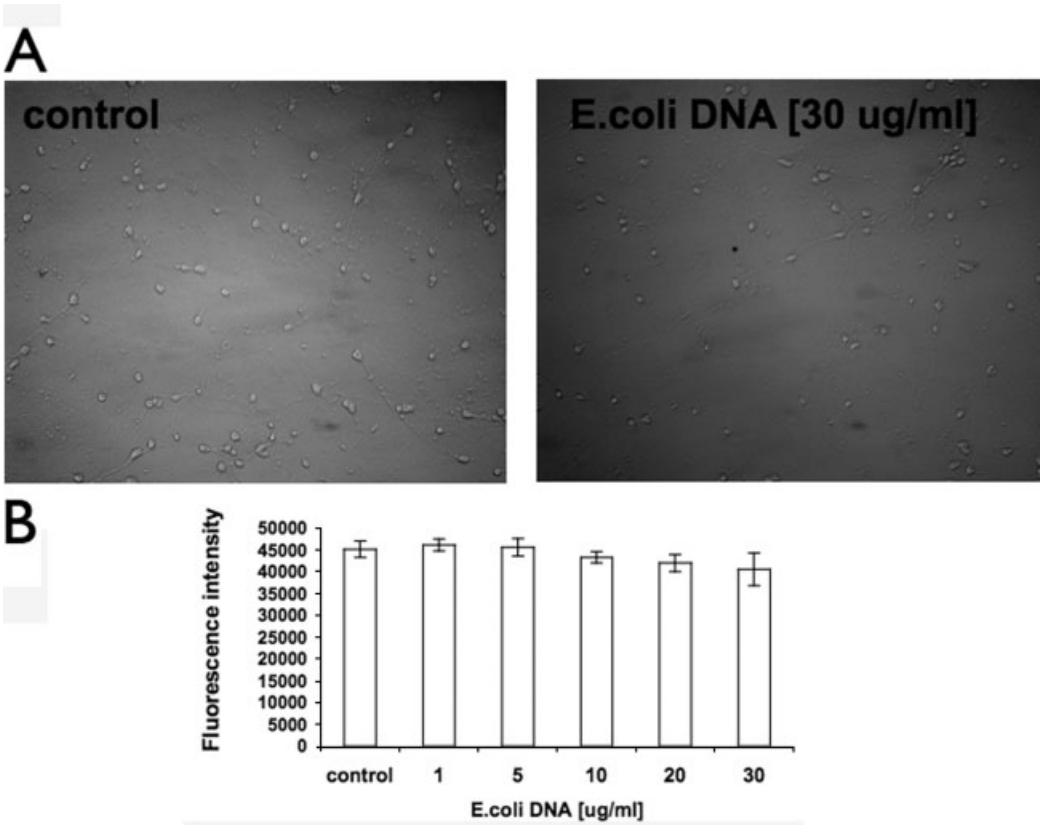

Fig. 3. *E. coli* DNA does not induce detachment phenotype on cortical neurons plated on PEI. **A:** Cortical neurons were plated on PEI-coated dishes. At DIV7, cells were treated with 30 μg/ml *E. coli* DNA. After 72 hr, cells were imaged. **B:** Viability measurements were conducted using the viability dye resazurin. There was no statistical difference between the different experimental groups.

also associated with a mild but significant decrease in viability of the cells. Intriguingly, caspase-3 is involved in the initiation of this process, insofar as a caspase-3 inhibitor was able to inhibit this phenotype significantly. The ability of other CpG PTO-ODNs and their control sequences as well as inhibitory sequences to induce the same effect implied that the effect of PTO-ODN on postmitotic neurons is nonspecific. This was further demonstrated when *E. coli* DNA, the natural ligand for TLR9, did not induce the same effect. Bacterial DNA does contain CG motifs but does not contain synthetically modified phosphothioates in the backbone of its sequence. A report by Iliev et al. (2004) showed that, at the same concentrations of PTO-CpG that we used, no such effect was observed. Because the only difference between the studies was the substrate used to induce attachment of the neurons, we compared the two cationic substrates, PEI and PLO, and laminin (a noncationic substrate that is also used to facilitate neuronal attachment). Insofar as the two cationic substances had opposite effects and because caspase-3 inhibition prevented the effect, charge effects between the negatively charged PTO-ODN and the positively charged PEI are not likely the cause of this effect. It remains to be determined why PEI and PLO induce such opposite effects on neuronal cultures when PTO-ODN is introduced to the cultures.

The observation that PTO-ODNs confer a nonspecific effect on PEI- and laminin-coated cultures is important because PEI and laminin are both widely used by many laboratories in the study of neuronal cultures. Antisense ODNs are widely used in the study of numerous cell types as well as neurons. TLRs in general are increasingly being studied in fields that are not related to the immune system. Synthetic CpG is not the only TLR ligand that is commercially synthesized using PTO nucleotides. Polyinosine-polycytidylic acid (poly-I:C) and dsRNA (both TLR3 ligands) and ssRNA (a TLR7 and TLR8 ligand) are also commercially synthesized using PTO-ODNs. It is therefore important to realize the interactions of PTO-ODN in general and PTO-TLR ligands in particular with different matrices used to prepare adhering cell cultures in general cultures and neuronal cultures in particular.

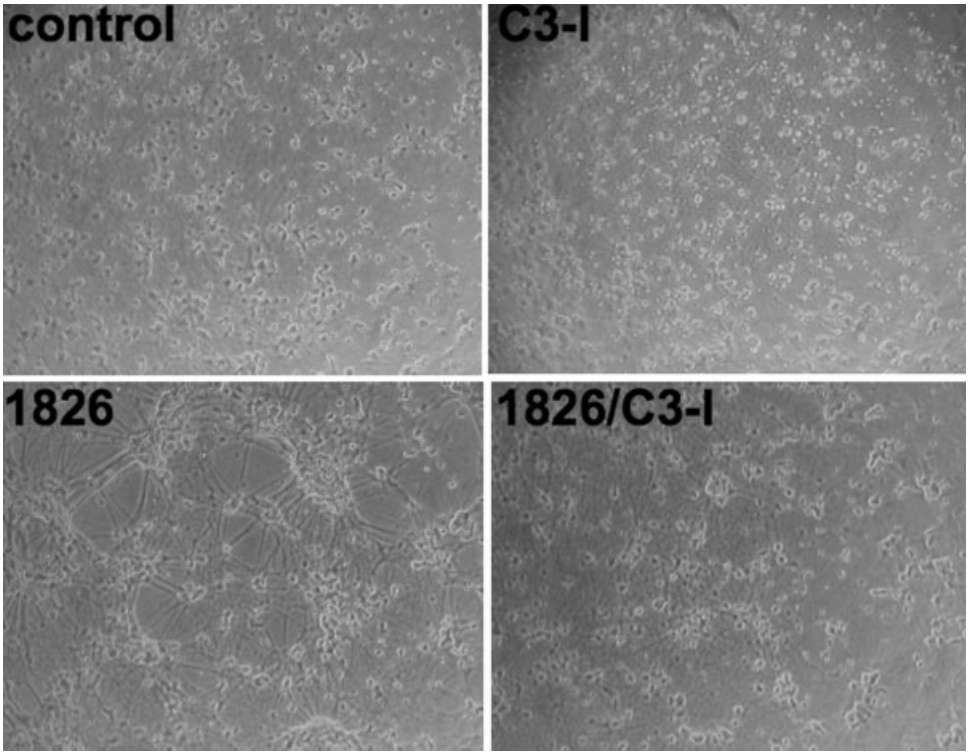

Fig. 4. Inhibition of caspase-3 prevents the detachment effect of P-ODN on cortical neurons plated on PEI. Cortical neurons were plated on PEI-coated dishes. At DIV7, cells were treated with caspase-3 inhibitor (Ac-DEVD-CMK, 100 μM) for 2 hours prior to addition of P-ODN 1826 (10 μM). After 72 hr, cells were imaged.

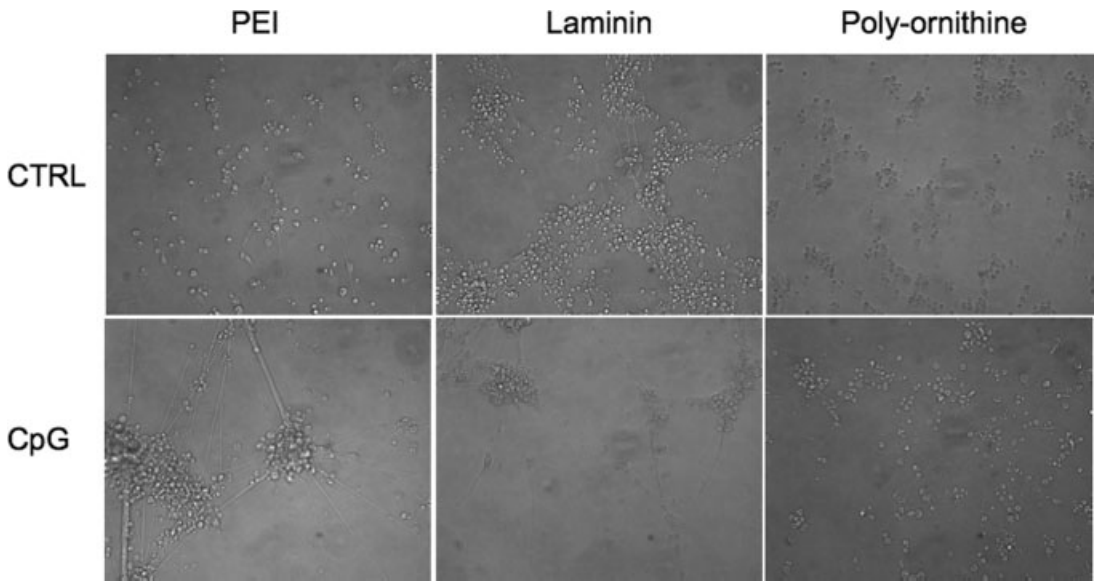

Fig. 5. Detachment phenotype occurs in neurons plated on PEI and laminin but not PLO-coated surfaces. Cortical neurons were plated on PEI (0.005%)-, laminin (10 μg/ml)-, or PLO (15 μg/ml)-coated 48-well plates. At DIV7, cells were treated with P-ODN (10 μM). After 72 hr, cells were imaged.

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