

—●— Technology Review —●—

Cyclic Olefin Polymers: Innovative Materials for High-Density Multiwell Plates

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Abstract: Extension of ultra-high-throughput experiment (UHTE) approaches to new assay methodologies is often limited by compromised data quality when samples are miniaturized. Overcoming this challenge requires attending to all components of an automated laboratory system contributing to assay variability. A key but often neglected source is the high-density multiwell platform or microtiter plate. Materials from which plates are fabricated may degrade or otherwise compromise an assay through a variety of sources, including structural weakness, distortion of optical signals, and chemical contamination. Cyclic olefin polymer (COP) resins (CAS Registry Number 26007-43-2, inclusive of polymers and copolymers, sometimes referred to as cyclo-olefin polymers or copolymers) are receiving attention for their structural strength, optical clarity, and biocompatibility. The physical and chemical properties of COP are reviewed for their ramifications on the performance of high-density multiwell plates. Cells known to be difficult to culture in standard plasticware thrive in miniaturized COP wells. In addition, cell-based assays whose data deteriorated when miniaturized in standard plastic reveal a robust recovery of data quality when miniaturized in COP. It is hoped that the material qualities and advantages of COP become better appreciated among the screening and biological communities.

Introduction

MINIATURIZATION OF BIOLOGICAL ASSAYS has enabled a wealth of information to be obtained in automated high-throughput and ultra-high-throughput experimental (UHTE) formats.¹⁻³ The utility of this information for drug discovery and larger questions of cellular biology hinges on the quality of the data that can be produced. Concerns have emerged, however, about decreases in the fraction of the dynamic range usable for determining statistically significant differences (Z' -factor or Z') when assays are scaled from 96- and 384-well plates to volumes less than 10 μ l in 1,536- and 3,456-well microtiter plates.⁴⁻¹¹ While all the avenues by which

data quality can be compromised have not been resolved, the existence of this miniaturization problem is becoming increasingly appreciated among the high-throughput community. This review seeks to allay these concerns by offering guide points to engineering smaller volume assays. Data quality is not necessarily expected to be sacrificed *per se* when sample volume is decreased, provided that the accuracy and precision of liquid handling used for assay composition and the sensitivity and range of assay signal readout are adjusted for the decreased dimensions of the assay samples.¹² Instead, attention is focused on the often neglected high-density multiwell plate. The materials used in its fabrication and the properties of those materials can contribute a myriad of factors with

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ABBREVIATIONS: COP, cyclic olefin polymer; DMSO, dimethyl sulfoxide; FRET, fluorescence resonance energy transfer; PCR, polymerase chain reaction; UHTE, ultra-high-throughput experiment.

direct bearing on assay performance. In this review, advantages afforded by the physical and chemical inertness of cyclic olefin polymer (COP) are compared with those of polystyrene and other plastics to suggest that this newer material offers a performance benchmark for improvements in assay miniaturization by the confluence of its properties. A short history of multiwell microtiter platforms reveals that data quality has always been an inextricable concern with parallel assays and suggests the time is right to reconsider the material used to fabricate the platform.

Origins of Multiwell Plates amid Concerns with Data

One of the earliest expressions of concern with data reliability arose during efforts to standardize microbiological analysis of pathogenic bacteria in milk and water—undiluted samples typically produced uncountable numbers of bacterial colonies on agar plates. Serial dilutions of a sample were shown to reduce colony numbers to ranges from which bacterial titer could be accurately estimated.¹³ However, risks of cross-contamination discouraged using multiple agar-containing wells disposed on the same platform, which were becoming prevalent as adoption of the germ theory of disease became widespread. Thus, the multiwell microtiter plate was not to originate from an obvious source, bacteriology. The first uses of multiwell platforms arose instead with the exigencies of the Dust Bowl during the Great Depression. The description of the “soil tester,” a rectangular array of cup-like depressions in a ceramic slab, captured the essence of the microtiter plate—“a simple, compact, efficient structure, such that different specimens will be tested uniformly.”¹⁴ Storing small quantities of chemical reagents in an arrangement of depressions in a plate was recognized as providing a way to format an assay,¹⁵ presaging not only the chemical storage plate, but also, when the depressions were interconnected, the microfluidic reactor. As microbiologists gradually overcame their objections, rows of simple covered boxes made of glass to enable observation of blood samples¹⁶ evolved to rows and columns of attached glass cups for staining viruses, bacteria, and blood cells with antibodies.¹⁷ The planar array of microreaction vessels made of sterilizable glass became a routine microbiological tool for the serial dilutions of Wassermann assays by introducing useful structural features to the plate, such as detachable lids and O-rings surrounding each well to prevent intermingling of contents between wells.¹⁸ The development of relative-moving boss molding for plastic sheets that maintained uniform thickness,¹⁹ however, was required to enable fabrication of multiply disposed microbiologi-

cal sample wells in a plate with a tight-fitting lid²⁰ and the now-familiar disposable microtitration plate manufactured with an 8- × 12-well array.²¹ With the standardization of dimensional format in the SBS/ANSI standards, systems of automated instruments can now be integrated with minimal concern for the origin of the plate and, perhaps more importantly, have enabled greater well densities to become routinely accommodated in the quest for realizing the scientific and economic benefits of miniaturization.^{1,3,11}

Linear Olefins and Polystyrene as Plate Materials

Plastics are used for modern plate fabrication because they can be economically synthesized and molded. A major concession is presented by the requirement for optical quality in high-content imaging assays, for which a glass plate bottom is glued to the plastic honeycomb. Plastics are matched to applications by particular physical or chemical properties. For example, resistance of polypropylene and polyethylene to degradation by solvents such as dimethyl sulfoxide (DMSO) makes these useful for storage of chemical compound concentrates in libraries. Even high-density formulations of these plastics are relatively flexible, however, as seen by comparison of their flexural and tensile moduli (see Appendix), which may be as high as 3.0 GPa, with their ultimate tensile strengths of 50 MPa or less. Thus, fine features with dimensions on the order of 1 mm, such as lid rims, plate flange edges, and the walls between wells in high-density arrangements such as the 32- × 48-well array of 1,536-well plates, have short life expectancies. Their relatively high melting points (>100°C) and thermal conductivities make them useful for the high temperatures encountered in thermal cycling, and so they have also found a place as disposable multiwell vessels for polymerase chain reaction (PCR).

Polystyrene has been successful as a material for assay plates because of its physical hardness, thermal resistance, and optical transparency. Its polymerization chemistries have been optimized to enable economical production of plates with different well shapes and other customized features that retain shape and withstand mechanical shock.²² For example, resistance to deformation, as denoted by a Young's modulus of 3.0–3.6 GPa, enables plates to withstand the rigors of gripping and clamping during automated handling. In polystyrene's most easily synthesized atactic form, phenyl rings are distributed randomly on both sides along the linear covalent polymer. Noncovalent interactions between polymers create a tightly bound vitreous mass that has no true crystalline melting temperature and a glass transition temperature >90°C, so that polystyrene plates withstand the

cycling temperatures encountered during PCR. The need for the polymerization chemistries to achieve desired uniformities of composition while simultaneously becoming more economical, however, has resulted in chemical tradeoffs of which biologists with a mind to miniaturization need to consider. Addition polymerization of styrene and linear olefins such as propylene and ethylene was established with Ziegler-Natta co-catalysis, for which Ziegler and Natta shared the Nobel Prize for Chemistry in 1963. Thermal and chemical initiation by halide derivatization or other initiators such as benzoyl peroxide or azoisobenzoylnitrile produced highly variable results. Ziegler and Natta replaced these unstable initiators with co-catalytic complexes of titanium chloride and aluminum dialkyl chlorides that enabled spacing the phenyl residues along the addition polymer chain with some control of stereospecificity.^{23,24} Because of their economy, the most widely used polymerization methods remain metal-assisted catalysts, which have been evolved to more sophisticated ferrocene, zirconocene, and molybdocene coordination structures.²² They catalyze stereoselective, syndiotactic placement of phenyl groups alternating on both sides of the linear polymer to create extremely hard crystalline resins with melting temperatures as high as 270°C. Their drawback is that these innovative and efficient catalysts are retained in the hardened plastic, and the metal content may exceed 5 mol%.^{22,25}

Biological Interactions of Polystyrene

While having proven their usefulness for biological applications since 1960, polystyrene and other linear olefin plastics are becoming discovered to exert biological effects of their own. In a study of substrate materials for tissue engineering, monocyte-derived macrophages exhibited spontaneous secretion of interleukin-1 β and tumor necrosis factor- α when cultured on tissue culture-grade polystyrene and polyethylene regardless of the presence of preadsorbed serum proteins.²⁶ In a model system for control of cell-substrate interactions, U937 cells on polystyrene released nearly 25% of their acid phosphatase and exhibited activated intracellular monocyte-specific and cholesteryl esterases nearly three times greater than cells cultured on hydrophobic polydimethylsiloxane or biodegradable polycarbonate-coupled urethanes.²⁷ Moreover, the culture of neural stem cells is found to be successful only on a highly restricted subset of tissue culture-grade plasticware,²⁸ suggesting variations in biocompatibility among tissue-grade polystyrene.

Plate effects are also becoming apparent in attempts to decrease the volume of assay samples for HTS and ultra-HTS. In cell-based calcium flux fluorimetric assays,

Z' has been observed to decrease from about 0.85 to 0.9 in 384-well plates to 0.7 or less simply on moving the assay to 1,536-well plates.^{4,5,7} While plate origin was not specified nor was dispensing error propagation analysis that might contribute to the decreased Z' presented in these studies, it is noteworthy that calcium assays would be expected to be susceptible to residual divalent transition metal cations leached from the polystyrene, and this heavy metal load could become substantial enough to overwhelm an assay at smaller volumes. Such effects are not confined to calcium flux studies and have been observed in a variety of assays, including ubiquitin ligase assembly enzyme-linked immunosorbent assay,⁹ nuclear factor κ B activation,⁸ and novel receptor transduction pathways.¹⁰ The accumulation of data suggests that progress in UHTE, particularly routine miniaturization to 3,456-well formats, may require control of the plate material as an intrinsic variable.

Modern Requirements for Microtiter Plates in UHTE

COP was selected as the base material for a new high-density multiwell plate by Aurora Biotechnologies (San Diego, CA) (when it was the Biophysics and Instrumentation Department of Aurora Biosciences Corp. before acquisition by Vertex Pharmaceuticals, Inc. in 2001 and spinoff as Aurora Discovery, Inc. in 2003) to meet a confluence of disparate plate requirements for UHTE¹²:

1. The mechanical stiffness of the material had to be large enough so that high-density multiwell (1,536 and 3,456) plates, having fine features such as thin well walls and bottoms but also conforming to industrial dimensional standards, could be fabricated that would maintain dimensional uniformity for close positioning to liquid dispensers and assay signal acquisition devices designed for the smaller wells, as well as for withstanding deformation by automated plate handling.
2. The melt viscosity of the working resin needed to be low during rapid injection molding to ensure exact replication of all the features in every manufactured plate, such as uniformly thin well walls and uniformly positioned well rims.
3. The hardened resin needed to be optically inert, ideally resembling glass of high-optical quality, by having high transmittance of ultraviolet and visible wavelengths of light and low autofluorescence especially when irradiated with ultraviolet light, to maintain a high signal dynamic range and low noise of fluorescence and other optical assay readouts.
4. The material required resistance to chemical degradation to enable storage of compound concentrate li-

TABLE 1. DIMENSIONS OF AURORA BIOTECHNOLOGIES PLATES³⁰

	<i>Plate well density</i>			
	<i>384-low</i>	<i>384-high</i>	<i>1,536</i>	<i>3,456</i>
Pitch (mm)	4.5	4.5	2.25	1.50
Well top diameter (mm)	3.07	3.6	1.70	1.13
Well bottom diameter (mm)	2.52	2.74	1.36	0.90
Top wall thickness (mm)	1.43	0.9	0.55	0.37
Bottom wall thickness (mm)	1.98	1.76	0.89	0.60
Well wall draft angle (° arc)	1.99	4.6	2.01	2.03
Well depth (mm)	7.9	12.35	4.85	3.25
Well volume (μ l)	48.94	126.39	9.03	2.66
Usable volume (μ l)	45	120	8.75	2.40

The 384-high base plate has square wells. All other plates have round wells.

baries, especially those in DMSO, and other reagents used in assay construction.

- Thermal resistance was required to enable experimental conditions, such as temperature cycling, and overall physical stability, such as low water vapor permeability and low water adsorption, was needed to mitigate sample loss during assays requiring long incubation periods.
- Biological inertness was required to avoid assay contamination and cell death, but the capability of derivatization was desired to enable attachment of extracellular matrix materials conducive to cell growth.

These requirements could not all be simultaneously satisfied by linear olefins or polystyrene. While polystyrene is mechanically very stiff and appears visibly clear, it is not optically inert, does not resist DMSO, and clouds relatively easily when sterilized by ethylene oxide or radiation. Polyethylene and polypropylene resist chemical degradation but are not optically clear and are porous to water. Polycarbonate and polymethylmethacrylate are brittle, susceptible to chemical degradation, and optically worse than polystyrene, despite their apparent visible clarity. Therefore, a new plate material was needed, and COP was better able to satisfy these requirements.²⁹ Aurora Biotechnologies developed high-density multiwell plates with three different well densities plates to meet both assay staging and chemical compound and reagent storage needs in UHTE.³⁰ The first is a 48- \times 72-well array plate (for a total of 3,456 sample wells) in which the working volume of each sample well is 2 μ l (Table 1). The second is a 32- \times 48-well array that provides 1,536 sample wells each having a working volume of 8 μ l. The basic designs have been customized for chemical storage (deep wells), evaporation control (perimeter wells and tight-fitting lid), and optical quality for imaging assays (bottom flatness) and are

also available in the high- and low-volume 384-well format. They are fabricated entirely of COP.

COP Synthesis Results in Lower Retained Metal Catalyst

COP is synthesized by addition polymerization of a variety of cycloalkyldiene and alkene precursors. Ring-opening metathesis by using ruthenium- or osmium-centered catalysts is followed by hydrogenation in a one-pot reaction.²² Several features make this synthesis easy to industrially scale while producing a superior plastic. While cyclohexane is the typical reaction solvent, it is recovered after product formation and reused, making it essentially a “green synthesis.” The synthesis rate is controlled by low amounts of catalyst (10 ppm) such that resulting average molecular mass of each polymer is optimally as small as 200 kDa. These short polymers remain slurried in the solvent and do not collapse into a large viscous colloidal mass, as is the case with polystyrene synthesis, which results in the separation of a hard-to-penetrate phase. This allows the polymerization and hydrogenation catalysts to be attached to solid supports stirred throughout the mixture that are recovered by simple filtration, and dissolved catalysts are removed by aqueous extraction. The resulting COP resin has <1 ppm residual Ru or Os in the ashed resin, as determined by plasma-atomic emission spectrometry,³¹ compared to the typical \sim 150 ppm residual Ti catalyst in polystyrene.³² Analysis of virgin resin powder by inductively coupled plasma mass spectrometry reveals a total metal impurity content of <60 ppb for Pd and Ni (the hydrogenation catalysts) in addition to Zr, Al, V, Cr, W, Fe, Mg, Ti, Zn, and Cd,³³ all of which are typical impurities found in industrial formulations of polymerization catalysts. These impurities are present at >1 ppm in polystyrene and poly-

TABLE 2. MECHANICAL AND THERMAL PROPERTIES OF PLATE MATERIALS

	<i>Plastic</i>		
	<i>COP</i>	<i>PS</i>	<i>PP</i>
Flexural modulus (GPa)	2.2	3.3	0.55–2.41
Flexural strength (MPa)	94	103	23–50
Tensile modulus (GPa)	2.4	3.4	0.5–7.6
Tensile strength (MPa)	61	80	12–43
Glass transition temperature (°C)	136	90–104	44–148

COP, Zeonor 1420R; PS, polystyrene, high-impact, injection mold grade; PP, polypropylene. Glass transition temperature includes zero-pressure deflection temperature. Data are from Zeon Corp.³³ See Appendix for definitions.

carbonate and often as high as 100–200 ppm.³² Analysis by inductively coupled plasma mass spectrometry of metal ions leaching into physiological saline exposed to hardened resin at 100°C for 24 h has shown no detectable amounts (<20 ppb) of Pb, Cu, Sn, or Ca,³³ whereas the amounts of the same metals leached from polystyrene were >10 ppm.³⁴ Therefore, COP is a material of very high purity and should allay concerns of biological sample contamination.

Mechanical Properties and Plate Stability

The relatively simple synthesis of COP results in a resin having key thermomechanical properties essential for high-density multiwell platforms. Critical to fabricating millimeter-scale features with fine dimensional tolerances is the melt viscosity of <10⁴ poise at 295°C, allowing the melted resin to fill a 100°C mold and cool in <1 min.³⁵ Overall plate dimensions are consistently fabricated within ±130 μm tolerances, whereas fine features, such as well rim locations, lid rests, and machine vision fiducials for robotic plate positioning, are within ±13 μm.³⁰

Plates face mechanical problems that are readily apparent to the practitioner in the automated laboratory—a plate is no match for an uncontrolled encounter with a robotic gripper or positioner weighing 10 kg. Even the spring clamps of PCR thermal cyclers often result in noticeable plate destruction by the end of an experiment due to the heat distortion temperatures of polystyrene (90–104°C) and polyethylene (60°C). Less appreciated are the internal plate instabilities imposed by the well densities needed for UHTE. An ultra-high-throughput benchmark of >100,000 assay samples per day necessitates more than 1,000 samples on each plate, simply to avoid having to handle more than 100 plates in a campaign. Integral subdivision of the 9-mm center-to-center

well spacing of the 8- × 12-well array, in conformity with the footprint and well dimensions specifications for 96-, 384-, and 1,536-well plates^{36–38} to enable compatibility with automated laboratory instrumentation from any supplier by easy adjustment of motion control, results in center-to-center spacings of 2.25 mm for 1,536-well plates and 1.5 mm for 3,456-well plates (Table 1). To enable retraction of the well molding pins from the honeycomb without distorting the thin well walls, the wells require a draft angle such that the top diameter of the well is greater than the bottom diameter.³⁵ This creates a mass gradient in the plate from bottom to top that imposes a tendency of the plate to bend. While this bending force is distributed evenly across the well array due to (1) the regular disposition of holes and (2) reinforcement by the extra plastic between the perimeter wells and the plate edge, the force has to be distributed uniformly around the circumference and all along the height of each well to prevent concerted buckling of the wells and bending of the plate. For example, the mass difference between the top and bottom of the 1,536-well plate results in a bending pressure of ~2.6 Pa.³⁹

The seemingly dire situation indicates the need for stiff plate materials, so that slight deformation imposes restoring forces sufficient to resist further bending. An ANSI/SBS-sized plate of 127.5 × 85.5 mm with a Young's modulus of 1.0 GPa and 5 mm thick exhibits about 4 μm of natural bend in the long dimension.³⁹ This may be compensated by a massive enough frame or flange. Nonetheless, intrinsic plate bending sets a lower limit on the distance between a plate surface and the heads of liquid handling and top-reading signal detection instruments. Plate materials having Young's moduli exceeding 1 GPa are stiff enough for the honeycomb to be thin (*e.g.*, 3 mm) to enable assay sample well volumes of <10 μl. Table 2 shows the mechanical and thermal constants of the COP resin used in Aurora Biotechnologies plates (Zeonor 1420R, Zeon Corp., Tokyo, Japan) and some other olefins for comparison.

The mechanical rigidity and flatness of COP plates allow dispenser tips and optical read assemblies to be brought to a distance between 0.2 and 0.5 mm of the plate surface when positioned over each well. While the plate standard allows a 0.75 mm tolerance in the top and bottom surface planarity of plates,^{36–38} the ability of COP to consistently meet the manufactured quality tolerance of 0.13 mm for Aurora plates enables active heads to be safely positioned within 0.2 mm of the plate surface. A design principle originally adopted for instrumentation was to move the plate under dispenser heads in a continuous scanning motion to avoid volumetric dispensing errors due to pressure fluctuations in system lines and flapping of the liquid–vapor interfaces of the dispenser tips.¹² Aurora plate flatness allows fixing the relative po-

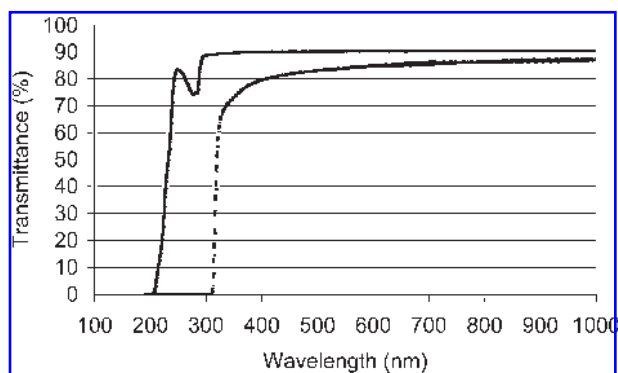


FIG. 1. Optical transmittance of 0.1-mm-thin sheets of optical grades of polystyrene (broken line) and Zeonex 480R COP (solid line) from ultraviolet through near-infrared wavelengths of light.

sition of the active head close to the plate surface to minimize dispensing and reading errors while avoiding head contact with the plate during rapid scanning.

Optical Properties of COP

Interactions of the plate material with light are some of the most critical properties of plate materials, especially those used for well bottoms. Assay signaling modalities have increased from fluorescence intensity, to fluorescence lifetime, fluorescence resonance energy transfer (FRET), time-resolved FRET, and polarization anisotropy, among others. With imaging in high-content and high-throughput formats becoming necessary for obtaining spatial localization information from cell-based assays, optical properties have become a crucial component of plate materials for understanding how they influence the signals obtained from assays. Light absorption decreases sensitivity in fluorescence assays by diminishing the intensity of the excitation illumination. Autofluorescence decreases sensitivity and dynamic range by contributing intensity to the emitted assay signal. Other effects such as polarization anisotropy through material strain may also diminish the illumination intensity by polarized sources such as lasers in time-resolved FRET and create unwanted background light depolarization and, hence, diminution of both incoming and outgoing light. Polystyrene was originally favored as a plate material over linear olefins not only because of its clarity, but also because it was only minimally clouded by gamma irradiation sterilization. Its noted absorbance of ultraviolet wavelengths (Fig. 1) and subsequent visible autofluorescence (Fig. 2), however, necessitated substitution of a glass bottom for obtaining high-quality fluorescence signals or images. In this regard, the Whatman (Maidstone, UK) Polyfiltronics 96-well plate used a sheet

of 177- μm -thick borosilicate glass (the same as a No. 1.5 coverslip) bonded to the plate bottom with epoxy cement, and Greiner Bio-One (Kremsmünster, Austria) has begun to offer plates with COP bottoms ($\mu\text{Clear}^{\text{®}}$) optimized for ultraviolet transmittance.

COP has very high transmittance from ultraviolet through infrared wavelengths of light compared to other plastics. It is much clearer than polystyrene (Fig. 1) and is closer to optical grades of crystalline or fused silica. Through a 0.1-mm-thin sheet polystyrene plate bottom, 1% transmittance is attained at a wavelength of 307 nm, and the transmittance reaches half of its maximum value of 87% at 317.7 nm. For a plate bottom of the same thickness of Zeonex 480R COP, from which Aurora fabricates the bottoms of its plates, 1% transmittance is attained at 206 nm, the maximum transmittance is 91%, and the half-maximum is reached at 229 nm. Thus, COP is very useful for measurements of DNA purity in high-throughput samples by 280 nm to 260 nm extinction ratios.

Autofluorescence is a vexatious difficulty with plastic plates by increasing the fluorescence background at wavelengths emitted by the most common visible fluorophores such as fluorescein, rhodamine, and their modern derivatives. Emission profiles of various plate bot-

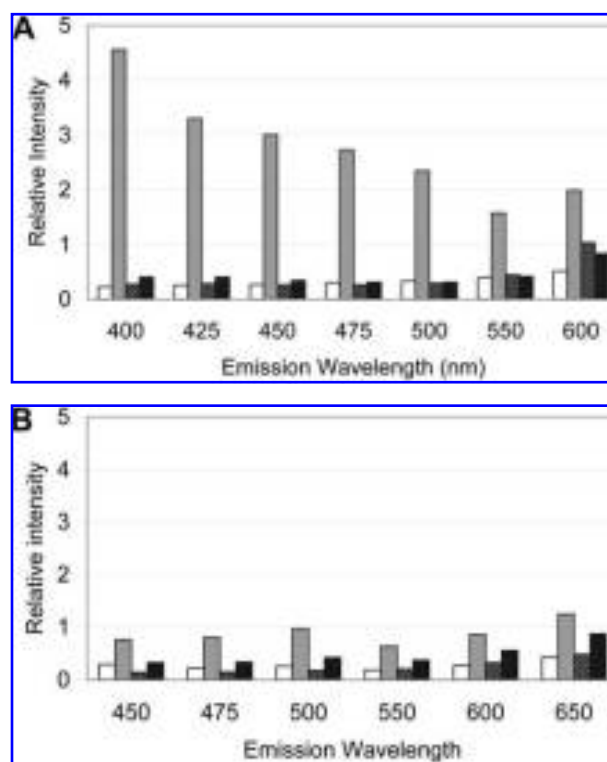


FIG. 2. Autofluorescence of 0.2-mm-thin sheets of materials used for plate bottoms with illumination by two excitation wavelengths: (A) $\lambda_{\text{ex}} = 315 \text{ nm}$ and (B) $\lambda_{\text{ex}} = 400 \text{ nm}$. □, fused quartz; ■, polystyrene; ▨, Aclar; ■, COP. Data are from Coassin *et al.*²⁹

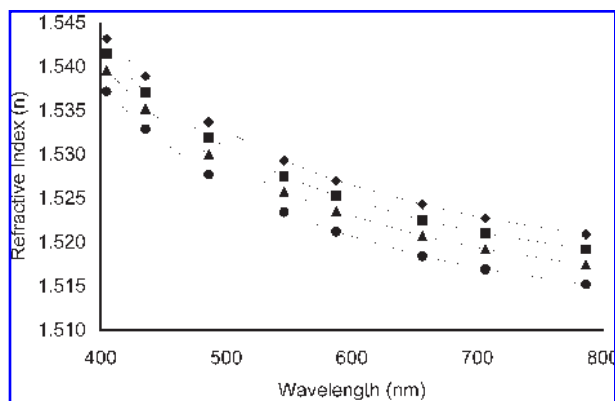


FIG. 3. Refractive indices of COP clear sheet as a function of wavelength at temperatures of 10°C (diamonds), 25°C (squares), 40°C (triangles), and 60°C (circles). Chromatic dispersion (see Appendix) is low, indicating that COP will not shift different colors in an image.

tom plastics are shown in Fig. 2 for two excitation wavelengths: 315 nm (Fig. 2A), to show the autofluorescence of polystyrene in response to ultraviolet absorptivity, and at 400 nm (Fig. 2B), the typical excitation wavelength for the blue fluorophores based on coumarin (2-chromenone). Polystyrene and COP are compared to other plate bottom materials used in optical-grade plates, including the Aclar fluorocarbon (Allied Signal, Morristown, NJ) used as an ultraviolet-quality plate bottom material, and Corning (Corning, NY) “Zinc-Titania” No. 210 coverslip glass, the original borosilicate grade with $n = 1.523$ used for standard coverslip manufacture. COP is noteworthy for its lower autofluorescence compared to polystyrene across the visible emission wavelengths, yielding lower plate background intensities for fluorescence measurements.

COP also exhibits refractive and birefringence properties useful for polarized light applications. The unstressed resin has no uniaxial or biaxial differences in indices of refraction (birefringence) as measured by circular dichroism, and applied stresses up to 2 MPa produce net birefringences $<10^{-4}$, amounting to an optical path difference <20 nm through a 3-mm-thick sheet.⁴⁰ This is important when considering that improvements in the optical properties of polystyrene have been realized by giving it a crystalline character, thus increasing its birefringence. The variation of refractive index with wavelength for COP and its temperature dependence are relatively small, characterized by an Abbe number of 56–58 (Fig. 3). Thus, COP is eminently suited as an optically inert material for new assay signaling modalities that incorporate light polarization.

An important consideration is the influence of the well bottom on an image. To achieve an optical-quality assay plate, a sheet of an optically superior grade of clear COP is stretched tight and welded to a well honeycomb molded

from a mechanically strong COP formulation containing a pigment such as carbon black, to decrease stray light and crosstalk between wells. The stretch decreases curvature of the well bottoms to less than $60 \mu\text{m}$, so that they do not impose a diverging lens element. Curvature of the well surface decreases the light intensities collected by objective lens (Fig. 4). The ability to create a flat bottom having minimum well curvature without inducing axial anisotropy enables COP plates to be useful for polarized light imaging modes such as differential interference and modulation contrast. Plates can also be pigmented white with titanium dioxide for top-read luminescence measurements. Titanium dioxide in the resin amplifies low levels of emitted light by metal-enhanced chemiluminescence for low quantum yield applications such as time-resolved fluorescence.

Biological Compatibility of COP

COP is exceptionally inert. The contact angle of water on virgin COP is 136° of arc,⁴¹ and that on Zeonor 1420R exposed to air is 94° of arc.³⁵ The surface is ef-

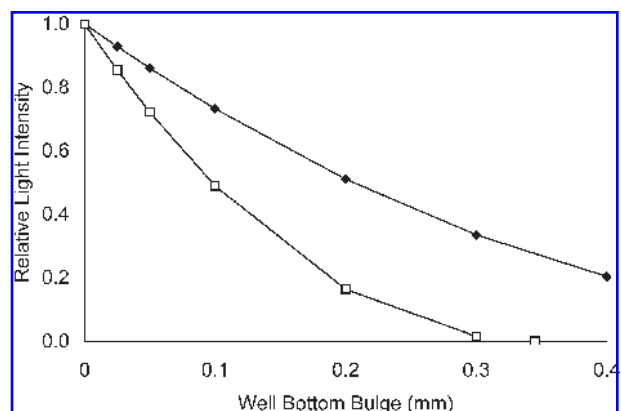


FIG. 4. Curvature of well bottoms decreases the light collected by objective lenses imaging well samples from below. Calculated results for two objectives, a 100 \times , numerical aperture (NA) 1.25 (solid symbols) and a 40 \times , NA 0.6 (open symbols) objective lens, are depicted for a 1.36-mm-diameter well (from a 1,536-well plate). The change in focal length of the compound lens system comprising the objective and well bottom was calculated as $(1/f_L + 1/f_{\text{well}})^{-1}$, where f_L is the focal length of the objective and f_{well} was calculated with the standard diverging lens equation $1/f_{\text{well}} = 2(n - 1)/R$ for well bottom bulges of the indicated depth. The refractive index was taken as $n = 1.523$ (COP and Corning 210 glass) with a sign convention of $R < 0$ for a diverging refracting surface. Focal length was taken as proportional to $1/NA$ and light intensity proportional to $(NA)^2$. Light intensity reaching the objective was calculated for each bulge relative to an ideally flat well bottom. Well bottom curvature increases the focal length of the imaging system, resulting in decreased NA, which is more pronounced for the lower NA objective.

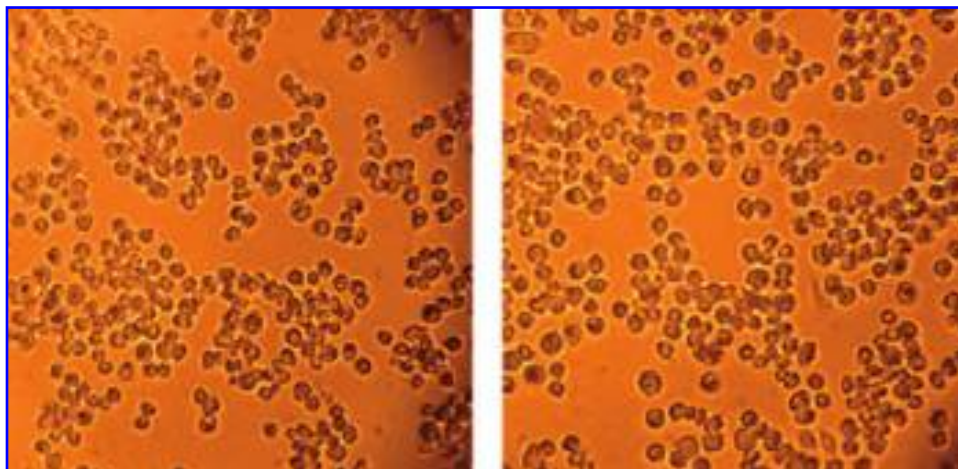


FIG. 5. Human embryonic kidney 293 cells cultured in a 3,456-well COP plate: (**left**) untreated and (**right**) corona-discharge-treated plate prior to cell culture. Cells were cultured in standard polystyrene plasticware, then trypsinized, pelleted, and diluted to 10^5 cells/ml in RPMI medium without serum. Each well was seeded with $1 \mu\text{l}$, and an additional $1 \mu\text{l}$ of medium was added. Cells were cultured at 37°C for 24 h with the plate lidded and then observed in bright-field microscopy.

fectively self-passivated and undergoes minimal uncontrollable oxidation when exposed to atmosphere. For comparison, it is noteworthy that the contact angle of water on acid-cleaned and neutralized polystyrene exposed to air is $65\text{--}80^\circ$ of arc and on clean fused silica is 30° of arc (authors' unpublished data). Low polarity provides a surface for the adsorption of hydrophobic molecules with low aqueous solubility, such as anthranoyl and diphenylhexatriene fluorophores, however, and small ($<10\text{-kDa}$) hydrophobic polypeptides such as stromal cell-derived factor- 1α adsorb as well (authors' unpublished data). Nonetheless, the major advantage of the inert COP surface is that its properties are ultimately under the control of the experimenter. Mild plasma treatment renders the surface hydrophilic with no decrease in optical clarity. Treatment for as little as 5 min in a pure oxygen plasma generated at 30 W power at the relatively high pressure of 0.1 atm with a small flow rate of 3 ml/min (scm) decreases the contact angle of water with Zeonor 1060R to 10° of arc.⁴² While this contact angle is not stable and triples in 2 days, the resulting surface reactivity can be functionalized and used for covalent attachment of macromolecules. Moreover, the large change in surface wettability with such a mild plasma treatment reveals that economical plasma treatments such as passing a corona-discharge arc over the plastic (*e.g.*, with a hand wand) will create sufficient reactivity for promoting the adherence and assembly of scaffolds. Aurora's plates suitable for cell culture are sterilized by gamma irradiation and corona-discharge-treated.

Cells grow readily on COP surfaces. Preliminary surface treatment may be used to prepare the substrate for self-assembly of extracellular matrix molecules, such as collagens, laminins, Matrigel (BD Biosciences, San Jose,

CA), chondroitins and other components. Cells whose culture is generally recognized as difficult thrive on COP even in minimal conditions. This is illustrated in Fig. 5, where human embryonic kidney 293 cells have been cultured on untreated and on corona-discharge-treated (but no further substrate preparation) 3,456-well COP plates. Under both conditions, the cells proliferated to similar densities on both treated and untreated surfaces. Other cell types may require extracellular matrix materials for

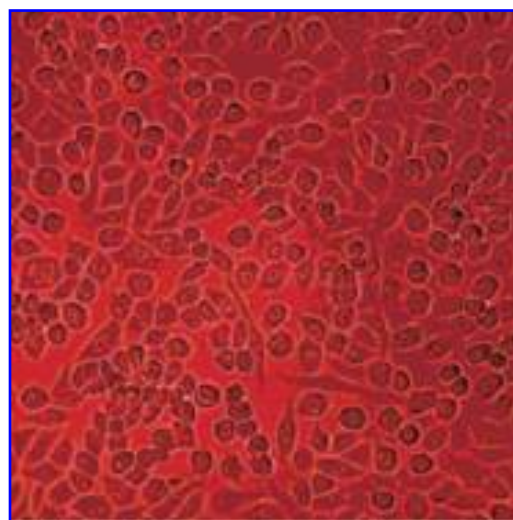


FIG. 6. THP-1 cells 24 h after culture in a corona-discharge-treated 3,456-well plate. The well was coated with $2 \mu\text{l}$ of undiluted Matrigel that was aspirated after 15 min and then inoculated with $2 \mu\text{l}$ of THP-1 cells at 10^5 cells/ml in RPMI medium supplemented with 10% fetal calf serum. The cells were incubated and viewed as in Fig. 5.

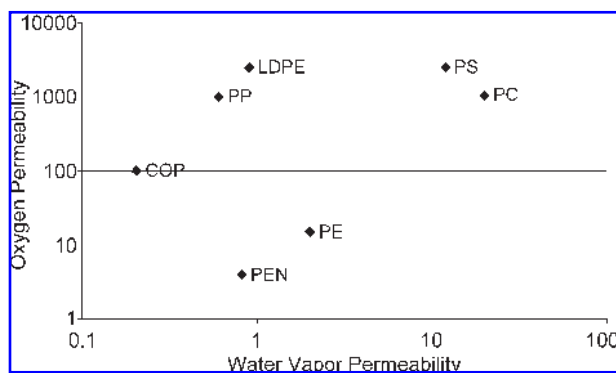


FIG. 7. Oxygen versus moisture permeability for different plate materials. Oxygen permeability (in ml/m² · 24 h at 1 atm) was measured for 80- μ m-thin sheets of the indicated material, and water vapor permeability (g/m² · 24 h) was measured for 0.3-mm-thick sheets by JIS Z 0280 (Japanese mechanical measurement standard). COP, Zeonor 1420R; PP, polypropylene; PEN, polyethylene naphthalate; LDPE, low-density polyethylene; PE, polyethylene; PS, polystyrene; PC, polycarbonate. Data from Zeon Corp.³³

optimal survival and growth on COP. The THP-1 monocyte line shown in Fig. 6 proliferated only on the presence of Matrigel basement membrane matrix allowed to adsorb to the well bottom prior to inoculation. This biocompatibility of COP makes it worthy for consideration as the new cell culture standard. The material lacks inherent protease and nuclease activities,⁴³ making it suitable for cell-free biomolecular assays as well.

Sample Stability in COP

The combination of several physical and chemical properties of COP makes it excellent for other applications in UHTE. For example, compound library storage is possible because the resin exhibits little penetration, softening, or other deterioration by DMSO, as well as al-

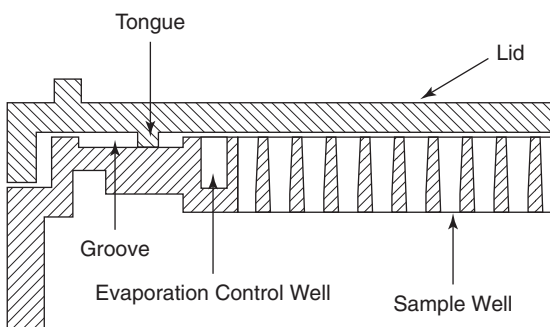


FIG. 8. Evaporation control well location and lid tongue-groove feature for sample evaporation control. The evaporation control wells contain the sample solvent and supply the headspace with vapor.

cohols shorter than *n*-butanol, ketones shorter than methylisobutyl ketone, aldehydes, peroxides, alkalis, and dilute acids.³³ COP is susceptible, however, to degradation by benzene, tetrahydrofuran, and ethers and to penetration by alkanes, so it has limited utility as a solid support for organic synthesis.

COP has a relatively low permeability to water vapor compared to other gases such as oxygen (Fig. 7). The low water permeability of the resin attenuates sample volume diminution by evaporation through the plate material, while the high oxygen permeability favors culture of respiring cells.

These permeability properties allow liquid samples in wells to be stabilized by incorporation of evaporation control wells and tight-fitting plate lids into the plate design. Evaporation control wells are a set of wells containing the sample solvent completely surrounding the perimeter of the array of sample wells. The solvent may be DMSO for stored library compounds, water for stored assay reagents or constructed assays in incubation, or some other liquid. The lid has a tongue completely surrounding the evaporation control and sample wells that fits into a groove on the top surface of the plate to create a continuous headspace and seal it from the outside atmosphere (Fig. 8).

The evaporation control wells penetrate only three-quarters of the way through the plate. They are peripheral features of the plate surface and do not decrease the number of sample wells in a plate (*i.e.*, a 1,536-sample well plate actually has 1,700 evaporation and sample

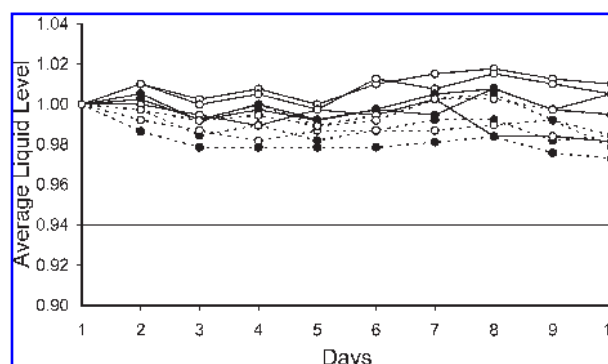


FIG. 9. Average well volume change per plate of 75:25 DMSO:water. Each sample well was filled with 7 μ l of solution, and each evaporation control well was filled with 4 μ l of the same solution. Each plate was either lidded (open symbols) or sealed (solid symbols) and then stored at room temperature (solid line) or at 4°C (broken line). The meniscus location in each well of every plate was determined once each day by echo timing with the EDC Biosystems HTS-01. The meniscus heights were averaged for each plate and compared to the initial level measured on the first day. There are no significant differences between sealing and lidding or storage at room temperature or in the cold. The largest volume decrease (−2.8%) was observed for a sealed plate stored at 4°C.

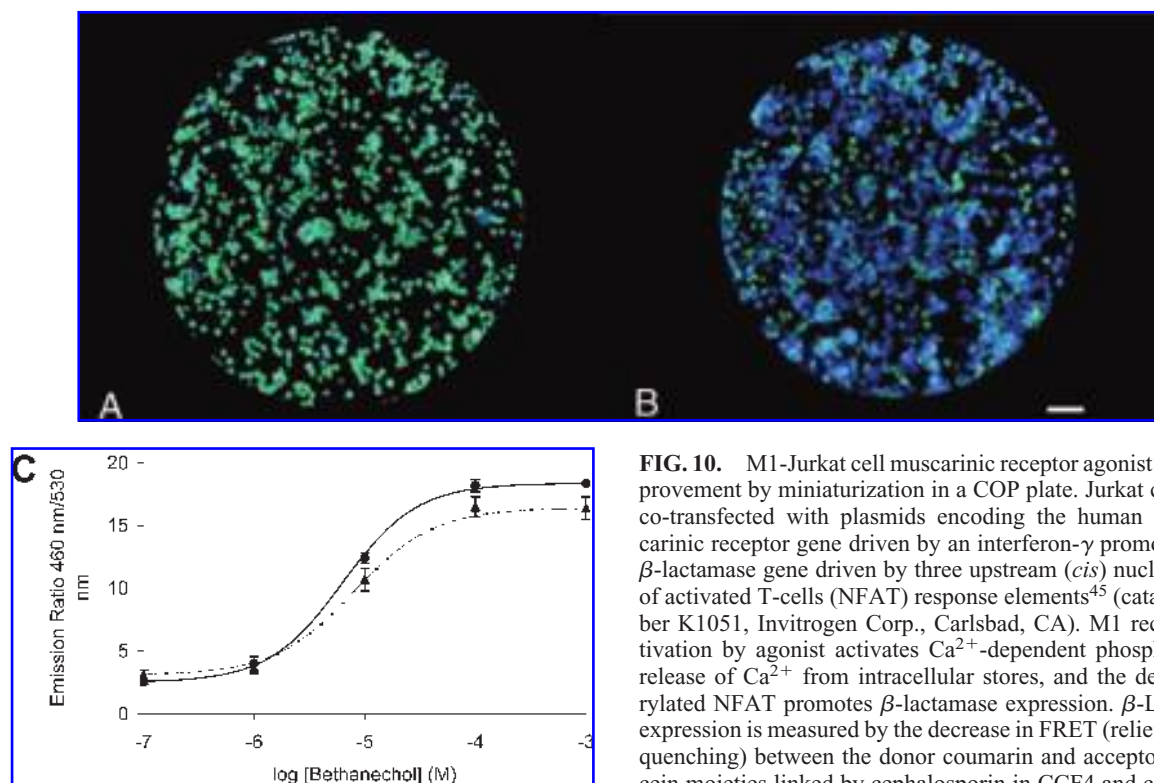


FIG. 10. M1-Jurkat cell muscarinic receptor agonist assay improvement by miniaturization in a COP plate. Jurkat cells were co-transfected with plasmids encoding the human M1 muscarinic receptor gene driven by an interferon- γ promoter and a β -lactamase gene driven by three upstream (*cis*) nuclear factor of activated T-cells (NFAT) response elements⁴⁵ (catalog number K1051, Invitrogen Corp., Carlsbad, CA). M1 receptor activation by agonist activates Ca^{2+} -dependent phosphatase by release of Ca^{2+} from intracellular stores, and the dephosphorylated NFAT promotes β -lactamase expression. β -Lactamase expression is measured by the decrease in FRET (relief of donor quenching) between the donor coumarin and acceptor fluorescent moieties linked by cephalosporin in CCF4 and cleaved by expressed β -lactamase. (A) Image of M1-Jurkat cells in a 3,456-

well plate loaded with CCF4-acetoxymethyl ester for 1 h and washed. Intracellular esterase cleaves the acetoxymethyl moiety from fluorescein, resulting in 530 nm emission on excitation of the coumarin with 400 nm light. (B) Cells activated by 1 μM carbachol. β -Lactamase cleavage of the FRET dye linker relieves donor quenching, which increases emission intensity at 460 nm. (C) Dose-response profiles for cells exposed to concentration series of bethanechol. The assay was run in a 384-well polystyrene plate in 25 μl total sample volume per well (triangles). Reagents were dispensed with a Beckman Coulter Biomek, and the assay signal was read with a PerSeptive Biosystems (Foster City, CA) CytoFluor[®]. $R^2 = 0.986$, $n = 1.2$, log 50% effective concentration = -5.1 , $Z' = 0.67$. Alternatively, the assay was run with 2 μl sample volume in an Aurora 3,456-well Nanoplate, with reagents dispensed by piezo- and solenoid-valve inkjet dispensers (Piezo-electric Sample Distribution Robot and Flying Reagent Dispenser), and the assay was read with a topology-compensated plate reader (diamonds). $R^2 = 0.999$, $n = 1.3$, log 50% effective concentration = -5.3 , $Z' = 0.96$. The lines are nonlinear least-squares fits of the logistic function to each set of points.

wells). Nonetheless, when the evaporation control wells are filled with the same solvent used in the sample wells, their volume is sufficient to provide enough solvent vapor in the headspace to block evaporation of the samples. In Fig. 9, liquid height data are shown for 10 1,536-well plates filled with 75:25 DMSO:water in both evaporation control and sample wells. Half the plates were sealed with an adhesive sheet of aluminized polyethylene, and the rest were lidded. Once each day, the liquid volume in each well was determined by sounding of the meniscus (HTS-01, EDC Biosystems, Inc., Milpitas, CA). The average sample volume for all the plates changed by only $1.18 \pm 0.74\%$ over a 10-day period, independent of storage temperature, sealing, or lidding.

COP may also be used to store aliquots of chemical compounds prespotted in an assay-ready format. In an interesting experiment,⁴⁴ submicroliter aliquots of 75:25

DMSO:water were spotted in 1,536- (20 nl) and 3,456-well (10 nl) Aurora ChemLib[®] plates and then stored for various periods of time at -20°C either with or without filling the evaporation control wells and with or without bagging. DMSO evaporation was determined by comparison of cytochrome P450 3A4 activity with a standard curve of enzyme susceptibility to DMSO. With filled evaporation control wells, prespotted 1,536-well plates produced enzyme activity of acceptable criterion for up to 7 months, whereas without filled wells, plates could be used for 4 months. For 3,456-well prespotted plates, filled evaporation control wells extended usability from 6 to 12 months compared to plate bagging alone.⁴⁴ DMSO volume stability is an important variable to consider in assay variability for screening compound libraries. Well-to-well variation in the amount of residual solvent may affect not only the intrinsic activity of the

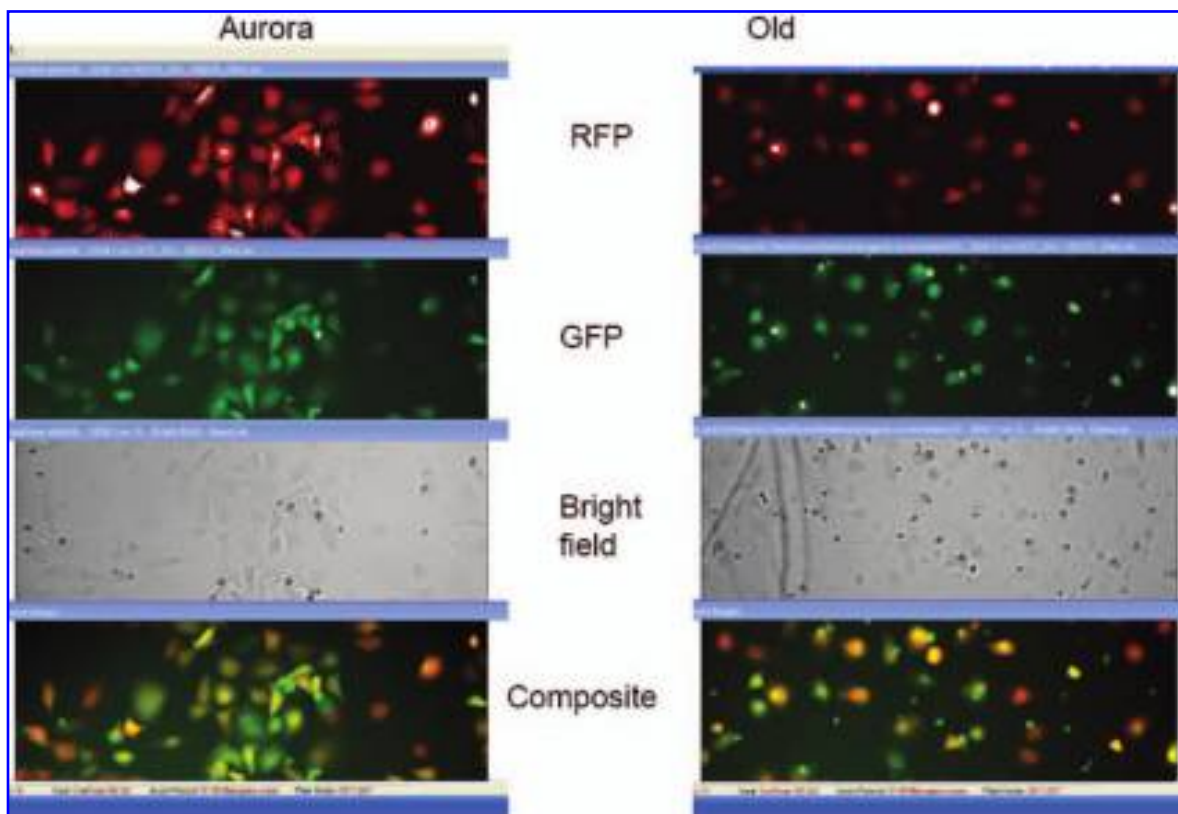


FIG. 11. Images of cells cultured in **(left)** Aurora Lo-Base[®] 1,536-well plates compared with **(right)** standard optical-quality plates obtained in an InCell 1000 (GE/Amersham Biosciences, Chalfont St. Giles, UK). The cells co-express green fluorescent protein (GFP) and red fluorescent protein (RFP). Note the cells grow larger in the COP Aurora plate and exhibit less evidence of death. Data from the NIH Chemical Genomics Center.

assay, but also the effective concentrations of chemical compounds having different polarities and, hence, aqueous solubilities. Thus, it was reassuring in this study that storage of prespotted COP plates at -80°C for a year produced no variation in assay quality over storage time, suggesting that prespotting and storage may be acceptable in eliminating compound distribution as a bottleneck in ultra-HTS.

Maintenance of Assay Quality Through Miniaturization in COP

In Aurora's experience, assay quality is maintained and even improved by miniaturization to 1,536- and 3,456-well formats in COP plates.³ Bethanecol dose-response curves are shown for a Jurkat cell-based M1 muscarinic receptor agonist assay in 384-well polystyrene and 3,456-well COP plates in Fig. 10. When the assay was deployed to a total sample volume per well of $25\ \mu\text{l}$ in polystyrene 384-well plates, Z' was only 0.67. Miniaturization to 2

μl in COP 3,456-well plates actually improved the assay parameters, with an increased Z' of 0.96 and better regression coefficient, largely by decreasing the variability within replicated agonist concentrations (Fig. 10).

The improvement in assay quality may be ascribed at least partly to the use of COP as the plate material. However, the ink-jet dispensing and plate reading instrumentation were specialized for the small well dimensions and assay volumes of 3,456-well plates. These specializations allowed the dispenser tips to be brought to within 0.25 mm of the well rim and the optical head to within 0.15 mm of the well bottoms, and the plate was moved while the active heads remained stationary. Instrumentation engineering exerts notable effects on assay quality.¹² For example, precision of solenoid valve dispensing was $>98\%$ compared to the 95% typically achieved by the contact-based piston-displacement dispensing of the Biomek[®] (Beckman Coulter, Fullerton, CA), which generally requires tip contact with the well contents to attain accurate and precise dispensing. Furthermore, the plate reader actively repositioned the optical head during the

read scanning to decrease signal variability caused by well bottom curvature. It is undoubted that all these factors contributed to the improved assay quality in addition to any decrease in the heavy metal load imposed on the assay by the COP relative to polystyrene. Nonetheless, given that intracellular calcium mobilization regulates the signal development in the assay, it is likely that the cleaner plate material contributed to the better quality.

Live-cell imaging also benefits from miniaturization in COP. Culture of the same genetically engineered cell lines in COP and polystyrene plates reveals more uniform expression of fluorescent proteins and improvement of the overall morphological quality in COP (Fig. 11).

Conclusions

In this review, some physical and chemical properties of COP, polystyrene, and other plastics are enumerated and described that may enable explication of some difficulties of assay miniaturization in high-density multiwell plates. While there is no certainty that heavy metal impurities of polystyrene underlie bioactivities contributing to these difficulties, the case is advanced that the generally recognized optical qualities and biocompatibility of COP contribute to assay quality. More general appreciation of the qualities of COP among members of the biological community may result in the successful extension of UHTE methodologies to new assays, including nucleic acid reactions such as PCR, RNA interference, and micro-RNA studies for target identification, and live-cell imaging, by adopting COP as the standard plate material.

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Appendix

Definitions of Thermal and Mechanical Constants⁴⁶

Tensile Modulus

The ratio of an applied stress to the resulting strain in a material. A constant that describes the force needed to compress or expand an object to increase or decrease its surface area by a unit amount. Other names include tangent or secant modulus of elasticity and Young's modulus. Unit is pressure.

Tensile Strength

The pressure applied in a linear direction along a surface of a material that results in fracture or other defect.

Flexural Modulus

The ratio of a stress applied in a curving direction to the bending strain induced in a material. A constant that

describes the force needed to bend an object by a unit curvature. Other names include bending modulus. Unit is pressure.

Flexural Strength

The pressure applied to a material as a bend or twist that results in fracture.

Glass Transition Temperature

The temperature above which a vitreous (noncrystalline) solid softens or melts and is able to flow, and below which a melt hardens and is unable to flow. The major difference between the glass transition temperature and crystal melting temperature is that melting of a vitreous solid occurs over a wider range of temperature com-

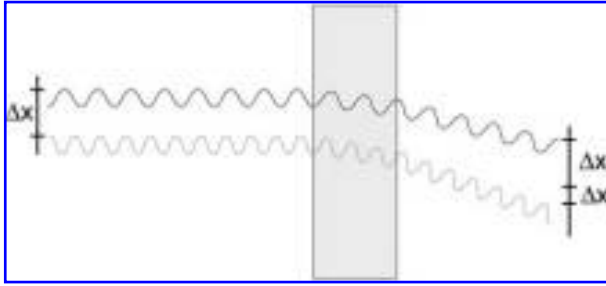


FIG. A1. Chromatic dispersion. The material block exhibits variation in index of refraction as a function of wavelength. Longer wavelengths (black) have a smaller index of refraction and hence are refracted by smaller angles on transmission into and out of the block than shorter wavelengths (gray). Thus, non-vergent light waves of different colors transmitted along paths initially separated by a distance Δx will be diverged on passing through the material, as illustrated by the increment in separation of $\Delta x'$. This results in spatial displacement of the focus locations of the two waves in the image relative to the object.

pared to a solid having crystalline structure. Applied mechanical pressure causes plastics to deform at lower temperature; hence, the glass transition temperature is often quoted in many sources as a zero-pressure deformation temperature.

Melt Viscosity

The viscosity (dynamic or kinematic) of a melted plastic resin above its glass transition temperature.

Chromatic Dispersion

The mechanism of chromatic aberration in an image in which the optical paths of light waves having different wavelengths are spatially displaced because of the light waves passing from one medium into a different medium have different indices of refraction for each wavelength (Fig. A1).