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Diversity-oriented fluorescence library approaches for probe discovery and development

Marc Vendrell[1], Jun-Seok Lee[2,3] and Young-Tae Chang[1,2]

Diversity-oriented fluorescence library approaches have significantly accelerated the development of new sensors. By making use of combinatorial chemistry and high-throughput screening, they can circumvent our limitations in designing probes for particular recognition processes. Combinatorial chemists have proved how to derivatize fluorogenic scaffolds, tune their photophysical spectra and adjust their properties (from cell permeability to quantum yields) to generate libraries of potential sensors. Several platforms (*in vitro* assays, cell-based imaging) have also been optimized to screen these libraries in a high-throughput manner, and with the recent progress in image acquisition and analysis, their scope has been expanded toward more diverse and demanding biological systems. Supported by successful examples of fluorescent sensors for biomolecules, proteins, or even phenotypes, this review (together with a video abstract) stresses the important role that diversity-oriented approaches will continue to play in probe development in the near future.

Addresses
[1] Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium, Agency for Science, Technology and Research (A*STAR), 11 Biopolis Way, #02-02 Helios Building, 138667 Singapore, Singapore
[2] Department of Chemistry & MedChem Program of Life Sciences Institute, National University of Singapore, 117543 Singapore, Singapore

Corresponding author: Chang, Young-Tae (chmcyt@nus.edu.sg)
[3] Current address: Korea Institute of Science & Technology (KIST), Life/Health Division, Integrated Omics Center 39-1 Hawolgok-dong, Sungbuk-ku, L2121 Seoul, Korea 136-791.

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Introduction

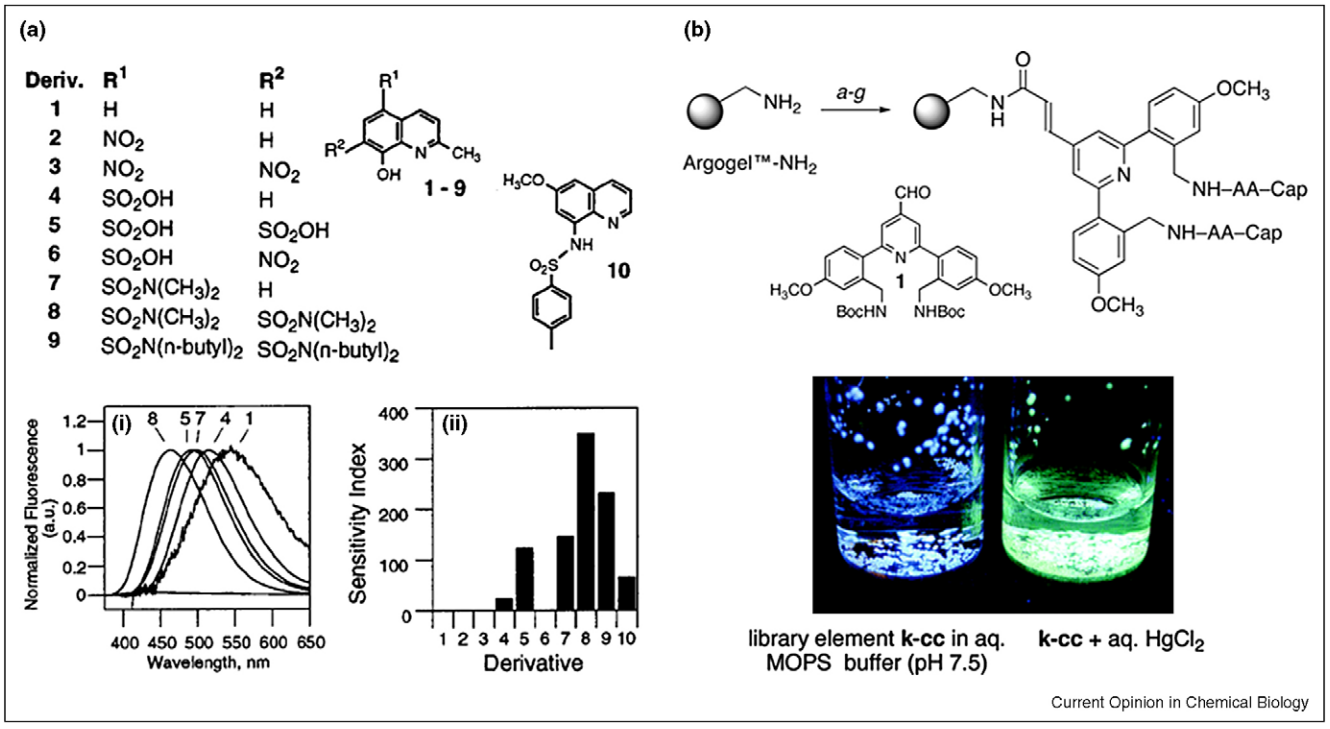
Antibodies, fluorescent proteins, and small molecule fluorophores are the most common labeling methods used in fluorescence bioimaging. Among them, small fluorescent molecules are the most versatile. Their low cost, cell permeability, and minimal technical limitations make them useful for multiple applications, from analytical sensors to live cell imaging probes [1–3]. However, such versatility often entails difficulties when designing probes for a particular aim. Despite recent advances in molecular recognition knowledge, our ability to *de novo* design fluorescent sensors for many contemporary scientific challenges (e.g. stem cells differentiation, cancer diagnosis) is still limited. Diversity-oriented fluorescence library approaches rely on high-throughput, rapid, and inexpensive tools to circumvent that limitation. Whereas some may compare this strategy to finding a 'needle-in-a-haystack', balanced approaches utilizing combinatorial chemistry and high-throughput screening (HTS) can significantly accelerate the development of novel fluorescent probes.

Combinatorial chemistry in fluorescent sensor development

Initial efforts in the development of fluorescent sensors through combinatorial chemistry were based on derivatizing well-known recognition structures (boronic acids for sugars [4], guanidines for nucleosides [5], polyamines for metals [6]) and including fluorescent reporters as signal amplifiers. Whereas such approaches rendered, and continue to render, successful fluorescent sensors [7], the discovery of novel probes depends on the preceding knowledge of a recognition motif.

A more versatile application of combinatorial chemistry in sensor development started with the derivatization of fluorogenic scaffolds. Taking advantage of the chelating properties of quinolines, Pearce *et al.* developed divalent zinc sensors on the basis of fluorogenic quinoline structure (Figure 1a) [8]. The chelation-induced effect was used to identify the best probes, and fluorophores were regarded as potential sensors rather than simple reporters. It was apparent that combinatorial chemistry could generate large numbers of potential fluorescent probes, but two remaining issues were still unresolved. First, how would the structural diversity affect the photophysical properties of the fluorophores? And second, how could successful probes be synthesized without incorporating a pre-established binding architecture in their structure? Initial efforts involving the synthesis of coumarin derivatives demonstrated that the fluorescent scaffold could accommodate structural diversity at different positions, and that such diversity may lead to a range of photophysical properties [9–11]. On the other hand, Mello and Finney discovered novel fluorescent Hg^{2+} sensors by making use of the binding-induced restriction of the biarylpyridine torsion without implementing any known recognition motif (Figure 1b) [12]. Hence combinatorial chemistry could not only compensate for the low capacity

Figure 1



First stages of combinatorial chemistry in fluorescent sensor development. (**a**) 8-Hydroxy-2-methylquinoline derivatives as potential Zn^{2+} sensors; (**b**) fluorescent library based on the biarylpyridine fluorophore and their application as Hg^{2+} sensors.Part (**a**) partly reproduced from [8] with permission; copyright 2001, American Chemical Society. Part (**b**) reproduced from [12] with permission; copyright 2005, American Chemical Society.

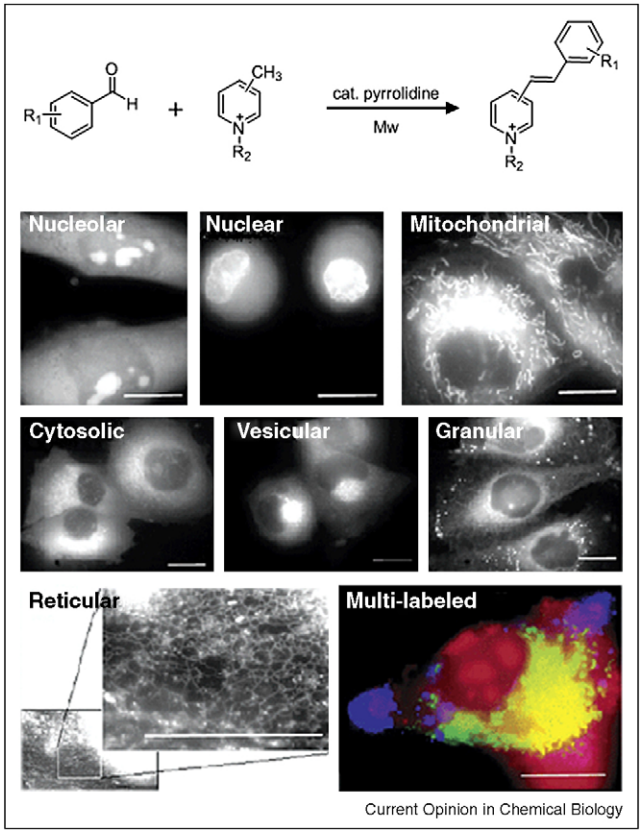
of predicting the photophysical behavior of new fluorophores [13,14] but also afford collections of potential fluorescent sensors in a diversity-oriented manner.

Combinatorial chemistry techniques to construct libraries of fluorescent sensors do not significantly differ from those developed for drug discovery or medicinal chemistry purposes. Parallel solution phase syntheses have been reported for a number of fluorescent scaffolds (xanthone, dapoxyl or isocoumarin, among others), which are typically derivatized with available building blocks following well-known organic procedures (e.g. condensation reactions, click chemistry, Pd-catalyzed couplings) [15–17]. Similar approaches have been carried out on solid-phase [18–20], which can be either used to facilitate the purification or to afford on-bead fluorescent sensors [21,22]. Finally, although less common, multicomponent or one-pot reactions can also be a source of fluorescent structures with large structural and spectral diversity [23,24•].

In light of the proven utility of combinatorial chemistry for the construction of diversity-oriented fluorescent libraries (DOFLs), Chang and co-workers pioneered the use of this methodology in biological systems [25], and stressed the molecular diversity, cell permeability and water solubility of the libraries of potential sensors. With a styryl-based library using commercially available aldehydes and pyridinium salts as building blocks, Rosania *et al.* reported combinatorial chemistry as a practical tool to evaluate the behavior of the fluorescent scaffolds in living cells. Notably, slight changes in the styryl structure redirected its subcellular localization (Figure 2) [26]. This information was used to evolve into probes for specific applications, such as DNA probes [27].

Having demonstrated the utility of DOFLs for the discovery of new fluorescent probes, more recent efforts have been focused on the use of scaffolds (e.g. rhodamine or BODIPY) with better inherent fluorescence properties, such as longer wavelengths and higher extinction coefficients. In such cases, combinatorial chemistry aims not only to implement broad structural diversity but also to modulate the quantum yields of the fluorescent cores. Compounds with moderate quantum yields are likely to exhibit fluorescence intensity fluctuations in an environment-dependent manner, and thus have a better potential for sensor development. Bearing this in mind, Ahn *et al.* redesigned the rhodamine core to synthesize 240 rosamine derivatives, on the basis of 12 xanthones and 33 Grignard reagents. The higher flexibility of the rosamine structure, which lacks the 2′-carboxylic acid, decreased

Figure 2



Styryl derivatives as multi-purpose fluorescent probes: (*upper*) general synthetic scheme; (*lower*) organelle localization study (scale bar: 10 μm).Reproduced from [26] with permission; copyright 2003, American Chemical Society.

the high quantum yield of the rhodamine scaffold, while maintaining its high extinction coefficient [28] (Figure 3a). Further screening of the rosamine library in different systems has rendered sensors for diverse applications, such as glutathione [29] or cell state sensors [30••].

Another example has been recently reported by Lee *et al.*, with the first DOFL based on the BODIPY structure [31]. Making use of the Knoevenagel condensation reaction, the authors optimized the combinatorial derivatization of a highly fluorescent BODIPY scaffold to generate 160 BODIPY dyes (Figure 3b). The broad structural and spectral diversities, and the optimum quantum yields as potential turn-on sensors were crucial to identify a selective glucagon probe, which was characterized *in vitro* and in live cells [32•].

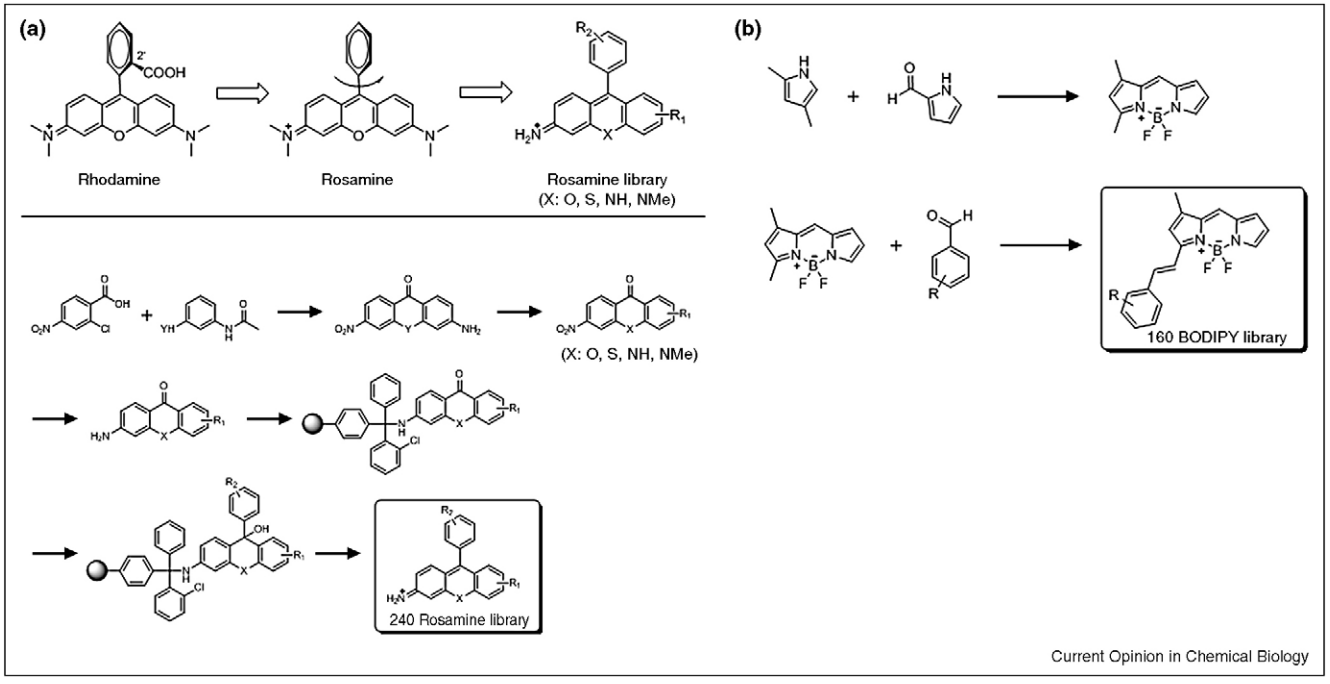
With combinatorial chemistry facilitating the preparation of chemically diverse sets of fluorophores, the discovery of novel fluorescent probes has been expanded to a broad range of biological systems. The high sensitivity of the fluorescence techniques enables monitoring small intensity and/or color changes of potential sensors even at low concentrations, where non-specific binding and toxicity can be minimized. Moreover, the high-throughput implementation and the improvement of the resolution limits have significantly upgraded the screening methodologies. The following sections will review the progress of different screening platforms, and how they have been adapted to the development of new probes for an increasing number of biological challenges.

In vitro screening of diversity-oriented libraries

The discovery of fluorescent probes from diversity-oriented libraries clearly depends on the design of the assay platform. The most conventional assay considers the *in vitro* examination of the fluorescence spectrum changes, either intensity or ratiometric, upon specific interaction with a particular analyte (Figure 4). In the early stages of fluorescent sensor development, DOFL compounds were screened against sets of biologically relevant molecules, which were typically selected according to the structural properties of the potential sensors (chelating properties, electrostatic charges, or environmental-sensitivity, among others). Fluorescent probes for heparin [33] or HSA (human serum albumin) [16] are some good examples. The simplicity of these experiments also favored the use of this screening method for targets that were not readily available. Li *et al.* applied this methodology to develop β-amyloid imaging sensors: 320 styryl compounds were *in vitro* evaluated using insulin fibrils with amyloidogenic properties [34•]. The inexpensive primary screening enabled the selection of 13 candidates, which were further examined in mouse brain tissue to eventually render probes with *in vivo* imaging application [35]. This work exemplifies how a conveniently designed *in vitro* primary screening can successfully narrow down large DOFLs to minimize costs and efforts in the search of probes for complex biological samples.

Once an effective sensor is identified from a given library, what happens to the remaining compounds? One of the major merits of DOFLs is that they are not restricted to detect a single analyte. One library can be screened against different sets of target molecules and successfully lead to different sensors for different applications. A representative example of such adaptability are the styryl dyes, which aside from its above mentioned application as β-amyloid trackers, have been used to develop selective RNA probes combining a RNA-selective *in vitro* response and a nuclear localization in live cells [36]. This versatility has also been exploited to perform more systematic analysis, so-called *in vitro* profiling. The systematic screening of a benzimidazolium library against all nucleotides and nucleosides enabled the discovery of **GTP**

Figure 3



Derivatization of highly fluorescent scaffolds: (**a**) (*upper*) structural differences between the rhodamine and rosamine scaffolds, (*lower*) general synthetic scheme of the rosamine library; (**b**) synthesis of BODIPY derivatives as fluorescent turn-on sensors.Part (**a**, *upper*) reproduced from [28] with permission; copyright 2003, American Chemical Society.

Green, a selective turn-on GTP-specific probe [37] (Figure 4). More recently, *in vitro* profiling strategies have been also applied to fluorescent nanoparticle libraries for the unbiased discovery of *in vivo* imaging probes for endothelial cells [38•].

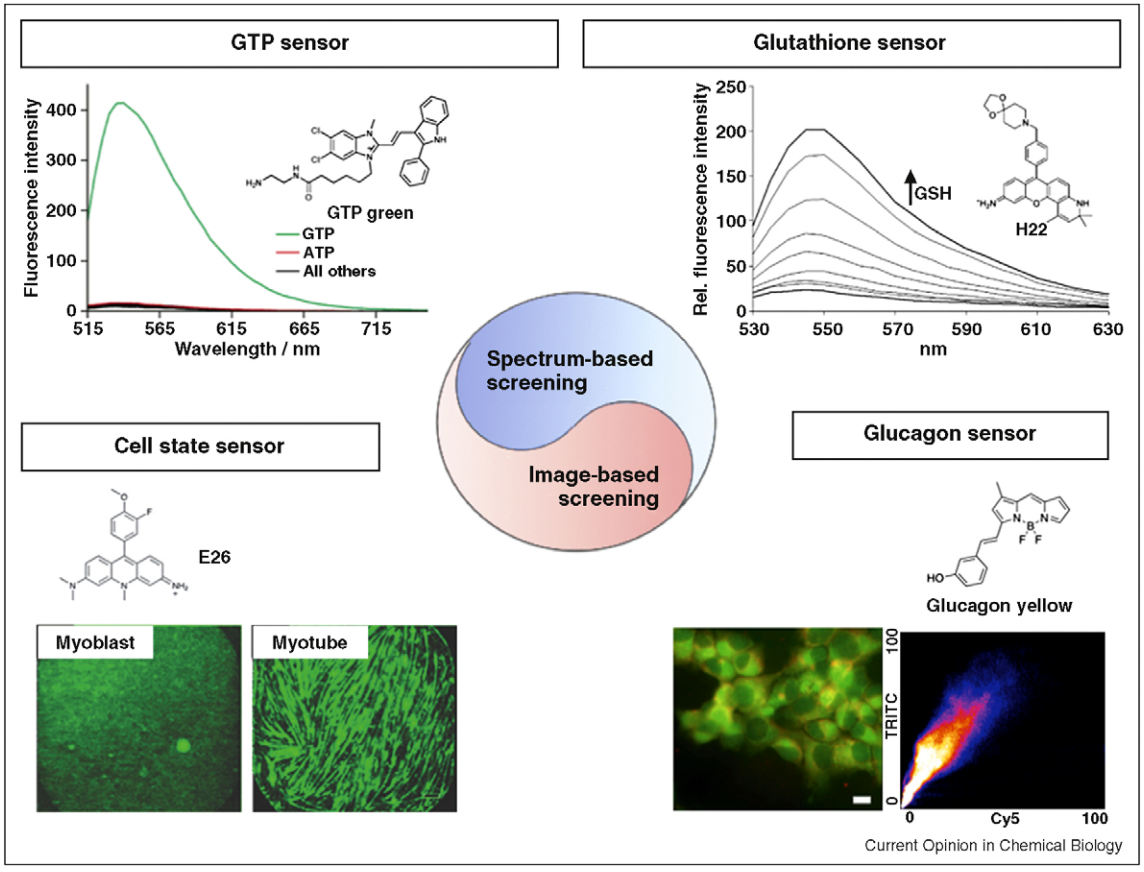
Toward image-based high-content screening

In spite of their obvious usefulness, *in vitro* screens are limited in that they do not consider factors such as permeability, cellular uptake, and localization, which may affect the interaction between a fluorescent probe and its target biomolecule in cells. Live cell or tissue-based screening (initially used to validate *in vitro* discovered probes) has appeared as a sensible alternative, and major technical improvements have been gradually implemented to evolve into high-content screening (HCS). HCS is a powerful tool to examine biological systems in an unbiased manner (Figure 4). Since the first report of a HCS in live cells [39], the progress in automated imaging microscopy [40,41] and image analysis [42,43,44•] has been remarkable. However, the scope of the HCS also relies on the diversity of probes, and despite the extensive use of Hoechst [45–47], the development of fluorescent molecules tracking proteins, organelles or phenotypes is necessary to expand HCS to multiple biological challenges [48].

Recently, HCS platforms have been aimed at tracking not single biomolecules but more challenging targets, such as phenotypes or cell states. Wagner *et al.* reported the first cell-based screening of a DOFL to discover fluorescent probes for a specific cell state (e.g. muscle cell differentiation) [30••]. Two differentiation stages of muscle cells were treated with a collection of fluorescent dyes, and analyzed by automated fluorescence microscopy. A rosamine compound (**E26**) was identified to selectively stain myotubes (Figure 4). Notably, since **E26** was discovered from a live cell platform, its application in bioimaging is straightforward. Even though the molecular target is unclear, the use of **E26** in muscle differentiation studies is of great interest, as it provides a practical and cheaper alternative to the antibody-based discrimination.

Image-based HTS has also been used to discover probes that track molecular targets by examining unique characteristics of different cell lines. A particular example has been reported with the screening of a large collection of BODIPY dyes in pancreatic α-cells and β-cells [32]. In this case, an α-cell selective staining compound was identified, and further examination revealed the glucagon secretion as the origin of its response. The viability of the probe (**Glucagon Yellow**) was eventually confirmed

Figure 4



Panel of HTS used in diversity-oriented libraries: (*upper*) *in vitro* HTS monitor fluorescence spectrum changes, either intensity or ratiometric, upon specific interaction with a particular analyte; (*lower*) cell-based screenings have recently succeeded in developing new imaging probes to discriminate cell lines or cell states.Reproduced from [37] (*upper left*), [28] (*upper right*), [29] (*lower left*) and [32] (*lower right*) with permission. Copyright 2006–2009, American Chemical Society.

by co-localization experiments with the respective antibodies (Figure 4).

Conclusions and outlook

In recent years, diversity-oriented approaches have led to a number of fluorescent sensors for metabolites, macromolecules, and even cell states. Although combinatorial chemistry, fluorescence microscopy, and HCS have enhanced their usefulness for these purposes, complex biological questions are on the horizon. Stem cells, lineage differentiation, or specific cancer biomarkers are in the need for tools that help their diagnostic and/or characterization. On the other hand, with the advances of HTS in whole organisms [49] and the emerging interest in small animal *in vivo* imaging (SAIVI), the discovery of fluorescent probes in such exigent fields will certainly become a challenge in the near future. We believe that, in conjunction with well-defined biological systems, diversity-oriented approaches will continue to play an important role in the development of high-performance fluorescent sensors for contemporary scientific problems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbpa.2010.02.020.

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