Mr. Alan Blake
Yorktown Technologies, LP
Austin, TX 78759

RE: Safety Assessment of Fluorescent Proteins

Dear Mr. Blake:

You have asked me to analyze and comment on the toxicity of fluorescent proteins that are commonly used in transgenic organisms. Specifically, your company plans to create and sell through retail channels transgenic ornamental fish containing genes, which express such fluorescent proteins, including green fluorescent protein (GFP) and red reef coral fluorescent protein (RFP).

The following analysis is based on the currently available information on the ecological and toxicological aspects of fluorescent proteins and transgenic organisms expressing such fluorescent proteins, as well as my knowledge of the fluorescent protein literature and scientific research in this area.

My own experience with fluorescent proteins and scientific and technical experience is summarized in the attached C.V. In brief, I have a Ph.D in Molecular Biology from the University of California, San Diego. I have been actively involved in scientific research on fluorescent proteins for approximately five years, and subsequently involved in intellectual property and business development aspects of the commercialization of fluorescent proteins for approximately three years. During this time I published several research articles on fluorescent proteins. I am also an inventor on a number of significant US patents directed to fluorescent proteins.

The available data summarized below suggests that fluorescent proteins are a low toxicological risk and are very unlikely to represent a health risk to either consumers or other organisms. Since fluorescent proteins naturally and harmlessly occur in the marine ecosystem, their presence in transgenic ornamental fish is unlikely to cause harm to the ecosystem.

I. FLUORESCENT PROTEINS HAVE BEEN WIDELY USED WITHOUT APPARENT TOXIC EFFECTS

Fluorescent proteins have been widely used and successfully expressed in a wide range of cell types without toxic side effects.

- Tsien, R.Y (1998) The green fluorescent protein. Annu. Rev. Biochem. 67 509-44.
- Hadjantonakis AK, *et al.* (2003) Technicolour transgenics: imaging tools for functional genomics in the mouse. Nat Rev Genet. Aug;4 (8):613-25.

Stable cells lines have been successfully obtained from virtually every cell type and organism so far tested, and transgenic animals that constitutively express fluorescent proteins have been reported as being healthy.

- Higashijima *et al.* (1997) High frequency generation of transgenic zebra fish which reliably express GFP in whole muscles or the whole body by using promoters of zebra fish origin. Dev. Biol. 192 289-299.

- Hadjantonakis, *et al.* (1998) Generating green fluorescent mice by germline transmission of green fluorescent ES cells. Mech. Dev. 76 79-90.

- Lois *et al.* (2002) Germline transmission and tissue specific expression of transgenes delivered by lentiviral vectors. Science 295 868-872.

- Tsai *et al.* (2001) Uniform GFP-expression in transgenic medaka (Oryzias latipes) at the F0 generation. Transgenic Research 10 303-315.

- Ekker, *et al.* (2001) Three-color imaging using fluorescent proteins in living zebrafish embryos. Biotechniques Jul 31(1) 66-70, 72.

Accordingly, the available data suggests that fluorescent proteins do not exert significant toxicological effects at the sub-cellular, or whole animal level.

II. FLUORESCENT PROTEINS ARE ALREADY PRESENT IN THE FOOD CHAIN

Fluorescent proteins are widely dispersed in the natural ecosystem and are found naturally in many oceanic species. Fluorescent proteins are particularly common within the phylum Cnidaria that includes medusae (jellyfish), sea anemones, corals, marine hydroids, fresh water hydras, and sea fans.

- Prasher (1995) Using GFP to see the light. Trends Genetics 11 (8) 320-326.

Accordingly, it is concluded that any fluorescent proteins added to the ecosystem through genetically modified fish would be of minor influence because fluorescent proteins are already widely present and harmless in the ecosystem and within the marine food chain.

III. FLUORESCENT PROTEINS DO NOT APPEAR TO SHARE SIGNIFICANT HOMOLOGY TO KNOWN ALLERGENS

Sequence analysis of fluorescent proteins reveals that these proteins do not share significant homology in amino acid sequence with any known food allergens.

- Richards *et al.*(2003) Safety Assessment of recombinant green fluorescent protein orally administered to weaned rats. J. Nutr. 133 1909-1912.

- Gendel (1998) Sequence databases for assessing the potential allergeicity of proteins used in transgenic foods. Adv. Food. Nutr. Res. 42 63-92

- Gendel (1998) The use of amino acid sequence alignments to assess potential allergenicity of proteins used in genetically modified foods. Adv. Food Nutr. Res. 42 45-62

Further, direct analysis of the effect of recombinant GFP fed to rats demonstrated no adverse effects of oral GFP administration suggesting that that GFP is not an allergen or irritant to the digestive tract.

- Richards *et al.*(2003) Safety Assessment of recombinant green fluorescent protein orally administered to weaned rats. J. Nutr. 133 1909-1912.

IV. FLUORESCENT PROTEINS WOULD BE PREDICTED TO ACT LIKE OTHER PROTEINS AND UNDERGO RAPID DIGESTION IN THE GUT

Fluorescent proteins are comprised of naturally occurring amino acids, which would be predicted to be rapidly degraded in the gut.

- Hammond, B.G., Vicini, J.L., Hartnell, G.F., Naylor, M.W., Knight, C.D., Robinson, E.H., Fuchs, R.L. & Padgette, S.R. (1996) The feeding value of soybeans fed to rats, chickens, catfish, and dairy cattle is not altered by genetic incorporation of glyphosphate tolerance. J. Nut. 126 717-727.

- Harrison, *et al.* (1996) The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvyl-shikimate-3-phosphate synthase from Agrobacterium sp. Strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. J. Nutr. 126 728-740.

Direct analysis of the effect of fluorescent proteins on animals fed recombinantly expressed fluorescent proteins, or diets containing transgenic canola expressing fluorescent proteins, demonstrates that such treatments do not affect the growth, food intake, relative weight of intestine or other organs, or the activities of hepatic enzymes in the serum.

- Richards *et al.*(2003) Safety Assessment of recombinant green fluorescent protein orally administered to weaned rats. J. Nutr. 133 1909-1912.

VI. CONCLUSION

It is concluded that there is no basis for believing that fluorescent protein expression in transgenic fish would represent a toxicological risk, to either the environment or consumers, if the fish should enter the ecosystem.

Sincerely,

Andrew Cubitt, Ph.D

BIOGRAPHICAL SKETCH AND BIBLIOGRAPHY

Andrew Bryan Cubitt

Education

Institution and Location	**Degree**	**Conferred**	**Field of Study**
University of Birmingham, UK	B.Sc	1984	Medical Biochemistry
University of Sheffield, UK	Ph.D.	1987	Biochemistry

Professional Qualifications

U.S. Patent Agent   Registration No. 45,452   1999

SBIR New Technology review member   1999 to 2002

Research and Professional Experience

2002 to present

X-Ceptor Therapeutics Inc. Associate Director, Intellectual Property

1999 to 2002

Aurora Biosciences Corporation, Senior Manager.

Responsible for the review, preparation and analysis of new technologies and patent applications. Responsible for overall management and worldwide prosecution of biotechnology patents at Aurora (approximately 120 pending applications). Responsible for technical and IP analysis of third IP and freedom to operate analysis for internal use and commercialization. Review of technology and IP issues for in-licensing. Response for internal IP due diligence analysis for potential M & A activities.

1998 to 1999

Aurora Biosciences Corporation, Manager, Technology and Intellectual Property

Prosecute biotechnology patent applications with review of General Counsel. Oversee technology transfer into and out of Aurora. Oversight and coordination of intellectual property databases. Develop external academic and internal networks to enable successful biotechnology IP acquisition and dissemination of intellectual information for internal review. Provide technical support for external licensing into and out of Aurora. Review of public disclosures.

1996 to 1998

Aurora Biosciences Corporation, Principal Biochemist

Responsible for the development of fluorescence based biochemical and cell based assays for internal and external research projects. Supervision and training responsibilities for technicians. Responsible for the development of improved green fluorescent mutants, and subsequent licensing. Provide technical support for IP counsel.

1994 to 1996

Howard Hughes Medical Institute, UCSD, Project Scientist.

Worked on the use of rational design and random mutagenesis of GFP to produce brighter and more spectrally distinguishable mutants in laboratory of Roger Tsien. Characterization of GFP photochemistry, chromophore formation and spectral properties. Initiated development of novel biosensors based on GFP fluorescence. Optimization of the production and purification of GFP for X-ray crystallography and NMR studies

1990 to 1994

Department of Biology, UCSD, Post-Doctoral Research Fellow
Molecular genetic analysis of signal transduction pathways in the developmental regulation of *Dictyostelium* in the laboratory of Richard Firtel.. Evaluated the effect of gene disruptions of G-alpha subunits on developmental progression on phospholipid metabolism, including the regulation of phospholipases A, C and D. Used expression of Apo-aequorin as a novel method to measure for the first time calcium measurements throughout the developmental progression of the multicellular stages of *Dictyostelium.*

1993 to 1994

Marine Biological Laboratory, Visiting Scientist
Collaborated on the measurement of calcium levels using aequorin expression in *Dictyostelium* using the ultra low level light imaging equipment in the laboratory of Lionel Jaffe and cell lines created at UCSD. Demonstrated for the first time waves of calcium moving through all multicellular stages of development. Established a correlation between developmental fate and free cytosolic calcium levels.

1987 to 1990

Cornell University Medical College, New York, post-doctoral fellow
Analysis of phospholipase C activity and the control of inositol lipid synthesis in rat GH3 cells in the laboratory of Marvin Gershengorn. Identified, characterized and purified a phosphatidylinositol synthatase enzyme activity from GH3 cells. Established a link between the size of the hormone responsive pool of inositol lipids and the rate of resynthesis of lipids during TRH stimulated phospholipid hydrolysis.

Publications

Seventeen journal articles: journals represented include Nature (2), Science (1), J. Biological Chemistry (2), Development (2) and Biochemical J. (6). Three invited reviews

1. Cubitt, A.B., Brown, B.L. and Dobson, P.R.M. Activation of dopamine D2 receptors does not influence phosphoinositide turnover in NCB-20 cells. J. Neurochem. 49 183-188 (1987)

2. Shears, S.B., Story, D.J., Morris, A.J., Cubitt, A.B. , Parry, J.B., Michell, R.H. and Kirk, C.J. Dephosphorylation of myo-inositol 1,4,5-trisphosphate and myo-inositol bisphosphate. Biochem. J. 242 373-402 (1987)

3. Cubitt, A.B. and Gershengorn, M.C. Characterization of a salt extractable phosphatidylinositol synthase from rat pituitary-tumour membranes. Biochem. J. 257 639-644 (1989)

4. Cubitt, A.B., Zhang, B. and Gershengorn, M.C. Analysis by base exchange of TRH responsive and unresponsive inositol lipid pools in rat pituitary tumor cells. J. Biol. Chem. 265 9707-9714 (1990)

5. Cubitt, A.B., Geras-Raaka, E. and Gershengorn, M.C. Thyrotropin-Releasing hormone receptor occupancy determines the fraction of the responsive pool of inositol lipids hydrolyzed in rat pituitary tumor cells. Biochem. J. 271 331-336 (1990)

6. Cubitt, A.B. and Gershengorn, M.C. 5'-CMP activates reversal of phosphatidylinositol synthase and base exchange by distinct mechanisms in rat pituitary GH3 cell. Biochem. J. 272 813-816 (1990)

7. Cubitt, A.B., Thaw, C.N. and Gershengorn, M.C. 5'-CMP stimulates phospholipase A mediated hydrolysis of phosphatidylinositol in permeabilized pituitary GH3 cells. Biochem. J. 278 831-834 (1991)

8. Cubitt, A.B. and Firtel, R.A. Characterization of phospholipase activity in Dictyostelium discoideum. Identification of a calcium dependent polyphosphoinositidase specific phospholipase C. Biochem J. 283 371-378 (1992)

9. Okaichi, K., Cubitt, A.B. and Firtel, R.A. Amino acid substitutions in the Dictyostelium Ga subunit Ga 2 produce dominant negative phenotypes and inhibit the activation of Adenylate cyclase guanylate cyclase and phospholipase C. Mol. Biol. Cell. 3 735-747

10. Powell, J.A., Schnitzler, G.R., Hadwiger, J.A., Howard, P., Esch, R.K., Cubitt, A.B., Okaichi, K., Gaskins, C., Mann, S.K.O., and Firtel, R.A. Spatial and temporal regulation of Dictyostelium development through signal transduction pathways. In: "Evolutional Conservation of Developmental Mechanisms" (ed

A.C. Spradling), N.Y. Wiley-Liss, Inc. pp 159-184

11. Cubitt, A.B., Carrel, F., Dharmarwardhane, S., Gaskins, C., Hadwiger, J., Howard, P., Mann, S.K.O., Okaichi, K., Zhou, K. and Firtel, R.A. Molecular genetic analysis of signal transduction pathways activated by cell surface receptors in Dictyostelium. Cold Spring Harbor Symp. Quant. Biol. LVII 177-192 (1992)

12. Cubitt, A.B., Dharmawardhane, Firtel, R.A. Developmentally regulated changes in 1,2-diacylglycerol in Dictyostelium: Regulation by light and G proteins. J. Biol. Chem. 268 17431-17439 (1993)

13. Dharmawardhane, S., Cubitt, A.B. and Firtel, R.A. Role of G□1 subunit in regulating cAMP receptor-mediated pathways and multicellular development in Dictyostelium. Development 120 3549-3561 (1994)

14. Heim, R., Cubitt, A.B. and Tsien R.Y. Improved green fluorescence. Nature 373 663-664 (1995)

15. Cubitt, A.B., Firtel, R.A., Fischer, G., Jaffe, L.F., and Miller, A.L. Patterns of free calcium in multicellular stages of Dictyostelium expressing jellyfish apoaequorin. Development 121 2291-2301 (1995)

16. Cubitt, A.B., Heim, R., Adams, S.R., Boyd, A.E., Gross, L.A. and Tsien, R.Y. Understanding, improving and using green fluorescent proteins. Trends in Biochem Sci. 20 448-455 (1995)

17. Ormo, M., Cubitt, A.B., Kallio, K., Gross, L.A., Tsien, R.Y. & Remington, S.J. Crystal structure of the Aequorea victoria green fluorescent protein. Science 273 1392-1395 (1996)

18. Dickson, R.M., Cubitt, A.B., Tsien R.Y. and Moerner, W.E. On / off blinking and switching behavior of single molecules of green fluorescent protein. Nature 388 355-358 (1997)

19. Cubitt, A.B., Reddy, I., Lee, S., McNally, J.G. and Firtel, R.A. Coexpression of a constitutively active plasma membrane calcium pump with GFP identifies roles for intracellular calcium in controlling cell sorting during morphogenesis in Dictyostelium. Dev. Biol. 196 77-94 (1998)

20. Cubitt, A.B., Leslie A. Woollenweber, and Roger Heim. Understanding Structure –function relationships in the *Aequorea victoria* Green Fluorescent Protein. Chapter 2 in Green Fluorescent proteins Ed. Sullivan and Kay Academic Press, 1999

Patents

1. U.S. Patent No. 5,981,200 Tandem fluorescent protein constructs. Inventors Tsien, R.Y., Heim, R & Cubitt, A.B.

2. U.S. Patent No. 5,912,137 Assays for protein kinases using fluorescent protein substrates. Inventors Tsien, R.Y. & Cubitt, A.B.

3. U.S. Patent No. 5,925,558 Assays for protein kinases using fluorescent protein substrates. Inventors Tsien, R.Y. & Cubitt, A.B.

4. U.S. Patent No. 6,248,550 Assays for protein kinases using fluorescent protein substrates. Inventors Tsien, R.Y. & Cubitt, A.B.

5. U.S. Patent No. 6,124,128 Long wavelength mutant fluorescent proteins. Inventors Tsien, R.Y., Cubitt, A.B., Ormo, M. & Remington, S.J.

6. U.S. Patent No. 6,054,321 Long wavelength mutant fluorescent proteins. Inventors Tsien, R.Y., Cubitt, A.B., Ormo, M. & Remington, S.J.

7. U.S. Patent No. 6,077,707 Long wavelength mutant fluorescent proteins. Inventors Tsien, R.Y., Cubitt, A.B., Ormo, M. & Remington, S.J.

8. U.S. Patent No. 6,046,925 Photochromic fluorescent proteins and optical memory storage devices based on fluorescent proteins. Inventors Tsien, R.Y., Heim, R., Cubitt, A. B., Dickson, R. M., and Moerner, W. E.

9. Pending Fluorescent Protein sensors of post-translational modifications. Inventor Cubitt, A. B.

10. Pending Methods of protein destabilization and uses thereof. Inventors Stack, J.H., Whitney, M., Cubitt, A.B. and Pollok B.A.