GOD cytochemistry

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The activity of glucose oxidase (GOD) is usually visualized with tetrazolium salts and intermediate electron carriers. Alternatively, staining of GOD can be performed by a coupled enzyme system and by the use of 3,3'diaminobenzidine (KUHLMANN WD, 1970; KUHLMANN WD and AVRAMEAS S, 1971). The cytochemical procedure involves a two-step enzyme method in which GOD (the enzyme as label) and HRP (the indicator enzyme) are preferentially coimmobilized onto the same cellular sites by immunological bridging or by other ligand binding principles such as avidin-biotin interaction (KUHLMANN WD and PESCHKE P 1986). In this coupled enzyme technique,

- H_2O_2 is generated by enzymatic reaction of GOD with D-glucose and, subsequently,
- generated H_2O_2 is serving as substrate for HRP in the oxidation of DAB (3,3'-diaminobenzidine tetrahydrochloride) or AEC (3-amino-9-ethylcarbazole).

For detailed descriptions of the various cytochemical enzyme substrates see chapter *Enzyme cytochemical substrate solutions*.*

Tetrazolium salt technique

Enzyme substrate (GOD)	**Cytochemical feature**
0.05% MTT*, 0.75% D-glucose and 0.01% PMS* • Working solution: 150 mg β-D-glucose dissolved in 20 mL phosphate buffer (0.1 M pH 6.8) *plus* 10 mg MTT add 2 mg PMS just prior to use, mix and filter rapidly [in order to avoid crystallisation of MTT on the slide, add 2 mg cobalt chloride to the substrate], staining is performed in the dark MTT can be replaced by NBT or INT tetrazolium salts * Methyl thiazolyl tetrazolium ** Phenazine methosulfate	**Blue color (MTT)** Other chromogenic substrates: (a) Nitroblue tetrazolium (NBT) to give purple-blue color (b) Tetranitroblue tetrazolium (TNBT) to give black color (c) Iodophenyl-nitrophenyl-phenyl tetrazolium (INT) to give violet color Enzyme substrates are useful in combination with other substrates for double stainings
General procedure for immunohistological staining (paraffin sections): – Deparaffinize in xylene — 2 x 5 min – rehydrate with ethanol — 2 x 5 min absolute ethanol; 1 x 1 min 95% ethanol	

* Chromogens and other cytochemical reagents can be toxic and carcinogenic. They must be handled with care

		1 x 1 min 70% ethanol
–	distilled water	2 x 1 min
–	washing buffer (e.g.PBS/BSA)	2 x 2min
–	blocking solution	background reactions
–	primary antibody	up to 24 hours depending on antibody concentration
–	washing buffer	3 x 5 min
–	GOD labeled secondary antibody (other detection principle, e.g. biotin-avidin)	30 min
–	PBS	3 x 5 min
–	enzyme substrate, chromogen	20-60 min (variable incubation)
–	PBS	2 x 2 min
–	distilled water	2 x 2 min
–	counterstain	optional
–	distilled water	2 x 2 min
–	mounting	glycerol/gelatine or other mounting

Two-step enzyme technique (GOD as label and HRP as indicator)

Enzyme substrate (GOD)	**Cytochemical feature**
0.05% Diaminobenzidine (DAB), 1% D-glucose containing 0.1-1 mg peroxidase (HRP) per mL solution • DAB stock solution: 50 mg DAB are dissolved in 10 mL distilled water, aliquoted and stored at -20ºC (stable for years) • β-D-Glucose stock solution: 1000 mg D-glucose dissolved in 10 mL distilled water (for a final 1% glucose concentration), aliquoted and stored at 4ºC • Peroxidase (HRP RZ 3) stock solution: 10 mg HRP dissolved in 10 mL 0.1 M phosphate buffer pH 7.3 (for 0.1 mg peroxidase per mL working solution) • Working solution: 1 mL DAB stock *plus* 3 mL 0.1 M phosphate buffer pH 6.8 *plus* 1 mL D-glucose stock solution *plus* 1 mL peroxidase stock solution *plus* 4 mL 0.1 M phosphate buffer pH 6.8	**Brown color** Enzyme substrate is useful in combination with other substrates for double stainings

General procedure for immunohistological staining (paraffin sections):		
–	Deparaffinize in xylene	2 x 5 min
–	rehydrate with ethanol	2 x 5 min absolute ethanol 1 x 1 min 95% ethanol

		1 x 1 min 70% ethanol
–	distilled water	2 x 1 min
–	washing buffer (e.g.PBS/BSA)	2 x 2min
–	blocking solution	background reactions
–	primary antibody	up to 24 hours depending on antibody concentration
–	washing buffer	3 x 5 min
–	GOD labeled secondary antibody (other detection principle, e.g. biotin-avidin)	30 min
–	washing buffer	3 x 5 min
–	enzyme substrate, chromogen	10-30 min (variable incubation)
–	washing buffer, distilled water	2 x 2 min, each
–	postfixation with 0.1% OsO_4	2-3 min (optional)
–	counterstain	haematoxylin or other (optional)
–	dehydration, mounting	ascending series of ethanol, xylene, mounting medium

Enzyme substrate (GOD)	**Cytochemical feature**
0.05% Diaminobenzidine (DAB), 1% D-glucose, 0.025% nickel ammonium sulfate containing 0.1-1 mg peroxidase (HRP) per mL solution • DAB stock solution: 50 mg DAB are dissolved in 10 mL distilled water, aliquoted and stored at -20ºC (stable for years) • β-D-Glucose stock solution: 1000 mg D-glucose dissolved in 10 mL distilled water (for a final 1% glucose concentration), aliquoted and stored at 4°C • Peroxidase (HRP RZ 3) solution: 10 mg HRP dissolved in 10 mL 0.1 M phosphate buffer pH 7.3 (for 0.1 mg peroxidase per mL working solution) • Nickel ammonium sulfate stock solution: 0.1 g $Ni(NH_4)_2$ $(SO_4)_2$ are dissolved in 10 mL distilled water • Working solution: 1 mL DAB stock *plus* 3 mL 0.1 M phosphate buffer pH 6.8 *plus* 1 mL D-glucose stock solution *plus* 1 mL peroxidase stock solution *plus* 3.75 mL 0.1 M phosphate buffer (0.01 M pH 6.8) *plus* 250 µL nickel ammonium sulfate stock	**Black color** Enzyme substrate is used for enhanced GOD stainings and useful in combination with other substrates for double stainings

General procedure for immunohistological staining (paraffin sections):		
–	Deparaffinize in xylene	2 x 5 min
–	rehydrate with ethanol	2 x 5 min absolute ethanol 1 x 1 min 95% ethanol

		1 x 1 min 70% ethanol
–	distilled water	2 x 1 min
–	washing buffer (e.g.PBS/BSA)	2 x 2min
–	blocking solution	background reactions
–	primary antibody	up to 24 hours depending on antibody concentration
–	washing buffer	3 x 5 min
–	GOD labeled secondary antibody (other detection principle, e.g. biotin-avidin)	30 min
–	washing buffer	3 x 5 min
–	enzyme substrate, chromogen	10-30 min at room temperature or 1-2 hours at 4ºC (optimal incubation time and temperature depend on experimental conditions)
–	washing buffer, distilled water	2 x 2 min, each
–	counterstain	haematoxylin or other (optional)
–	dehydration, mounting	ascending series of ethanol, xylene, mounting medium

Two-step enzyme technique (GOD as label and HRP as indicator) in the coimmobilized GOD-HRP method

In this enzyme-amplification technique, H_2O_2 generated by the reaction of GOD with D-glucose serves as substrate for HRP in the oxidation of chromogens such as 3,3'-diamino-benzidine (DAB). Coimmobilization of both *marker enzyme* (GOD) and *secondary system amplifier* (HRP) in close proximity proved to be of great advantage in immunohistology because the unfavorable properties of soluble HRP in substrate mixtures are avoided. This method appears to be better than the use of soluble HRP in the substrate mixture (KUHLMANN WD and PESCHKE P, 1986).

The coupled GOD-HRP principle was developed for the use of GOD as marker enzyme in immunohistology because no such endogenous enzyme activity is known in mammalian cells and to profit from the general usefulness of DAB as cytochemical stain. Hence, the coupled GOD-HRP principle can be superior to conventional immunoperoxidase labeling for the localization of biomolecules in tissues which are rich in endogenous peroxidase activitites.

Enzyme substrate (GOD) (coimmobilized GOD-HRP method)	**Cytochemical feature**
0.05% Diaminobenzidine (DAB), 0.2-1% β -D-glucose 0.04% ammonium chloride (optional) • *0.5% Cobalt acetate for pretreatment is optional: 0.5 g cobalt acetate dissolved in 100 mL Tris buffer pH 7.6* • *Ammonium chloride stock solution is optional: 0.4 g NH_4Cl dissolved in 100 mL distilled water* • DAB stock solution: 50 mg DAB are dissolved in 10 mL distilled water,	**Brown color, dark blue/black color** GOD-HRP method as alternative to immunoperoxidase staining, useful in combination with other markers and substrates

aliquoted and stored at -20ºC (stable for years) • β-D-Glucose stock solution: 200 mg D-glucose dissolved in 10 mL distilled water (for a final 0.2% glucose concentration), aliquoted and stored at 4°C • Working solution: 1 mL DAB stock *plus* 5 mL mL phosphate buffer pH 7.3 *plus* 1 mL glucose stock *plus* 3 mL 0.1 M phosphate buffer pH 7.3 (*alternatively, 1 mL ammonium chloride stock and 2 ml phosphate buffer are added*)	

General procedure for immunohistological staining (paraffin sections):

–	Deparaffinize in xylene	2 x 5 min
–	rehydrate with ethanol	2 x 5 min absolute ethanol 1 x 1 min 95% ethanol 1 x 1 min 70% ethanol
–	distilled water	2 x 1 min
–	washing buffer (e.g.PBS/BSA)	2 x 2min
–	blocking solution	background reactions
–	primary antibody	up to 24 hours depending on antibody concentration
–	washing buffer	3 x 5 min
–	GOD labeled secondary antibody (other detection principle, e.g. biotin-avidin)	30 min
–	washing buffer	3 x 5 min

DAB intensification by metal salts such as cobalt acetate or nickel chloride can be useful. In this case, the incubation sequence is as follows:

–	wash in PBS	2 x 15 min
–	wash in 0.05 M Tris buffer pH 7.6	1 x 15 min
–	Sections are rinsed twice in PBS and once in 0.05 M (15 min in each)	
–	0.5% cobalt acetate solution	10 min
–	wash in 0.1 M Tris buffer pH 7.6	3 x 10 min
–	wash in 0.1 M phosphate pH 7.3	2 x 10 min
–	enzyme substrate, chromogen	10-30 min at room temperature or 1-2 hours at 4-20°C (optimal incubation time and temperature depend on experimental conditions)
–	wash in 0.1 M phosphate pH 7.3	3 x 10 min
–	wash in distilled water	2 x 2 min
–	counterstain	haematoxylin or other (optional)
–	dehydration, mounting	ascending series of ethanol, xylene, mounting medium

References for further readings

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Full citation of publications is given in chapter *References*
link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf

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